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

12/03
8 October 2003

FINAL ASSESSMENT REPORT

APPLICATION A484

FOOD FROM INSECT PROTECTED MON863 CORN

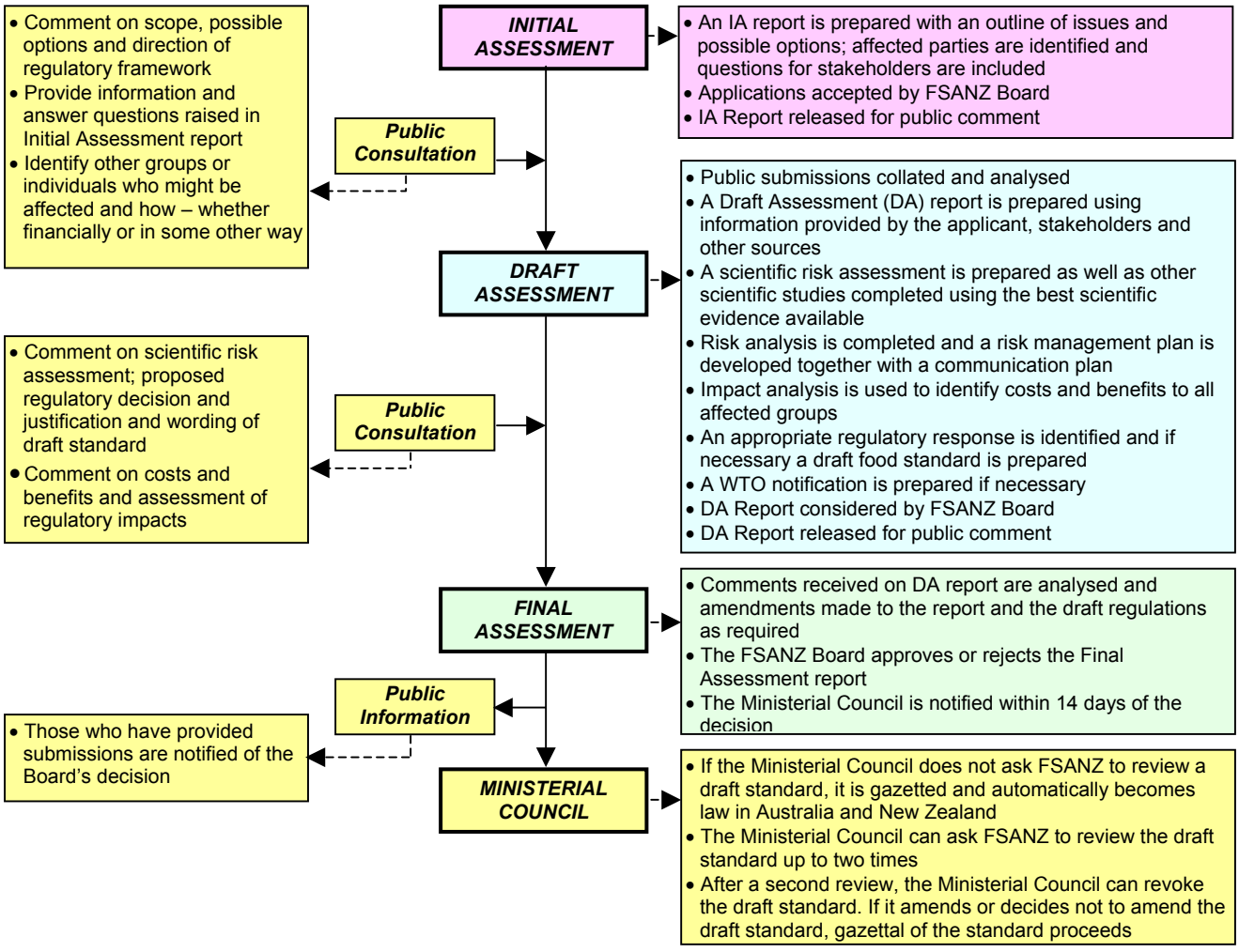
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

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

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

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

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



Final Assessment Stage

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

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

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

Further Information

Further information on this Application and the assessment process should be addressed to the FSANZ Standards Liaison Officer at one of the following addresses:

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

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Executive Summary and Statement of Reasons

An Application was received from Monsanto Australia Limited on 4 December 2002 seeking approval for food derived from insect-protected MON863 corn under Standard 1.5.2 – Food Produced Using Gene Technology of the *Australia New Zealand Food Standards Code* (the Code). Standard 1.5.2 requires that genetically modified (GM) foods undergo a pre-market safety assessment before they may be sold in Australia and New Zealand.

The new genetic trait in MON863 corn confers protection against the corn rootworm, a significant pest of corn crops in certain regions of the United States and Canada. Protection is conferred by expression in the plant of a bacterially derived protein (a *Bt* protein) that is toxic to beetle larvae. MON863 corn also contains a new gene encoding resistance to the antibiotic neomycin and related aminoglycoside antibiotics.

MON863 corn has been developed for cultivation in the United States and Canada only, but food derived from this corn could enter the market in Australia and New Zealand via imported products, once it is grown on a commercial scale.

Safety assessment

FSANZ has completed a comprehensive safety assessment of food derived from MON863 corn as required under the standard. The assessment included consideration of: (i) the genetic modification to the plant; (ii) the safety of any transferred antibiotic resistance genes; (iii) the potential toxicity and allergenicity of any new proteins; and (iv) the composition and nutritional adequacy of the food, including whether there had been any unintended changes.

No potential public health and safety concerns were identified in the assessment of food derived from MON863 corn. Therefore, on the basis of all the available evidence, including detailed studies provided by the applicant, it has been concluded that food derived from MON863 corn is as safe and wholesome as food derived from other corn varieties.

Labelling

Food derived from MON863 corn will require labelling if novel DNA and/or protein are present in the final food. Ingredients derived from corn likely to satisfy this criterion are starch, modified starch ingredients, meal/semolina and flour. Other ingredients, such as refined oil, glucose syrups and high fructose syrups are unlikely to contain either novel DNA or protein.

Impact of regulatory options

Two regulatory options were considered in the assessment: either (1) no approval; or (2) approval of food derived from MON863 corn contingent on a satisfactory safety assessment. Following an assessment of the potential impact of each of the options on the affected parties (consumers, the food industry and government), Option 2 is the preferred option as it potentially offers significant benefits to all sectors with very little associated negative impact. The proposed amendment to the Code, giving approval to food from MON863 corn, is therefore considered necessary, cost effective and of net benefit to both food producers and consumers.

Consultation

Two rounds of public consultation were undertaken. Four submissions were received in the first round and a further seven in the second round. The submissions were mixed in their response to the application. A number expressed support for the application, contingent on a satisfactory safety assessment, whereas other submissions were strongly opposed. The food safety concerns raised in submissions have been addressed in this Final Assessment Report. Where appropriate, reference to other government agencies has been provided in relation to issues beyond the legal responsibilities of FSANZ.

Conclusion/Statement of Reasons

An amendment to the Code to give approval to the sale and use of food derived from MON863 corn in Australia and New Zealand is agreed for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce insect-protected MON863 corn;

- food derived from MON863 corn is equivalent to food from other commercially available corn varieties in terms of its safety for human consumption and nutritional adequacy;

- labelling of certain food fractions derived from MON863 corn will be required if novel DNA and/or protein is present in the final food;

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

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

The variation to the Code will come into effect on the date of gazettal.

1. Introduction

An application was received from Monsanto Australia Limited on 4 December 2002 seeking approval for food derived from insect-protected corn event MON863 (referred to herein as MON863 corn) under Standard 1.5.2 - Food Produced Using Gene Technology.

The genetic modification involved the transfer of the following bacterial genes:

- the *cry3Bb1* gene from *Bacillus thuringiensis* subspecies *kumamotoensis*, which expresses an insect-specific protein toxin called Cry3Bb1 (a *Bt*-toxin); and

- the *nptII* gene from *Escherichia coli*, expressing the enzyme neomycin phosphotransferase II (NPTII) which confers resistance to particular aminoglycoside antibiotics.

2. Regulatory Problem

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

Monsanto Australia Limited has developed a new GM variety of insect-protected corn, known as MON863 corn, primarily for agronomic purposes. Before food derived from MON863 corn can enter the food supply in Australia and New Zealand it must first be assessed for safety and an amendment to the Code must be approved by the FSANZ Board, and subsequently be notified to the Australia and New Zealand Food Regulation Ministerial Council (ANZFRMC). An amendment to the Cod*e* may only be gazetted, once the Ministerial Council process has been finalised.

Monsanto Australia Limited has therefore applied to have Standard 1.5.2 amended to include food derived from insect-protected MON863 corn.

3. Objective

The objective of this Application was to assess whether it would be appropriate to amend the Code to approve the use of food derived from MON863 corn 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 10 of the FSANZ Act. These are:

- the protection of public health and safety;

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

- the prevention of misleading or deceptive conduct.

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

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

In addressing the issue of approving the sale and use of food derived from MON863 corn, the key objectives were the protection of public health and safety and the provision of adequate information to consumers. In fulfilling these objectives, FSANZ also had regard for the need for standards to be based on risk analysis using the best available scientific evidence and the desirability of an efficient and internationally competitive food industry.

4. Background

MON863 corn has been genetically modified to produce an insecticidal protein that is selectively toxic to certain Coleopteran (beetle) insects in the larval stage. The insecticidal protein (Cry3Bb1) is one from a family of proteins produced by the soil bacterium *Bacillus thuringiensis* (otherwise known as *Bt*). Cry3Bb1 is derived from the subspecies *kumamotoensis*. *Bt* formulations are widely used as bio-pesticides on a variety of cereal and vegetable crops grown either organically or under conventional agricultural conditions. Formulations specifically containing Cry3Bb1 as one of the active ingredients have been in commercial use in the United States since 1995.

The main purpose of the genetic modification is to confer protection against the corn rootworm (*Diabrotica* spp). Corn rootworm larvae damage corn by feeding on the roots, reducing the ability of the plant to absorb water and nutrients from the soil, and causing harvesting difficulties due to plant lodging. According to the applicant, corn varieties containing transformation event MON863 are afforded a level of protection from corn rootworm feeding damage that is comparable or superior to that offered by currently commercially available organophosphate, carbamate and pyrethroid insecticides. The superior performance of corn hybrids containing event MON863 is expected to result in a significant yield benefit of between 1.5 and 4.5%. There is also expected to be associated benefits resulting from the reduced use of chemical insecticides.

MON863 corn is also resistant to neomycin and the related aminoglycoside antibiotics through the expression of the enzyme NPTII. NPTII functions as a dominant selectable marker in the initial laboratory stages of plant cell selection and has no function in the final plant/crop.

It is intended that MON863 corn will be used in conventional breeding programs to produce corn hybrids tolerant to corn rootworm. Corn seed containing event MON863 has been developed for cultivation in the United States and Canada. The applicant has indicated that they do not intend to commercialise corn hybrids containing event MON863 for planting in Australia or New Zealand, as the corn rootworm pest is not present in either country. Food

derived from MON863 corn will therefore be entering the Australian and New Zealand food supply as imported, largely processed, food products only.

Domestic production of corn in Australia and New Zealand is supplemented by the import of a small amount of corn-based products, largely as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand. Such products are processed into breakfast cereals, baking products, extruded confectionery and corn chips. Other corn products such as cornstarch are also imported and used by the food industry for the manufacture of dessert mixes and canned foods.

Corn varieties containing event MON863 have been cleared for food and feed use in the United States, Canada and Japan. An application has also been submitted to the European Union.

5. Relevant Issues

5.1 Safety assessment of food derived from MON863 corn

Food derived from MON863 corn has been evaluated according to the safety assessment guidelines prepared by FSANZ[1]. The safety assessment included the following:

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

The applicant submitted a comprehensive data package in support of their application and provided studies on the molecular characterisation of event MON863, the potential toxicity and allergenicity of Cry3Bb1, compositional analyses of food derived from MON863 corn, and animal feeding studies to demonstrate the nutritional adequacy of the food. In addition to information supplied by the applicant, the evaluation also had regard to other available information and evidence, including from the scientific literature, general technical information, independent scientists, other regulatory agencies and international bodies.

No potential public health and safety concerns were identified in the assessment of food derived from MON863 corn. Therefore, on the basis of all the available evidence, including detailed studies provided by the Applicant, it has been concluded that food derived from MON863 corn is as safe and wholesome as food derived from other corn varieties. The full safety assessment report is at **Attachment 2** to this document.

5.2 Labelling

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

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

The Applicant has indicated that novel DNA is likely to be present in starch, modified starch ingredients, meal/semolina and flour derived from corn hybrids containing event MON863 but is unlikely to be present in refined oils, glucose syrups, high fructose syrups, maltodextrins and dextrose. This suggests that a number of food products containing ingredients derived from MON863 corn will require labelling.

5.3 Issues arising from public submissions

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

Duplication of overseas safety assessments

In the first round of public comment the Australian Food and Grocery Council (AFGC) noted that food derived from MON863 corn has already received approval in the United States and Japan and submitted that it is unfortunate that FSANZ has not negotiated "equivalence agreements" with the US or Japan which would permit FSANZ to accept overseas safety assessments. They added that they considered it unlikely that FSANZ would come to any different conclusions from those of the overseas authorities who have approved the use of food derived from MON863 corn.

Response

Standard 1.5.2 Food Produced using Gene Technology states that FSANZ will assess the safety for human consumption of each food prior to its inclusion in the Table to clause 2 of Standard 1.5.2 and that the safety assessment will be done in accordance with the Authority's approved safety assessment criteria. FSANZ always notes if regulatory approvals exist for a particular food elsewhere, however, it is important for the overall rigour of the safety assessment process that FSANZ undertake its own independent assessment using data and other information that conforms to the requirements in Australia and New Zealand. The fact that two or more regulatory bodies have reached the same or similar conclusions following independent and separately conducted safety assessments can provide a greater level of confidence that the conclusions reached are sound. There is also an expectation by Australian and New Zealand consumers that standards are established on the basis of an independent assessment of data, irrespective of decisions made by overseas regulators.

6. Regulatory Options

Two regulatory options were considered:

1. maintain the *status quo* by not amending the Code to approve the sale of food derived from MON863 corn; and

2. amend the Code to permit the sale and use of food derived from MON863 corn, with or without listing special conditions in the Table to clause 2 of Standard 1.5.2.

[2] www.foodstandards.gov.au/mediareleasespublications/factsheets/factsheets2002/index.cfm

7. Impact Analysis

7.1 Affected Parties

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

The Applicant has indicated that they do not intend to undertake cultivation of MON863 in either Australia or New Zealand, principally because the pest (corn rootworm) is not present in either country therefore neither Option is likely to have an impact (either positive or negative) on primary producers and the environment. If planting in Australia or New Zealand ever became likely, a comprehensive environmental risk analysis would be required by various government agencies such as the Office of the Gene Technology regulator (OGTR), Australian Pesticides and Veterinary Medicines Authority (APVMA) (formerly the National Registration Authority) and Environment Australia (EA), in addition to the Environmental Risk Management Authority (ERMA) and the Ministry of Agriculture and Fisheries (MAF) in New Zealand.

7.2 Impact of regulatory options

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

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

Option 1: Maintain the status quo and not approve food derived from MON863 corn

There may be a cost to consumers in terms of a possible reduction in the availability of certain food products, given that MON863 corn is permitted for use in several other countries that do not require segregation of products according to GM status. Similarly, there may be a cost to consumers associated with the segregation of GM and non-GM corn, where the necessity for segregation may contribute to higher retail prices to consumers in general. There would be no direct impact on those consumers who wish to avoid GM foods, as MON863 corn is not currently permitted in the food supply.

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

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

Option 2: Amend the Code to approve food derived from MON863 corn

Consumers may benefit from lower prices, to the extent that savings from production efficiencies are passed on. There may also be benefits in terms of access to a greater range of products including imported food products containing MON863 corn. There may be a cost to consumers wishing to avoid GM food by a potential restriction of choice of products, or increased prices for non-GM foods however the totality of this impact will be minimal as there are a number of other GM corn varieties already permitted in the food supply.

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

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

Discussion

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

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

The proposed amendment to the Code, giving approval to food derived from MON863 corn, is therefore considered necessary, cost effective and of net benefit to both food producers and consumers.

8. Consultation

8.1 Public submissions

The Initial Assessment of this Application was advertised for public comment between 18 December 2002 and 29 January 2003. A total of four submissions were received during this period and a summary of these is included in **Attachment 3** to this report.

Following the first round of consultation, FSANZ carried out an assessment of the application, including a safety evaluation of the food, taking into account the public comments. Specific issues relating to food derived from MON863 corn were addressed in the

Draft Assessment Report, which was release for public comment between 21 May and 2 July 2003.

In response to the release of the Draft Assessment Report, a further seven submissions were received. These are summarised in **Attachment 3** to this report.

8.2 World Trade Organization (WTO)

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

A notification of the proposed regulatory measures for food derived from MON863 corn was made to the WTO on 20 June 2003. The final date for member nation comments was 25 August 2003. No comments were received.

9. Conclusion and Recommendation

An amendment to the Code to give approval to the sale and use of food derived from MON863 corn in Australia and New Zealand is agreed for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce insect-protected MON863 corn;
- food derived from MON863 corn is equivalent to food from other commercially available corn varieties in terms of its safety for human consumption and nutritional adequacy;
- labelling of certain food fractions derived from MON863 corn will be required if novel DNA and/or protein is present in the final food;
- a regulation impact assessment process has been undertaken that also fulfils the requirement in New Zealand for an assessment of compliance costs. The assessment concluded that the amendment to the Code is necessary, cost effective and of net benefit to both food producers and consumers; and
- the proposed draft amendment to the Code is consistent with the section 10 objectives of the FSANZ Act and the regulatory impact assessment.

The proposed variation to the Code is provided in **Attachment 1**.

10. Implementation and Review

The commencement date of the variation to the Code should be the date of gazettal.

ATTACHMENTS

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

ATTACHMENT 1

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

To commence: on gazettal

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

Food derived from insect-protected corn event MON863

ATTACHMENT 2

SAFETY ASSESSMENT

APPLICATION A484: FOOD DERIVED FROM INSECT-PROTECTED MON863 CORN

SUMMARY AND CONCLUSIONS

Background

Food derived from MON863 corn has been assessed for its safety for human consumption. MON863 corn has been genetically modified for protection against corn rootworm and has been developed for cultivation in the United States and Canada. MON863 corn is not intended for cultivation in either Australia or New Zealand due to the absence of the corn rootworm pest. Food derived from MON863 corn will therefore be entering the Australian and New Zealand food supply as imported, largely processed, food products only.

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

History of Use

Corn (*Zea mays L)*, otherwise known as maize, is the world's third leading cereal crop, behind wheat and rice, and is grown in over 25 countries worldwide. Corn-derived products are routinely used in a large number and diverse range of foods and have a long history of safe use. Products derived from MON863 corn may include flour, breakfast cereals, high fructose corn syrup and other starch products.

Description of the Genetic Modification

MON863 corn was generated through the transfer of the *cry3Bb1* and *nptII* genes to an inbred corn line, A634.

The *cry3Bb1* gene is derived from the soil bacterium *Bacillus thuringiensis* subspecies *kumamotoensis* and encodes the Cry3Bb1 protein, which is selectively toxic to certain Coleopteran insects in the larval stage. The actual *cry3Bb1* gene transferred to MON863 was a variant of the native coding sequence. The variant *cry3Bb1* gene was designed to encode a protein – the Cry3Bb1 variant protein – with enhanced insecticidal activity against corn rootworm. The *nptII* gene is derived from the *Escherichia coli* transposon Tn5 and encodes the enzyme neomycin phosphotransferase II (NPTII), which confers resistance to particular aminoglycoside antibiotics. NPTII is used as a dominant selectable marker in the initial laboratory stages of plant cell selection and does not perform any function in the final plant or crop.

Detailed molecular and genetic analyses of MON863 corn indicate that the transferred genes are stably integrated into the plant genome as single copies at a single insertion site and are stably inherited from one generation to the next.

The assessment also considered the likelihood of transfer of the *nptII* gene from MON863 corn to bacteria in the human digestive tract. Such a transfer was considered an extremely remote possibility because of the number and complexity of steps that would need to take place consecutively. Moreover, in the highly unlikely event that the *nptII* gene were transferred the human health impacts were considered to be negligible because the *nptII* gene is already commonly found in bacteria in the environment as well as inhabiting the human digestive tract and the antibiotics to which it confers resistance have very little, if any, clinical use in Australia and New Zealand.

Characterisation of Novel Protein

MON863 corn expresses two novel proteins – the Cry3Bb1 variant protein and NPTII. The Cry3Bb1 variant protein is virtually identical in amino acid sequence to the native Cry3Bb1 protein with the exception of a small number of amino acid changes that were deliberately introduced to enhance insecticidal activity.

Both novel proteins are expressed at relatively low levels in most tissues of MON863 corn with concentrations ranging from 10-81 μg/g fresh weight for the Cry3Bb1 variant protein and <0.076 (non-detectable)-1.4 μg/g fresh weight for NPTII. NPTII was below the limit of detection in kernels and the mean concentration of the Cry3Bb1 variant protein in kernels was 70 μg/g fresh weight.

The novel proteins were evaluated for their potential toxicity and allergenicity. The safety of NPTII has been assessed on numerous previous occasions and is well documented in the peer reviewed scientific literature. In all instances it has been concluded that NPTII is non-toxic to humans and has limited potential as a food allergen. In addition, protein expression analyses indicate that NPTII is below the limit of detection in kernels from MON863 corn therefore exposure to the protein, through consumption of food derived from MON863 corn, would be minimal.

In considering the potential toxicity and allergenicity of the Cry3Bb1 variant protein it is worth noting that *Bt* formulations containing the Cry3Bb1 protein have been used safely since 1995. An acute toxicity study in mice using the Cry3Bb1 variant protein has confirmed the absence of mammalian toxicity. It has also been shown that processing, involving heat treatment, renders the Cry3Bb1 variant protein non-functional (i.e. unable to exert a toxic effect in insects). Bioinformatic studies have confirmed the absence of any significant amino acid similarity with known protein toxins and allergens and digestibility studies have demonstrated that the Cry3Bb1 variant protein would be rapidly degraded in the stomach following ingestion. Taken together, this indicates there is very limited potential for the Cry3Bb1 variant protein to be either toxic or allergenic to humans.

Comparative Analyses

Compositional analyses were done to establish the nutritional adequacy of MON863 corn, and to compare it to non-transformed control lines and commercial varieties of corn. The

constituents measured were protein, fat, carbohydrate, ash, moisture, fibre, fatty acids, amino acids, vitamins, minerals and the anti-nutrients phytic acid and trypsin inhibitor.

No differences of biological significance were observed between MON863 corn and its non-GM counterpart. Several minor differences in key nutrients and other constituents were noted however the levels observed were within the range of natural variation for commercial corn hybrids and do not indicate an overall pattern of change that would warrant further investigation. On the whole, it was concluded that grain from MON863 corn is equivalent in composition to that of other commercial corn varieties.

Nutritional Impact

The detailed compositional studies are considered adequate to establish the nutritional adequacy of the food and indicate that food derived from MON863 corn is equivalent in composition to food from non-GM corn varieties. The introduction of MON863 corn into the food supply is therefore expected to have minimal nutritional impact. The nutritional adequacy of food derived from MON863 corn was also confirmed using a feed study in rapidly growing broiler chicks, which demonstrated that MON863 corn is equivalent to non-GM corn 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 MON863 corn. On the basis of the data provided in the present application, and other available information, food derived from MON863 corn can be considered as safe and wholesome as food derived from other corn varieties.

1. INTRODUCTION

Monsanto Australia Limited has submitted an application to FSANZ to vary Standard 1.5.2 – Food Produced Using Gene Technology in the *Food Standards Code*, to include food from a new genetically modified (GM) corn. The GM corn is known as MON863 corn.

MON863 corn has been genetically modified for protection against corn rootworm (*Diabrotica* spp). Corn rootworm larvae damage corn by feeding on the roots, reducing the ability of the plant to absorb water and nutrients from the soil, and causing harvesting difficulties due to plant lodging.

Protection against corn rootworm is achieved through expression in the plant of the Cry3Bb1 protein, encoded by the *cry3Bb1* gene from the *kumamotoensis* subspecies of the spore-forming soil bacterium *Bacillus thuringiensis*. Cry3Bb1 is selectively toxic to certain Coleopteran insects, such as the corn rootworm, in the larval stage.

Field research indicates that corn varieties expressing Cry3Bb1 are protected from corn rootworm feeding damage to a degree that is comparable or superior to that offered by currently commercially available organophosphate, carbamate and pyrethroid insecticides.

The genetic modification in MON863 corn also involved the transfer of the *nptII* gene encoding the enzyme neomycin phosphotransferase II (NPTII), which confers resistance against neomycin and other aminoglycoside antibiotics. NPTII is used as a dominant selectable marker in the initial laboratory stages of plant cell selection and does not perform any function in the plant or crop.

MON863 corn will be used in conventional breeding programs to produce corn hybrids tolerant to corn rootworm. Corn seed containing event MON863 has been developed for cultivation in the United States and Canada and is not intended for cultivation in either Australia or New Zealand as the corn rootworm pest is not present in either country. Food derived from MON863 corn will therefore be entering the Australian and New Zealand food supply as imported, largely processed, food products only.

Domestic production of corn in Australia and New Zealand is supplemented by the import of a small amount of corn-based products, largely as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand. Such products are processed into breakfast cereals, baking products, extruded confectionery and corn chips. Other corn products such as cornstarch are also imported and used by the food industry for the manufacture of dessert mixes and canned foods.

Corn varieties containing event MON863 have been cleared for food and feed use in the United States, Canada and Japan. An application has also been submitted to the European Union. Corn seed containing event MON863 will be marketed in the United States and Canada under the brand name *YieldGard® Rootworm Corn Seed*.

2. HISTORY OF USE

2.1 Donor Organisms

Bacillus thuringiensis

The source of the *cry3Bb1* gene is the bacterium *B. thuringiensis* subsp. *kumamotoensis*. *B. thuringiensis* is a member of the genus *Bacillus*, a diverse group of gram-positive, rod-shaped, aerobic or facultative anaerobic, spore-forming bacteria consisting of more than 20 species. The species *thuringiensis* is characterised by the production of one or more parasporal protein crystals in parallel with spore formation. The protein crystals are delta-endotoxins that are generally toxic to a variety of insects.

The delta-endotoxins are commonly referred to as *Bt* proteins or Cry proteins, and are encoded by the *cry* genes. Over 100 *cry* genes have now been cloned and sequenced (Nester et al 2002). The *cry* genes are carried on transferable genetic elements (plasmids), which can be readily moved from one isolate to another, regardless of which subspecies they belong to.

More than 60 serotypes and hundreds of different subspecies of *B. thuringiensis* have been described. Several of these subspecies have been extensively studied and commercially exploited as the active ingredients in a number of different insecticide products for use on agricultural crops, harvested crops in storage, ornamentals, bodies of water and in home gardens. The majority of described *B. thuringiensis* strains have insecticidal activity predominantly against Lepidopteran insects (moths and butterflies) although a few have activity against Dipteran (mosquitoes and flies), Coleopteran (beetles), and Hemipteran (bugs, leafhoppers etc) insects. Other Cry proteins with toxicity against nematodes, protozoans, flatworms and mites have also been reported (Feitelson et al 1992, Feitelson 1993). The subspecies that served as the source of the *cry3Bb1* gene expressed in MON863 corn is selectively active against the larvae of certain Coleopteran insects, such as corn rootworm larvae.

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

More specifically, the Cry3 class of proteins, to which Cry3Bb1 belongs, have been registered for use in the United States and other countries for a number of years and formulations containing Cry3Bb1 as one of the active ingredients have been in commercial use in the United States since 1995 (Baum et al 1996).

Escherichia coli

The source of the *nptII* gene is the transposon Tn5 from the bacterium *Escherichia coli*. *E. coli* belongs to the Enterobacteriaceae, a relatively homogeneous group of rod-shaped, Gram-negative, facultative aerobic bacteria.

Members of the genus *Escherichia* are ubiquitous in the environment and found in the digestive tracts of vertebrates, including humans. The vast majority of *E. coli* strains are harmless to humans, although some strains can cause diarrhoea in travellers and *E. coli* is also the most common cause of urinary tract infections. More recently, a particularly virulent strain of *E. coli*, belonging to the enterohaemorrhagic *E. coli* group, known as 0157:H7, has come to prominence as a food-borne pathogen responsible for causing serious illness. This particular group of pathogenic *E. coli* are however distinct from the strains of *E. coli* (the K-12 strains) that are used routinely in laboratory manipulations and which were used as the source of the *nptII* gene. The K-12 strains of *E. coli* have a long history of safe use and are commonly used as protein production systems in many commercial applications (Bogosian and Kane 1991).

2.2 Host Organism

Corn (*Zea mays L)*, otherwise known as maize, is the world's third leading cereal crop, behind wheat and rice, and is grown in over 25 countries worldwide (OECD 2002). Worldwide production of maize is 500 million tons a year, with the United States and China being the major producers.

The majority of grain and forage derived from maize is used as animal feed, however maize also has a long history of safe use as food for human consumption. The grain is also processed into industrial products such as ethyl alcohol (by fermentation), and highly refined starch (by wet-milling) to produce starch and sweetener products. In addition to milling, the maize germ can be processed to obtain corn oil and numerous other more minor products (White and Pollak 1995).

Corn plants usually reproduce sexually by wind-pollination. This provides for natural out-crossing between plants, but it also presents an opportunity for plant breeders to produce hybrid seed by controlling the pollination process. Open pollination of hybrids in the field leads to the production of grain with properties derived from different lines and, if planted, would produce lower yields (Canadian Food Inspection Agency 1994). Instead, by controlling the cross-pollination of inbred lines from chosen genetic pools (using conventional techniques), the combining of desired genetic traits into a controlled hybrid line results in improved agronomic performance and increased yields. This inbred-hybrid concept and resulting yield response is the basis of the modern seed industry in several food commodities including corn.

The commercial production of corn has seen many improvements, particularly since the 1920's when corn varieties were developed by conventional breeding between progeny of two inbred lines to give hybrid varieties that were known to be superior to open-pollinated varieties in terms of their agronomic characteristics. In present agricultural systems, hybrid corn varieties are used in most developed countries for consistency of performance and production.

The corn germplasm that was used to generate event MON863 is a publicly available inbred line of corn, A634. This inbred line responds well to the particular method of gene transfer used (particle bombardment) and tissue culture regeneration. Inbred A634 was released in 1965 by the University of Minnesota and in the 1980's was among the five most popular public inbred lines used in hybrid corn production in the United States. It has also been widely used to develop new inbred lines by conventional breeding methods.

3. DESCRIPTION OF THE GENETIC MODIFICATION

3.1 Method used in the genetic modification

Corn event MON863 was generated by the transformation of corn callus tissue, derived from the inbred corn line A634, using particle acceleration technology. A purified linear DNA fragment containing the *cry3Bb1* and *nptII* genes, together with essential regulatory elements, was used in the transformation process. The DNA fragment of 4691 base pairs (bp) was isolated from the plasmid vector PV-ZMIR13 by restriction digestion with *Mlu* I and was designated ZMIR13L. This DNA fragment contained only the genes of interest. No additional plasmid DNA was used in the transformation process.

Following transformation, the callus tissue was incubated on tissue culture medium containing 2,4-D, which supports callus growth, and paromomycin, an aminoglycoside antibiotic. Only tissue into which the *nptII* gene had been successfully transferred would be able to survive and grow in the presence of paromomycin. Plants were regenerated from paromomycin-tolerant callus tissue and assayed for the presence of the Cry3Bb1 protein.

3.2 Function and regulation of novel genes

The purified *Mlu* I fragment derived from PV-ZMIR13 used in the transformation to produce MON863 corn is illustrated in Figure 1. The 4691 base pair DNA fragment consists of two adjacent gene cassettes for the expression of the two novel proteins, Cry3Bb1 and NPTII. The genetic elements associated with each of the gene cassettes are described in Table 1.

The cry3Bb1 gene

The bacterial *cry3Bb1* gene is derived from a novel *B. thuringiensis* strain (EG4691) reported to display insecticidal activity against the southern corn rootworm as well as other Coleopteran larvae (Rupar et al 1991). Strain EG4691 belongs to the subspecies *kumamotoensis*. The gene encoding the crystal protein responsible for corn rootworm insecticidal activity was subsequently cloned and sequenced (Donovan et al 1992, GenBank Accession No. M89794). The nucleotide sequence of the native bacterial gene has been modified to optimise its expression in monocotyledonous plants such as corn. To facilitate linkage of the modified *cry3Bb1* gene to a plant-effective promoter, a new restriction site was introduced at the 5' end of the coding sequence. This was accomplished by the insertion of the nucleotides GCC at positions 4, 5 and 6 of the coding sequence and resulted in the introduction of an alanine residue at position 2 of the encoded protein.

The native *cry3Bb1* gene sequence has also been modified to enhance its insecticidal activity against corn rootworm. The Cry3Bb1 variant protein is virtually identical in structure to the native Cry3Bb1 protein with the exception of a small number of strategically placed amino acid substitutions that impact insecticidal activity (English et al 2000). The Cry3Bb1 variant protein expressed in MON863 corn is reported to be approximately eight times more effective at destroying corn rootworm larvae than the native protein.

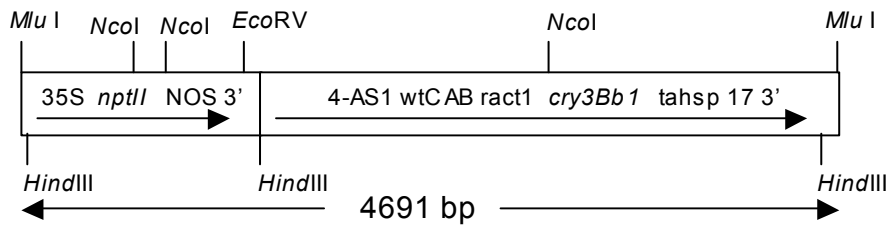

Figure 1: Arrangement of genetic elements in ZMIR13L

Table 1: Genetic elements present in the *Mlu* I restriction fragment ZMIR13L

Genetic Element	Size (kb)	Function
***cry3Bb1* gene cassette:**		
4-AS1	0.22	Promoter for the cry3Bb1 gene in MON863 corn. The promoter consists of four tandem repeats of activating sequence-1 (AS1)(Lam and Chua 1990) and a single portion of the 35S promoter (Odell et al 1985) both derived from cauliflower mosaic virus (CaMV). AS1 is a 21 base pair element associated with the 35S promoter, which has been linked with high levels of protein expression in roots (Lam et al 1989).
wt CAB	0.06	The 5' non-translated leader sequence of the wheat chlorophyll a/b binding protein. This leader sequence facilitates mRNA translation (Lamppa et al 1985).
ract 1 intron	0.49	The first intron from the rice actin 1 gene, which enhances DNA transcription (McElroy et al 1990).
cry3Bb1	1.96	The coding sequence for the Cry3Bb1 variant protein produced in *Bacillus thuringiensis* subsp. *kumamotoensis*.
tahsp 17 3'	0.23	The 3' nontranslated region of the coding sequence for wheat heat shock protein 17.3, which ends transcription and directs polyadenylation (McElwain and Spiker 1989).
Selectable marker:		
35S	0.35	The 35S promoter from CaMV (Odell et al 1985).
nptII	0.97	Coding sequence for gene encoding the enzyme neomycin phosphotransferase II from *Escherichia coli* transposon Tn5 (Beck et al 1982). The DNA derived from *E. coli* also includes a 153 base pair segment of the bleomycin binding protein gene (*ble*). The fragment of *ble* is located 20 base pairs downstream of the *nptII* stop codon.
NOS 3'	0.26	The 3' nontranslated region of the nopaline synthase gene of Agrobacterium tumefaciens T-DNA, which ends transcription and directs mRNA polyadenylation (Bevan et al 1983).

The nptII gene

The *nptII* gene is widely used as a selectable marker in the transformation of plants and is derived from *E. coli* transposon Tn5 (Beck et al 1982). The gene functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation (Horsch *et al* 1984, DeBlock *et al* 1984). It codes for the enzyme neomycin phosphotransferase II (NPTII) and confers resistance to the aminoglycoside antibiotics, including neomycin, paromomycin, kanamycin, and geneticin (G418). The *nptII* gene was transferred along with the *cry3Bb1* gene, enabling those plant cells successfully transformed with the *cry3Bb1* gene to grow in the presence of paromomycin. Those cells that lack the *nptII* gene, and hence the *cry3Bb1* gene, will not grow in the presence of paromomycin.

3.3 Breeding history of MON863 corn

The breeding history of MON863 corn is illustrated in Figure 2 below.

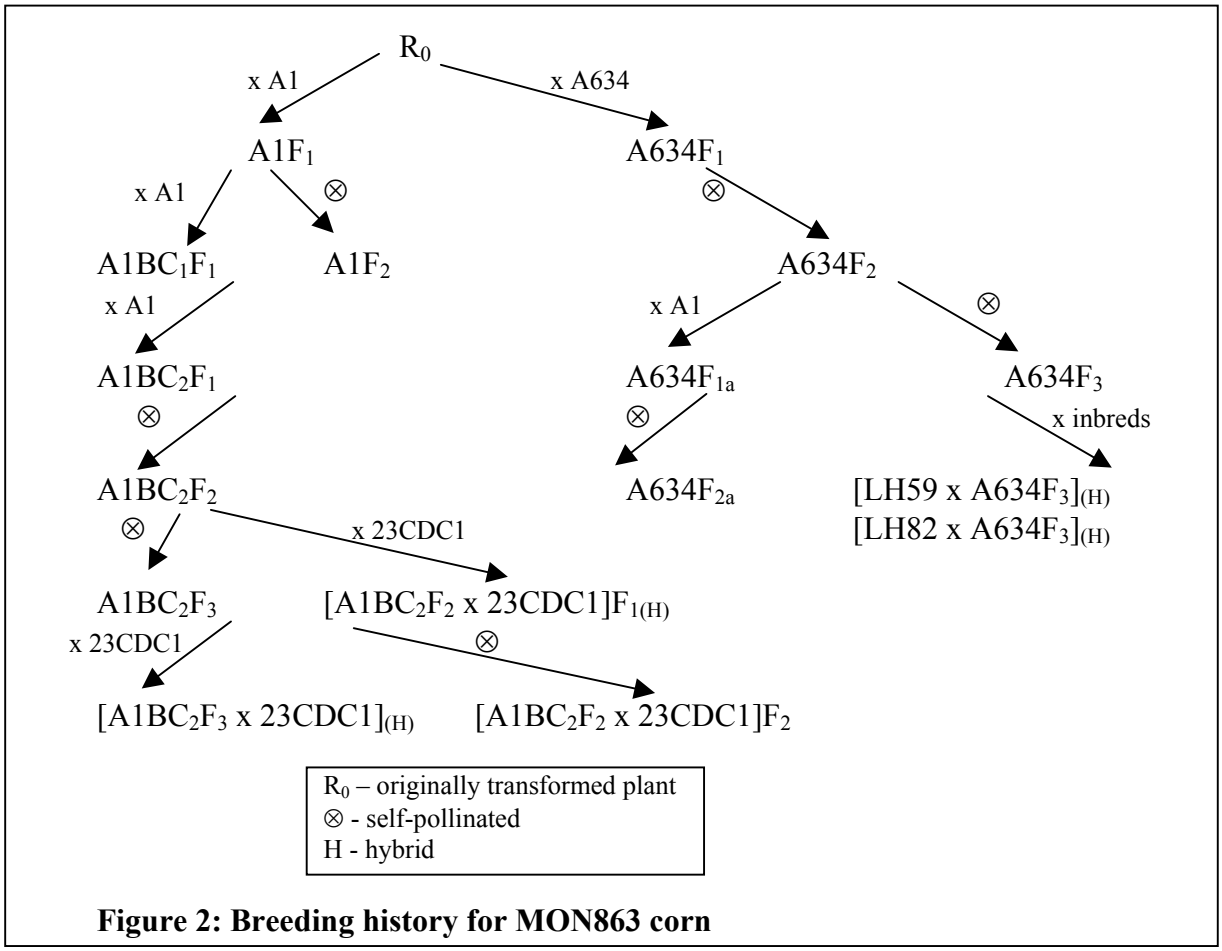

Figure 2: Breeding history for MON863 corn

A1 and A634 are inbred corn lines used for hybrid production.

Segregation analysis was done on generations $A1F_1$, $A1F_2$, $A1BC_1F_1$ and $A1BC_2F_2$. Molecular stability was analysed using generations $A1F_1$, $A1F_2$, $A1BC_1F_1$, $A1BC_2F_2$, $A634F_{2a}$, $A643F_3$ and the hybrids $A1BC_2F_3$x23CDC1 and LH82x$A643F_3$. The molecular characterisation was done using generation $A634F_{2a}$. Gene expression and compositional analyses were done using generations $A634F_{1a}$ and $A634F_{2a}$.

3.4 Characterisation of the genes in the plant

Genomic DNA from corn event MON863 (generation $A634F_{2a}$) was isolated and analysed using Southern hybridisation to determine the number of insertion events, the copy number of the inserted DNA, the integrity of the inserted cassettes and the presence or absence of plasmid backbone sequences. Polymerase chain reaction (PCR) analysis and DNA sequencing were used to further characterise the insert DNA and insert-to-plant junction regions and to confirm the results of the Southern hybridisation. Genomic DNA from non-transformed corn line MON846 (A1 x A634) was used as the control material. The reference material was plasmid PV-ZMIR13 from which the DNA fragment (ZMIR13L) used in the original plant transformation was derived.

DNA extracted from MON863 corn was digested with a variety of restriction enzymes then subjected to Southern hybridisation analysis. In every experiment, control genomic DNA was digested with the same restriction enzymes.

The molecular characterisation also included a determination of whether a second open reading frame (ORF) present in the *nptII* cassette, encoding a portion of the bleomycin binding protein gene (*ble*)(see Table 1), was translated to produce a protein.

Studies submitted:

Cavato, T.A., Rigden, E.C., Mittanck, D.W. and Lirette, R.P. (2001). Amended report for MSL-15505: Molecular analysis of corn event MON863. Monsanto Company, study number 99-01-39-27, MSL-17152.

Cavato, T.A. and Lirette, R.P. (2001) PCR analysis and DNA sequence analysis of the insert in corn rootworm event MON863. Monsanto Company, study number 01-01-39-02, MSL-17108.

Hillyard, J.R., Deng, M.Y., Cavato, T.A. and Lirette, R.P. (2000). Molecular analysis to determine the genetic stability of corn rootworm event MON863 across additional generations. Monsanto Company, study number 00-01-39-28, MSL-17063.

Silvanovich, A., Karunanandaa, K., Thoma, R.S., Blasberg, J. and Astwood, J.D. (2001). The absence of detectable *ble* translation products in corn grain containing event MON863. Monsanto Company, study number 01-01-39-42, MSL-17449.

Hileman, R.E. and Astwood, J.D. (2001). Additional characterisation of the Cry3Bb1 protein produced in corn event MON863. Monsanto Company, MSL-17137.

Insert number

The number of inserts was evaluated by digesting the test and control DNA with the restriction enzyme *Nde* I, which does not cleave within the DNA fragment used for the plant transformation. This enzyme should release a fragment containing the inserted DNA and adjacent plant genomic DNA. The blot, containing the separated DNA fragments, was then probed with ZMIR13L, the linear DNA fragment used for the plant transformation. The number of fragments detected by this probe would therefore indicate the number of inserts that are present in the corn genome.

The analysis produced a single band of approximately 5.0 kilobases (kb) indicating that MON863 contains a single insert.

Copy number

The number of copies at the single insertion site was determined by digesting the genomic DNA with the restriction enzyme *Eco*RV, an enzyme that cuts only once in the linear DNA fragment used to generate the event (see Figure 1). The blot was probed with the entire plasmid from which the DNA used to transform the corn was derived. If the event contains one copy of the transformation cassette, two bands should be produced, representing the two predicted border fragments. Each of these should contain a portion of the transformation cassette and flanking corn sequence.

The analysis produced two bands of approximately 3.7 and 9.6 kb indicating that MON863 corn contains only one copy of the inserted DNA at the locus of integration.

Integrity of inserted DNA

The integrity of the *cry3Bb1* and *nptII* cassettes was determined by digestion with the restriction enzyme *Hind*III, which cleaves at the 5' and 3' ends of each cassette (see Figure 1). Individual Southern blots were probed with the respective promoter fragments, the *cry3Bb1* or *nptII* coding region fragments, or the respective terminator fragments. The presence of a band representing the expected size of the *cry3Bb1* or *nptII* cassette indicates that each cassette and each of its elements are intact.

MON863 DNA probed with the *cry3Bb1* promoter fragment produced a band of approximately 3.2 kb, which is slightly larger than the expected band size. Sequence data from the genomic flanking region indicates that approximately 10 bp, including the *Hind*III site and *Mlu* I site, are missing at the 3' end of the insert. However, there is a *Hind*III site approximately 175 bp downstream from the 3' end of the insert. Therefore, the expected size of the band for the inserted *cry3Bb1* cassette is approximately 3.2 kb. No unexpected bands were detected, indicating that event MON863 does not contain any additional promoter elements, other than those associated with the intact *cry3Bb1* cassette. MON863 DNA probed with the *cry3Bb1* coding region fragment and the *cry3Bb1* terminator fragment both produced a 3.2 kb band, as would be expected from the missing *Hind*III site at the 3' end of the insert, indicating the MON863 does not contain any additional *cry3Bb1* coding or terminator regions. Genomic sequencing of the 3' end of the insert indicates that while the *Hind*III site at the 3' end is missing, the entire tahsp17 3' polyadenylation sequence is present in MON863 corn.

MON863 DNA probed with the promoter, coding region and terminator fragments for *nptII* produced the expected 1.6 kb band on each occasion, which corresponds to the correct size of an intact *nptII* cassette. No unexpected bands were detected, indicating that MON863 does not contain any additional *nptII* cassette sequences.

Analysis for plasmid backbone

The backbone of plasmid PV-ZMIR13 consists of a second *nptII* gene under the control of a bacterial promoter plus the plasmid origin of replication (*ori*). This region was not part of the ZMIR13L fragment used for plant transformation, therefore neither the *nptII* bacterial promoter nor the *ori* region would be expected to be present in MON863 corn. Genomic DNA from MON863 corn was digested with *Hind*III and probed with two PCR-generated probes to confirm the absence of backbone sequences.

One of the probes would detect the bacterial promoter region of the second *nptII* gene in PV-ZMIR13, while the second probe would detect the *ori* region located downstream (3') of the second *nptII* gene.

The analysis of MON863 corn DNA did not produce any hybridising bands with either of the PCR-generated probes, indicating that no plasmid backbone sequences were transferred to the corn during the transformation process.

PCR and sequence analysis

Overlapping PCR products spanning the entire length of the insert DNA in MON863 and the 5' and 3' junction regions with plant genomic DNA were generated and subsequently sequenced

The sequence data confirmed the results of the Southern blot analyses by demonstrating the linkage of the elements contained within the insert, as well as confirming that each genetic element is intact.

This sequence data also showed that there had been two unexpected changes to the inserted DNA. Firstly, the sequencing revealed that 10 bp of DNA from the 3' end of ZMIR13L had been lost during the transformation process, resulting in the deletion of two restriction sites – *Mlu* I and *Hind*III. However, the tahsp17 3' transcription termination sequence at the 3' end of the insert is intact and this small deletion has not affected the overall integrity of the insert DNA. Secondly, the nucleotide sequencing revealed that there had been a single amino acid change to the sequence of the encoded Cry3Bb1 protein. The plant expressed Cry3Bb1 protein – the Cry3Bb1 variant protein – contains an arginine residue at amino acid position 349, instead of a glutamine residue.

Detection of ble translation products

Due to the use of a unique restriction enzyme site for the excision of *nptII* from Tn5, this gene cassette also contains a 153 bp portion of the 378 bp bleomycin binding protein gene (*ble*) (Figure 3). This fragment of *ble* is located 20 bp downstream of the *nptII* stop codon and is joined to the NOS 3' terminator sequence. The mRNA transcribed from the *nptII* cassette in MON863 would therefore contain two tandem ORFs. The proximal ORF is the complete *nptII* coding sequence, while the distal ORF encodes approximately 40% of the bleomycin binding protein (BLE). If translated, this distal ORF would give rise to a 10.25 kDa protein containing amino acids 1 – 51 of the BLE protein linked to four amino acid residues encoded by the DNA polylinker, followed by 34 amino acid residues encoded by the NOS 3' terminator. This *putative* protein has been designated BLE 10.25.

It is expected that the distal ORF of the *nptII* mRNA would not be translated *in planta* because the signals that would be required to facilitate such internal initiation by ribosomes are absent from the DNA sequence. However, protein immunoblotting techniques using rabbit antiserum capable of detecting BLE 10.25 were done to confirm the absence of any *ble* translation products.

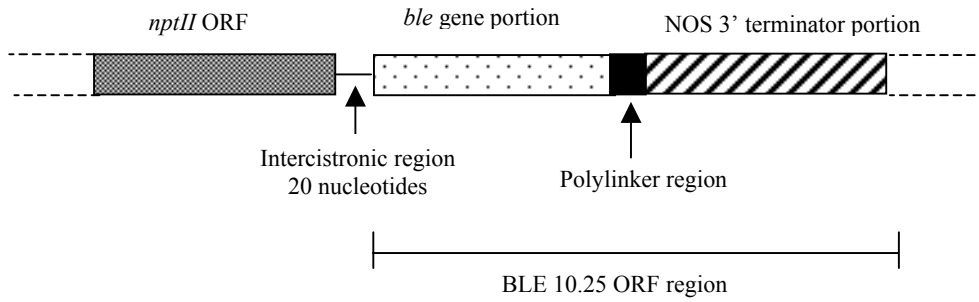

Figure 3: Diagram of putative *ble* ORF

Protein was extracted from grain tissue from MON863 corn and electrophoretically separated on a denaturing polyacrylamide gel, then blotted onto a membrane for probing with BLE 10.25-specific rabbit antibody. *E. coli* produced BLE 10.25, identical to the plant putative BLE 10.25, was used as a positive control. The limit of detection of the immunoblotting technique was determined by spiking known quantities of the reference protein (*E. coli* expressed BLE 10.25) into protein extracts of the test material. The lowest amount of BLE 10.25 that could be detected was 1.7 µg of BLE 10.25 protein per gram fresh weight of corn grain tissue, or 1.7 ppm.

When protein extracted from MON863 corn was probed with BLE 10.25 specific antibody, no visible bands were detected of the expected molecular weight where *E. coli* produced BLE 10.25 was readily detected. These results confirm the absence of any detectable *ble* translation products in MON863 corn above the limit of detection of 1.7 ppm.

Conclusion

Detailed molecular analysis indicates that a single copy of the ZMIR13L fragment, containing the *cry3Bb1* and *nptII* gene cassettes, has been inserted at a single genomic locus in MON863 corn. Both genes are intact, although during the transformation process, a small mutation occurred in the coding sequence of the *cry3Bb1* gene, resulting in a single amino acid substitution in the expressed protein. The plant expressed Cry3Bb1 variant protein will therefore contain an arginine residue at amino acid position 349, instead of a glutamine residue.

3.4 Stability of the genetic changes

A number of analyses were done to demonstrate the stability of the genetic changes in MON863 corn. Southern fingerprint analysis was used to demonstrate the stability of the inserted DNA across three self-pollinated R_0 generations, segregation analysis was used to determine the heritability and stability of the *cry3Bb1* gene across five generations derived from a R_0 x A1 cross (see Figure 2) and Southern fingerprint analysis was used to determine the stability of the inserted DNA across nine generations derived from R_0 x A1 and R_0 x A634 crosses.

Stability of inserted DNA in self-pollinated generations

The restriction enzyme *Nco*I generates a unique Southern hybridisation pattern fingerprint for MON863 corn when probed with the *nptII* coding region (see Figure 1 for position of restriction sites within ZMIR13L). Genomic DNA from the F_2 generation (a self cross of the first R_0 cross), and another F_2 generation (two generations removed from the first F_2 generation), was used in the Southern fingerprint analyses.

Genomic DNA from two different self-crossed generations of MON863 produced the expected fingerprint bands at 0.4 kb and 8.0 kb. The 0.4 kb band represents an internal segment of the insert while the 8.0 kb band represents a border fragment at the 5' end of the insert. No difference in banding pattern between the two different F_2 generations was observed.

Segregation analysis

Chi square analysis of Mendelian inheritance data over five generations was done to determine the heritability and stability of the *cry3Bb1* gene in corn varieties containing event MON863. Genotype frequencies were compared by means of a Chi square test. Expected and observed segregation frequencies of MON863 progeny positive for the corn rootworm protected phenotype are presented in Table 2.

Table 2: Comparison of expected and observed segregation frequencies for MON863 progeny.

	Observed		**Expected**		
Generation	+	-	+	-	χ^2
$A1F_1$	41	36	38.5	38.5	0.21†
$A1F_2$	89	23	84	28	0.96†
$A1BC_1F_1$	18	15	16.5	16.5	0.12†
$A1BC_2F_1$	931	1040	985.5	985.5	5.92*
$A1BC_2F_2$	322	110	324	108	0.03†

† - not significant at $p \leq 0.05$ (Chi square = 3.84, 1 degree of freedom)
* - significant at $p \leq 0.05$ (Chi square = 3.84, 1 degree of freedom)
no significant differences observed at $p \leq 0.01$ (Chi square = 6.63)

With only one exception, χ^2 values were less than the critical value of 3.84, indicating no significant differences between expected and observed frequencies for the corn rootworm protected phenotype across five generations of MON863 corn. The unusual results obtained for the $A1BC_2F_1$ generation are anomalous with the findings for the previous generation ($A1BC_1F_1$) and most importantly are inconsistent with the segregation pattern of the subsequent generation ($A1BC_2F_2$). These results therefore most likely indicate a failure in the detection method being used for the Cry3Bb1 protein, rather than any inherent instability of the *cry3Bb1* gene.

The applicant reports that at the time trials with the $A1BC_2F_1$ generation were being conducted, their field researchers were reporting a 10% failure rate of the ELISA kit being used for the detection of the Cry3Bb1 protein in the field. Apart from this anomalous result for one of the generations, the results of the segregation analysis are consistent with the finding of a single active site of insertion of the *cry3Bb1* gene that segregates according to Mendelian laws of genetics. This stability is demonstrated across three generations of cross fertilisation and two generations of self-pollination.

Stability of inserted DNA in cross-fertilised generations

Genomic DNA from a total of nine different cross-fertilised generations was analysed using Southern fingerprint analysis. The lines from which genomic DNA was extracted were $A1F_1$, $A1F_2$, $A1BC_1F_1$, $A1BC_1F_2$, $A1BC_2F_2$, $A634F_{2a}$ (the line used for the molecular characterisation), $A643F_3$ and the hybrids $A1BC_2F_3$x23CDC1 and LH82x$A643F_3$ (see Figure 2). The control DNA was derived from the non-transformed corn lines A1x23CDC1, A1, A634 and LH82xMON863-/$A634F_3$. Plasmid PV-ZMIR13 spiked into genomic DNA extracted from the non-transformed corn line A1 was used as the reference material. As per the previous Southern fingerprint analysis done for the self-fertilised generations, the extracted genomic DNA was digested with *Nco*I and then probed with the *nptII* coding region DNA.

All corn lines tested, which included the line used in the original molecular characterisation, exhibited the expected hybridising bands of 0.4 and 8.0 kb, following probing with the *nptII* coding region DNA and no differences in banding pattern were observed between any of the lines. This indicates that the inserted DNA is stably inherited across multiple self-pollinated and cross-fertilised generations.

Conclusion

The results of the segregation analysis are consistent with a single site of insertion for the *cry3Bb1* gene and confirm the results of the molecular characterisation. Molecular analysis of both self-pollinated and cross-fertilised lines, representing a total of nine different generations, indicates that the inserted DNA is stably inherited from one generation to the next.

3.5 Antibiotic resistance genes

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes in the laboratory or in the field. It is generally accepted that there are no safety concerns with regard to the presence in the food of the antibiotic resistance gene DNA *per se* (WHO 1993). There have been concerns expressed however that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to microorganisms present in the human digestive tract and that this could compromise the therapeutic use of some antibiotics. This section of the report will therefore concentrate on evaluating the human health impact of the potential transfer of antibiotic resistance genes from corn rootworm-protected MON863 corn to microorganisms present in the human digestive tract.

The genetic modification generating MON863 involved the transfer of the *nptII* gene, which confers resistance to aminoglycoside antibiotics such as kanamycin, neomycin, and geneticin. Neomycin, and related aminoglycoside antibiotics have only a very limited clinical use because of their toxic side effects (WHO 1993) and because resistance to these antibiotics is already quite widespread. These antibiotics have now largely been replaced by more effective aminoglycoside antibiotics, which are not themselves substrates for the NPTII enzyme (Nap et al 1992).

The first issue that must be considered in relation to the presence of the *nptII* gene in MON863 corn is the probability that this gene would be successfully transferred to and expressed in microorganisms present in the human digestive tract. The following steps are necessary for this to occur:

1. a fragment of DNA, containing the coding region of the *nptII* gene, would have to be released, probably as a linear fragment, from the DNA in the GM food;

2. the DNA fragment would then have to survive exposure to various nucleases excreted by the salivary glands, the pancreas and the intestine;

3. the DNA fragment would have to compete for uptake with dietary DNA and would have to be available at a time and place in which competent bacteria develop or reside;

4. the recipient bacteria would have to be competent for transformation;

5. the DNA fragment would have to be stably integrated into the bacterium, either as a self-replicating plasmid or through a rare recombination event with the bacterial chromosome;

6. the *nptII* gene would have to be expressed, that is, would have to be integrated into the bacterial chromosome in close association with a promoter or would need to already be associated with a promoter that will function in the recipient bacterium;

7. the *nptII* gene would have to be stably maintained by the bacterial population.

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

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

In the case of the potential transfer of the *nptII* gene, the human health impacts are considered to be negligible. The *nptII* gene occurs naturally in bacteria inhabiting the human digestive tract therefore the additive effect of an *nptII* gene entering the human gastrointestinal flora from a genetically modified plant would be insignificant compared to the population of kanamycin resistant microorganisms naturally present.

Conclusion

It is extremely unlikely that the *nptII* gene would transfer from MON863 corn to bacteria in the human digestive tract because of the number and complexity of steps that would need to take place consecutively. If transfer of the *nptII* gene did occur, the human health impacts would be negligible because the *nptII* gene is already commonly found in bacteria in the environment, including in the human digestive tract, and the antibiotics to which it confers resistance have virtually no clinical use in Australia and New Zealand.

4. CHARACTERISATION OF NOVEL PROTEIN

4.1 Biochemical function and phenotypic effects

Cry3Bb1

MON863 corn expresses a variant of the native *B. thuringiensis* Cry3Bb1 protein. The native Cry3Bb1 protein is one of a number of different crystal proteins from *B. thuringiensis* that have been identified as having insecticidal activity. The Cry3 class of crystal proteins are toxic to Coleopteran insects (Hofte and Whiteley 1989) and Cry3Bb1 has been identified as having specific activity against corn rootworm larvae (Von Tersch et al 1994). The Cry3Bb1 protein was previously named CryIIIB2 (or Cry3B2) as well as Cry3Bb or CryIIIC. According to the most recent and accepted nomenclature, the protein is now referred to as Cry3Bb1 (Crickmore et al 1998).

A variant of the native *cry3Bb1* coding sequence (Donovan et al 1992, GenBank Accession No. M89794) was designed to encode a protein with enhanced insecticidal activity against corn rootworm (English et al 2000). This *cry3Bb1* coding sequence variant was used to create recombinant *B. thuringiensis* strain EG11098. Expression of the *cry3Bb1* coding sequence variant in *B. thuringiensis* results in the production of a protein, called Cry3Bb1.11098, which contains a total of five amino acid differences from the native Cry3Bb1 protein. The *cry3Bb1* coding sequence was then further manipulated to enhance expression in plants and then placed in a plasmid vector used for the transformation of corn. The resultant transformation event, MON863 corn, produces a protein that differs from the native Cry3Bb1 protein by seven amino acids, and from the Cry3Bb1.11098 protein by two amino acids.

The protein produced in MON863 is referred to as the Cry3Bb1 variant protein and consists of 653 amino acids with a predicted molecular mass of 74 kDa. Of the two additional amino acid changes to the Cry3Bb1.11098 protein, one is due to the presence of a new restriction site at the 5' end of the coding sequence (as described in Section 3.2) and results in a protein that is one amino acid longer than the native Cry3Bb1 protein (652 amino acids). The second amino acid change was unintentional and occurred during the plant transformation process.

The complete amino acid sequence of the Cry3Bb1 variant protein in MON863 has been provided. The amino acid changes, which have been introduced to generate the variant protein, are detailed in Table 3.

Table 3: Summary of amino acid changes to Cry3Bb1

Original amino acid	Position	New amino acid
-	2	Alanine
Aspartic acid	166	Glycine
Histidine	232	Arginine
Serine	312	Leucine
Asparagine	314	Threonine
Glutamic acid	318	Lysine
Glutamine	349	Arginine*

* unintended amino acid change

The mode of action of *Bt* toxins, such as Cry3Bb1, is to function as a midgut toxin in the insect larvae only after the protein has been ingested. The ingested Cry proteins are processed by proteases in the gut of the insect to yield an active core toxin. This activated core protein then binds to specific receptors on the midgut epithelium of the larvae, forming pores which lead to the loss of transmembrane potential, cell lysis, leakage of midgut contents, paralysis, and eventually death of the larvae (Nester et al 2002). Death usually takes hours to days to occur. Insects that develop resistance to the *Bt* toxins most commonly exhibit decreased or altered receptor binding, although altered proteolytic activation has also been reported (Nester et al 2002).

Neomycin phosphotransferase II

Neomycin phosphotransferase II (NPTII) is an enzyme with a molecular weight of 29 kDa and catalyses the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group on the aminohexose moiety of aminoglycoside antibiotics, thereby inactivating them (Davies et al 1986).

Many aminoglycosides are phosphorylated by NPTII but NPTII does not confer resistance to all aminoglycosides because of widely different phosphorylation rates for the different substrates (Redenbaugh et al 1994). NPTII confers resistance to neomycin, kanamycin, geneticin and paromomycin, but not more clinically important aminoglycoside antibiotics such as amikacin and gentamicin B.

4.2 Protein expression analysis

Studies submitted:

Dudin, Y.A., Tonnu, B-P., Albee, L.D. and Lirette, R.P. (2001). Amended report for MSL-16559: *B.t.* Cry3Bb1.11098 and NPTII protein levels in sample tissues collected from corn event MON863 grown in 1999 field trials. Monsanto Company, MSL-17181.

Hileman, R.E., Holleschak, G., Furner, L.A., Thoma, R.S., Brown, C.R. and Astwood, J.D. (2001). Characterisation and equivalence of the Cry3Bb1 protein produced by *E. coli* fermentation and corn event MON863. Monsanto Company, MSL-17274.

Thoma, R.S., Holleschak, G., Hileman, R.E. and Astwood, J.D. (2001). Primary structural protein characterisation of corn event MON863 Cry3bb1.11098 protein using N-terminal sequencing and MALDI time of flight mass spectrometric techniques. Monsanto Company, MSL-17154.

Protein characterisation

The physicochemical and functional properties of the Cry3Bb1 variant protein were characterised using SDS-PAGE analyses, immunoblot analyses, mass spectrometric analysis, N-terminal sequencing, amino acid composition analyses, glycosylation analysis, and insect bioassays. These studies were also used to determine the equivalence of two separate Cry3Bb1 variant protein preparations used for the characterisations. One of the preparations consisted of Cry3Bb1 variant protein purified from a crude protein extract of grain from MON863 corn and the other preparation consisted of Cry3Bb1 variant protein produced using a heterologous *E. coli* protein expression system. The *E. coli*-produced protein is identical in amino acid sequence to the Cry3Bb1 variant protein produced in MON863, including the unintended glutamine to arginine change that occurred during the plant transformation process.

The expression of the Cry3Bb1 variant protein in *E. coli* enables the production of large quantities of protein, whereas it is only possible to purify very small amounts of the Cry3Bb1 variant protein directly from corn tissue.

SDS-PAGE and immunoblot analysis

Densitometric analyses of *E. coli*- and corn-produced proteins, separated using SDS-PAGE, were done to estimate molecular weight and purity of each protein extract. Both protein extracts were subject to immunoblot analyses using both polyclonal and monoclonal antibodies. A molecular mass of 74 kDa was expected for the intact (653 amino acid) protein. Multiple immunoreactive bands were observed with an apparent electrophoretic mobility ranging from 66 kDa to 74 kDa, indicating that both the *E. coli*- and corn-produced proteins were partially degraded.

Mass spectrometric analysis

The identity of the *E. coli*- and corn-produced proteins was also confirmed using Matrix Assisted Laser Desorption Ionisation (MALDI) – Time of Flight (TOF) mass spectrometry techniques and N-terminal sequence analysis. MALDI-TOF is an indirect means of establishing protein identity because it is based on the number of experimentally derived mass fragments (derived from tryptic digestion) matched to computer generated expected mass fragments. The more mass fragments that match, the greater likelihood the correct protein has been identified. A protein can typically be identified from 10-15 mass fragments.

The protein bands identified in the protein extracts as being derived from Cry3Bb1 were excised from the gel and subjected to an in-gel trypsin digest. After digestion, the peptides were extracted from the gel matrix and prepared for mass analysis. In protein purified from MON863, 50 tryptic digest fragments matched expected mass fragments, and in protein purified from *E. coli*, 42 matching fragments were identified. In *E. coli*, a fragment mass of 685.48 Da was observed, which corresponds to the N-terminal fragment of the *E. coli*-produced protein minus the methionine residue. The N-terminal methionine is often processed in proteins (Bradshaw et al 1998). This mass was not observed in the corn protein digest. Instead, a mass of 727.48 Da was observed, which is 42 Da greater than that observed in the *E. coli* digest, and corresponds to an acetylation of the N-terminal alanine residue. Post-translational modifications, such as N-terminal acetylation, are commonly observed in eukaryotic organisms (Tsunasawa and Sakiyama 1984). Masses that were designated as matches were used to build a coverage map for the entire protein. Approximately 69% and 72% of the Cry3Bb1 amino acids were identified for *E. coli* and corn protein extracts, respectively. These data unambiguously identified peptides that included the arginine for glutamine substitution at position 349, as well as both the N- and C- termini. Coverage of >50% is considered sufficient for confirming the identification of a protein.

N-terminal sequencing

N-terminal sequence analysis was then used to further assess and confirm the identity of the proteins expressed on MON863 corn and *E. coli*. Confirmation of identity requires that the observed sequence match the expected sequence. Two sequences, starting at positions 2 and 32, were determined for the *E. coli* produced protein and three sequences, starting at positions 19, 25 and 36, were determined for the corn-produced protein.

These data confirmed the identity of the proteins and also indicated that these proteins had truncated N-termini. The truncated N-termini are most likely the result of partial degradation from exposure to proteases during the protein purification process. The sequencing data also confirmed that the *E. coli*-produced protein was missing the N-terminal methionine. This corroborates the results of the MALDI-TOF mass spectrometry where a peptide mass was observed that corresponded to an N-terminal peptide minus methionine. No corresponding N-terminal sequence was observed from the corn-produced protein. The earliest observed sequence started at position 19. The results of the MALDI-TOF mass spectrometry suggest that the N-terminus of the corn-produced protein is acetylated and would therefore be refractory to N-terminal sequencing.

Amino acid composition

The amino acid composition was determined for the *E. coli* produced protein but could not be determined for the corn-produced protein because the purity of the protein extract was too low (53.9%) to make a meaningful comparison. The observed amino acid composition of the *E. coli*-produced protein was comparable to the theoretical amino acid composition and consistent with the identity of the test substance.

Glycosylation analysis

The amino acid coding sequence of the Cry3Bb1 variant protein expressed by MON863 contains five potential N-glycosylation consensus sites (defined as [Asp-X-Thr/Ser] where X can be any amino acid). No consensus sequence for O-glycosylation has been defined. Because the potential for post-translational glycosylation exists, the *E. coli*- and corn-produced proteins were analysed for covalently N- or O-linked carbohydrate moieties.

No bound carbohydrates were observed for the *E. coli*-produced proteins, as would be expected as prokaryotic organisms rarely glycosylate proteins and are generally thought to lack the cellular machinery to do so. The corn-produced protein yielded multiple reacting bands indicating the detection of bound carbohydrates, however none of these reacting bands appeared in the region corresponding to 66-75 kDa, indicating that the Cry3Bb1 variant protein expressed by MON863 corn is not glycosylated.

Insect bioassays

Functional activity of the *E. coli*- and corn-produced proteins was assessed using Colorado potato beetle larvae fed artificial diets containing varying amounts of the purified proteins. The purity corrected dose concentrations used were approximately 5.73, 2.86, 1.43, 0.72, 0.36 and 0.18 µg/ml for *E. coli*-produced protein and 9.62, 4.81, 2.40, 1.20, 0.60 and 0.30 µg/ml for the corn-produced protein. Larvae were scored for survival after 7 days. A dose-response was observed for each replicate assay, indicating that both proteins have insecticidal activity. The estimated LC_{50} values (the concentration required to kill 50% of larvae) for the *E. coli*- and corn-produced proteins were 0.76 µg/ml (0.57-0.92 µg/ml) and 0.63 µg/ml (0.48-0.77 µg/ml), respectively. Given the considerable overlap in the results, the two proteins are considered to be functionally equivalent.

Additional characterisation of the Cry3Bb1 variant protein

Initial attempts to obtain primary structural data on the Cry3Bb1 variant protein purified from MON863 corn using N-terminal sequencing techniques failed to produce sequence information corresponding to the N-terminus of the protein. This suggests that the N-terminus of the protein is blocked. Further analyses were therefore done to try and confirm the N-terminal sequence of the Cry3Bb1 variant protein, as expressed in MON863 corn. Cry3Bb1 variant protein was purified directly from MON863 corn tissues using immunoaffinity chromatography and was subjected to both N-terminal protein sequencing and MALDI-TOF mass spectrometry techniques.

Purified protein was initially submitted for N-terminal sequence analysis as four SDS-PAGE lanes of 74 kDa and 66 kDa Coomassie Blue stained bands from an immunoblot. No N-terminal sequence could be discerned from the 74 kDa full length protein sample. N-terminal sequence was however obtained for the 66 kDa band. The data from this band indicated the presence of a ragged N-terminus starting at positions 47, 50 and 61. Based on the sequence data, it could be established that the 66 kDa band contained a mixture of truncated Cry3Bb1 variant proteins.

MALDI-TOF mass spectrometry was then used to try and confirm the identity of the full length Cry3Bb1 variant protein. Both the 74 kDa and 66 kDa proteins were digested with trypsin, as in the previous MALDI-TOF mass spectrometry analysis described. The mass spectrum for the 74 kDa trypsinised mixture produced 34 masses. An additional 3 masses were observed using a different mode on the spectrometer. Each observed mass was compared to the theoretical trypsin digest map of the Cry3Bb1 variant protein. Less than 1.0 mass unit difference between experimental and predicted digestion products was observed for 24 out of 37 masses. These results strongly suggest the identity of the purified 74 kDa band to be the Cry3Bb1 variant protein. The mass spectral data from the 74 kDa digest resulted in the identification of 34.5% (225 amino acids out of 653) of the Cry3Bb1 variant protein expressed in MON863. This included three fragments from the N-terminal region of the protein. A mass was identified of 727.4 Da, corresponding to the N-terminal sequence of the Cry3Bb1 variant protein having the terminal methionine cleaved and the alanine residue at position 2 acetylated. No other combination of amino acid sequence from the Cry3Bb1 variant protein was found to match this experimental mass.

The mass spectral data from the 66 kDa digest was nearly identical to that from the 74 kDa digest. This represents supporting evidence that the 66 kDa polypeptide is derived from the full length 74 kDa Cry3Bb1 variant protein. The only observed differences were three missing masses of 727.4, 829.4 and 3445.7 Da. All three masses corresponded to the N-terminal region of the Cry3Bb1 variant protein, at positions 2-7, 8-14 and 15-44, respectively.

Protein expression levels

Validated ELISA methods were used to estimate the levels of Cry3Bb1 variant and NPTII proteins in tissues from MON863 corn and non-transformed corn.

MON863 seed from generation $A634F_{1a}$ was planted to produce tissues for the analysis. Tissue samples were collected from plants grown in four field trials conducted in the United States during the 1999 growing season and three additional sites in Argentina were used for harvesting of pollen during the winter of 2000.

Collectively, these sites provided a variety of environmental conditions representative of regions where corn rootworm protected corn lines would be grown as commercial products. Tissue samples from non-transformed plants of comparable genetic background to MON863 (MON846) were used as controls and were analysed for the presence of both proteins. Both MON863 and MON846 were planted in four replicate plots at each location.

Composite samples of young leaf (V4 stage), forage, mature root and grain were collected from each replicate at the four US sites. Only one replicate from each site was analysed. At three of the US sites, single plot composite samples of leaf, whole plant and root were collected throughout the growing season and evaluated. A composite sample of silk was evaluated from one US site. Composite samples of pollen were evaluated from one US site and from twelve plots planted at three sites in Argentina. Cry3Bb1 variant protein levels were measured in all tissues. NPTII protein levels were evaluated only in samples of young leaf, forage and grain taken from all four sites. The identity of MON863 tissue samples collected from all sites was confirmed throughout various stages of the study by an event-specific PCR assay. Molecular analysis also confirmed the absence of *cry3Bb1* and *nptII* coding sequences from the control plants. The results of the analyses are presented in Tables 4 and 5. Cry3Bb1 variant and NPTII protein levels in control tissues were below the limit of detection and are not reported.

The mean concentrations of Cry3Bb1 variant protein in MON863 corn were highest in leaf (81 µg/g), followed by grain (70 µg/g), pollen (62 µg/g), root (41 µg/g), forage (39 µg/g) and silk (10 µg/g). Mean levels of Cry3Bb1 variant protein declined during the growing season in leaf tissue, whole plant and root tissue. Mean levels in root tissue ranged from a high of 58 µg/g in young plants to a low of 24 µg/g in senescent plants. The Cry3Bb1 variant protein levels in root tissue were sufficient to confer protection from corn rootworm larvae feeding damage during the critical early periods of plant development.

NPTII protein levels in all tissues tested ranged from non-detectable (<0.076 µg/g) to 1.4 µg/g, with the levels in grain being below the limit of detection in all samples analysed.

Table 4: Summary of Cry3Bb1 variant and NPTII protein levels measured in MON863 tissue samples collected from multiple field sites

Tissue (days post-planting)	Parameter	Cry3Bb1 variant protein † (µg/g fresh weight)	NPTII † (µg/g fresh weight)
Young leaf [1] (21 days)	Mean ± SD Range (n)	81 ± 11 65 – 93 (4)	0.98 ± 0.27 0.74 – 1.4 (4)
Forage [2] (90 days)	Mean ± SD Range (n)	39 ± 10 24 – 45 (4)	0.19 ± 0.03 0.17 – 0.23 (4)
Mature root [2] (90 days)	Mean ± SD Range (n)	41 ± 13 25 – 45 (4)	Not analysed
Grain [3] (125 days)	Mean ± SD Range (n)	70 ± 17 49 – 86 (4)	< 0.076 n/a (4)
Silk [4] (58 days)	Mean ± SD Range (n)	10 n/a (1)	Not analysed
Pollen [5] (60 days)	Mean ± SD Range (n)	62 ± 18 30 – 93 (13)	Not analysed

[1] Samples were a pool of tissues ranging from 37 to 50 plants collected from each site at approximately V-4 stage.
[2] Forage (above ground portion only) and mature root were a composite of two plants collected from each site at early dent stage.
[3] Process grain samples were composited from 28-41 corn ears collected from each site at plant maturity and dried to about 15% moisture content (n=4).
[4] Silk was composited (n=1) from five plants at about 50% pollen shed from one field site.
[5] In the US, one sample of pollen tissue was composited over a period of 7 days (about 60 days post planting or about 50% pollen shed). Samples of pollen from Argentina were composited as four replicates per site (three sites total) and collected about 65 days post-planting over about 5 days (n=13).
[†] Limit of detection for NPTII in corn grain = 0.076 µg/g fresh weight and for the Cry3Bb1 variant protein ranges from 0.08 µg/g in silk to 0.76 µg/g in root tissues.

Table 5: Cry3Bb1 variant protein levels in MON863 over the growth of the plant

Days Post-planting	Parameter	Leaf (µg/g fresh weight)	Whole plant [1] (µg/g fresh weight)	Root (µg/g fresh weight)
21 days	Mean ± SD Range (n)	81 ± 14 65 93 (3)	Not collected	Not collected
35 days	Mean ± SD Range (n)	79 ± 6.4 72 – 84 (3)	46 ± 7.8 38 – 54 (3)	58 ± 10 46 – 66 (3)
49 days	Mean ± SD Range (n)	43 ± 18 30 – 56 (2)	31 ± 3.3 28 – 33 (2)	57 ± 3.8 54 – 59 (2)
90 days	Mean ± SD Range (n)	Not collected	37 ± 12 24 – 45 (3)	37 ± 11 25 – 47 (3)
126 days	Mean ± SD Range (n)	Not collected	25 ± 11 13 – 35 (3)	24 ± 18 3.2 – 36 (3)

[1] Only the above ground portion of the plant was included in the sample

4.3 Potential toxicity of novel proteins

Cry3Bb1 variant protein

Studies submitted:
Hileman, R.E., Holleschak, G., Furner, L.A., Thoma, R.S., Brown, C.R. and Astwood, J.D. (2001). Characterisation and equivalence of the Cry3Bb1 protein produced by E. coli fermentation and corn event MON863. Monsanto Company, MSL-17274.

Bonnette, K.L. and Pyla, P.D. (2001). An acute oral toxicity study in mice with *E. coli* produced Cry3Bb1.11098(Q349R) protein. Monsanto Company, MSL-17382.

Hileman, R.E., Rice, E.A., Goodman, R.E., Astwood, J.D. (2001). Bioinformatics evaluation of the Cry3Bb1 protein produced in corn event MON863 utilising allergen, toxin and public domain databases.

History of use

Formulations of *B. thuringiensis*, expressing a number of different Cry proteins, have been used safely and effectively over the last 40 years for the control of a wide variety of insect pests. The Cry3 class of proteins, to which Cry3Bb1 belongs, have been registered for use in the United States and other countries for a number of years and formulations containing Cry3Bb1 as one of the active ingredients have been in commercial use in the United States since 1995 (Baum et al 1996). The deduced amino acid sequence of the Cry3Bb1 variant protein expressed in MON863 corn is >98.9% identical to the Cry3Bb1 protein contained in

the commercialised biopesticide product.

In addition, the Cry3Bb1 protein also shares approximately 67% amino acid identity to Cry3Aa4, which provides control of the Colorado potato beetle and has been used commercially in various insecticidal sprays since 1989.

Specificity

The Cry proteins are a highly specific group of toxins. Their toxicity towards Lepidopteran, Dipteran and Coleopteran insect larvae is well documented. A critical step in the mechanism of action of the Cry proteins is their binding to specific receptors in the target organism (Wolfersberger 1990, Ferré et al 1991). Without such receptor binding, no toxic effect can be exerted. No receptors for the Cry proteins have been identified on the intestinal cells of mammalian species to date (Noteborn et al 1993), which explains the absence of similar toxic effects in other species.

Acute oral toxicity study

An acute oral toxicity study using CD-1 mice was conducted to examine the potential toxicity of the Cry3Bb1 variant protein. The scientific basis for using an acute test is that, if toxic, proteins are known to act via acute mechanisms and laboratory animals have been shown to exhibit acute toxic effects from exposure to proteins known to be toxic to humans (Sjoblad *et al* 1992).

It was not possible to isolate sufficient quantities of pure Cry3Bb1 variant protein from MON863 corn for use as the test material in the toxicity study therefore the test material had to be produced using a heterologous *E. coli* fermentation system. The identical *cry3Bb1* variant coding sequence, as present in MON863 corn, was cloned into the protein expression vector pET24d(+)/25097 then introduced into *E. coli* for large-scale protein production. The equivalence of the *E. coli*- and MON863 corn-produced proteins was established using a range of methods, including MALDI-TOF mass spectrometry, N-terminal sequencing, immunoblotting, insect bioassay, and glycosylation analysis (see Section 4.2).

The only biochemical difference between the two proteins is the acetylation of the alanine residue at position 2 of the MON863 corn-produced protein. This sort of post-translational modification does not occur in prokaryotes, therefore is absent in the *E. coli*-produced protein. This difference is not predicted to affect the outcome of the toxicity studies, as the N-terminal portion of the Cry3Bb1 variant protein has been shown to be relatively sensitive to degradation by proteases. Hence, once the MON863 plant tissue is disrupted, the Cry3Bb1 variant protein would become N-terminally truncated and would no longer retain the acetylated alanine residue. These studies have therefore established the physicochemical and biological equivalence of the *E. coli*- and MON863 corn-produced proteins and support the use of *E. coli*-produced protein as a surrogate for corn-expressed Cry3Bb1 variant protein.

E. coli-produced Cry3Bb1 variant protein was administered by gavage to CD-1 mice (10/sex/group) as two separate oral doses administered approximately 4 hours apart. The dose levels used were 300, 900 and 2700 mg/kg body weight (bw). The vehicle control consisted of phosphate buffer and the protein control was bovine serum albumin dissolved in phosphate buffer. The protein control was administered at the dose of 2700 mg/kg bw. Following dosing, the animals were observed daily for 14 days for any clinical signs or

mortality. Animals were weighed at the beginning of the study and then weekly thereafter.

On day 14 of the study, the animals were killed and examined for gross necropsy and any abnormalities recorded.

No animal deaths occurred during the course of the study and no significant clinical observations were noted. No statistical differences were observed in the body weight or body weight gain data. Slight body weight loss was noted for a few animals: one female during day 0 to 7 given the protein control, one male during day 7 to 14 given 2700 mg/kg bw Cry3Bb1 variant protein, one female given 300 mg/kg bw Cry3Bb1 variant protein and one female given 900 mg/kg bw Cry3Bb1 variant protein. These animals however all exceeded their initial body weight by study termination (day 14) and body weight gain was noted for all other animals during the test period. The 300 mg/kg bw dosed males and the 900 mg/kg bw dosed males had a significant increase in food consumption compared to the vehicle control group during the 0 to 7 day food consumption interval. No significant gross internal findings were observed at necropsy on day 14.

In conclusion, no adverse effects were observed in the mice that could be attributed to the Cry3Bb1 variant protein at doses up to 2700 mg/kg bw.

Similarities with known protein toxins

Bioinformatic analyses were done to assess the Cry3Bb1 variant protein for any similarity with known protein toxins. Protein sequence databases were assembled for this purpose and the FASTA[3] sequence alignment tool was used to assess structural similarity. Although the FASTA program directly compares amino acid sequences and thus is mainly used to assess primary protein structure, the alignment data may also be used to infer secondary and tertiary structure of proteins.

The toxin (TOXIN4) sequence database was assembled from public domain databases GenBank[4] and EMBL[5] release 108, PIR[6] release 56, the NRL3D[7] (release 56) of RCSB PDB[8] and SwissProt[9] release 36. The ALLPEPTIDES sequence database was used to represent all currently known publicly available protein sequences and consisted of SwissProt release 38 and GenBank release 116. Although it may have been redundant to search both the TOXIN4 and ALLPEPTIDES databases for potential similarity to protein toxins, the ALLPEPTIDES database search was used to assess for potential similarity to other pharmacologically active proteins, such as prions, which may not have been annotated with the keyword "toxin".

The deduced amino acid sequence from the DNA sequence obtained from the variant *cry3Bb1* gene coding sequence in MON863 corn was compared to the amino acid sequences in the databases using the FASTA sequence alignment tool. The extent of similarity was

[3] Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences.
[4] A public genetic database maintained by the National Centre for Biotechnology Information at the National Institutes of Health, Bethesda, Maryland, USA
[5] A public genetic database maintained by the European Molecular Biology Laboratory at the European Bioinformatics Institute, Hinxton, England.
[6] Protein Information Database.
[7] National Research Laboratory's protein 3-dimensional protein database founded at Brookhaven National Library and maintained by the Research Collaboratory for Structural Bioinformatics (RCSB).
[8] Protein Database.
[9] Translated sequences from the EMBL database.

evaluated by visual inspection of the alignment, the calculated percent identity and *E* score value.

The *E* score (expectation score) reflects the degree of similarity and the value depends on the overall length of the sequence alignment, the quality (percent identity, similarity) of the overlap and the size of the database. A larger *E* score value indicates a lower degree of similarity between the query sequence (the Cry3Bb1 variant protein amino acid sequence) and the sequence from the database.

As expected, the best similarity observed was to the *B. thuringiensis* Cry3Bb1 protein (E score value of 2.7×10^{-228}), with a 99.1% identity being calculated. Inspection of the other sequence alignments between the TOXIN4 database and the Cry3Bb1 variant protein revealed that almost all of the 169 entries were structurally related sequences from the Cry family of proteins. No other significant sequence similarities with known toxins were detected.

Structural similarities between the Cry3Bb1 variant protein and all publicly available proteins were evaluated using the FASTA sequence alignment tool. Once again, the best similarity observed was to the *B. thuringiensis* Cry3Bb1 protein (*E* score value of 5.7×10^{-259}). A further 267 sequence alignments were identified, with 266 of these being structurally related sequences from the Cry family of proteins. The poorest scoring entry (*E* score value of 8.3) corresponded to an uncharacterised protein from *Drosophila melanogaster*.

Heat stability

A study was done to assess the immuno-detectability and bioactivity of the Cry3Bb1 variant protein in grain from MON863 corn following heat treatment similar to that used in the manufacture of corn flakes.

Grain from MON863 corn plants grown in the field and corresponding control lines MON847 and MON846 were ground to a fine powder and baked at 204˚C for 30 minutes to simulate the heat step used in food processing. Unbaked and baked grain samples from MON863 and control lines were extracted with two different buffer solutions – a relatively mild aqueous extraction buffer at physiologic ionic strength and pH, and a denaturing and reducing extraction buffer, and then analysed by immunoblotting and ELISA to determine the immuno-detectability of the Cry3Bb1 variant protein. An insect bioassay, using Colorado potato beetle, was also done with the baked and unbaked samples.

Cry3Bb1 variant protein could not be detected in grain samples following heat treatment using either immunoblot analysis or ELISA. In contrast, the Cry3Bb1 variant protein was readily detected in the unbaked samples using both methods. These results were also reflected in the insect bioassay, where baked samples exhibited a significant reduction in their insecticidal activity, from 93.75-100% mortality with unbaked sample down to 0-6.25% mortality for baked sample.

These results indicate that processing of the grain, involving heat treatment, renders the Cry3Bb1 variant protein non-functional.

Neomycin phosphotransferase II

The potential toxicity of neomycin phosphotransferase II (NPTII) has been investigated previously where acute oral toxicity studies in mice have been evaluated.

The safety of this protein has also been considered on numerous occasions in the peer reviewed scientific literature (Flavell *et al* 1992, Nap *et al* 1992, Fuchs *et al* 1993a, Fuchs *et al* 1993b). In all instances it has been concluded that NPTII is non-toxic to humans. This conclusion also applies to NPTII expressed in MON863 corn, which is identical to the NPTII assessed for toxicity on previous occasions. Furthermore, although NPTII is expressed in MON863 corn, its expression levels are below the limit of detection in grain, the only part of the plant consumed as food by humans.

4.4 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 similarity between the novel protein and that of known allergens, and the structural properties of the novel protein, including susceptibility to degradation in simulated digestion models. Applying such criteria systematically provides reasonable evidence about the potential of the newly introduced proteins to act as an allergen.

Studies submitted:

Holleschak, G., Hileman, R. and Astwood, J.D. (2001). Amended report for MSL-16597: Immuno-detectability of Cry3Bb1.11098 and Cry3Bb1.11231 proteins in the grain of insect protected corn events MON863 and MON853 after heat treatment. Monsanto Company, MSL-17223.

Hileman, R.E., Rice, E.A., Goodman, R.E., Astwood, J.D. (2001). Bioinformatics evaluation of the Cry3Bb1 protein produced in corn event MON863 utilising allergen, toxin and public domain databases.

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Cry3Bb1 variant protein

Source of protein

The Cry3Bb1 variant protein is >98.9% identical to the Cry3Bb1 protein obtained from *B. thuringiensis*. *B. thuringiensis* has been used as the active ingredient in insecticidal sprays for the last 40 years and during that period has not been associated with any reported allergic reactions associated with its use. Humans using the insecticidal sprays have been shown to develop antibodies to the expressed Cry proteins but in no case has the presence of these antibodies been linked with any acute or chronic disease (Nester et al 2002).

Similarity to known allergens

Bioinformatic analyses were done to assess the Cry3Bb1 variant protein for any similarity with known allergens. A protein sequence database (ALLERGEN3) was assembled for this purpose and consisted of known allergen and gliadin amino acid sequences. Gliadins are a specific class of proteins that are suspected of causing gluten-sensitivity enteropathy (celiac disease).

A preliminary list of sequences was compiled using STRINGSEARCH (keyword = allergen). The resulting list was compared to previously compiled allergen and gliadin databases. Sequences present in the previous databases and missing in the STRINGSEARCH list were added to the list. The list was finalised by adding additional allergen sequences identified by: (i) comparison of the list to allergens listed on a publicly available list located on the Internet; and (ii) performing a search of the current literature using the publicly available PubMed and Entrez information retrieval systems. Newly identified allergens were assembled from the same public domain databases used to compile the TOXIN4 sequence database (see Section 4.3). The final database consisted of 659 separate protein sequences.

As with the determination of similarity to known protein toxins, the FASTA sequence alignment tool was used to assess structural similarity. The extent of similarity was evaluated by visual inspection of the alignment, the calculated percent identity and *E* score value. A second bioinformatics tool (IDENTITYSEARCH) was used to identify matches of 8 linearly contiguous amino acid identities between the Cry3Bb1 variant protein sequence and sequences within the ALLERGEN3 database. A sequence length of 8 contiguous amino acids was chosen as a target to identify potential epitopes. IDENTITYSEARCH may be more accurate than FASTA for identification of immunologically relevant epitopes because it only requires that the specified window size (8 amino acid residues) match. While FASTA is a powerful tool for identification of sequence similarity, it is more appropriately used to assess structural similarity.

The strongest similarity observed using the FASTA alignment tool was to the Bermuda grass pollen allergen, Cyn d 1 (23.6% identity, 55 amino acid overlap, *E* score value of 3.8). In this alignment the overlap of 55 amino acids contained two gaps and was relatively short compared to the length of the allergen (>246 amino acids), suggesting that the Cry3Bb1 variant protein does not share homologous structure with Cyn d 1. Inspection of the remaining alignments showed poor E score values and did not suggest homologous structure or function. No immunologically relevant sequences (8 contiguous amino acid identities) were detected when the Cry3Bb1 variant protein sequence was compared to the ALLERGEN3 sequence database. Combined, these data indicate that the Cry3Bb1 variant protein expressed in MON863 corn does not share relevant structural or immunological sequence similarities with either known allergens or gliadins.

In vitro digestibility

Typically, most food allergens tend to be stable to the peptic and acidic conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergic response (Kimber et al 1999; Astwood et al 1996; Metcalfe et al 1996). The Cry3Bb1 variant protein was therefore investigated for its digestibility in simulated digestion models.

Two studies were done – one to investigate the digestibility of the Cry3Bb1 variant protein in

simulated gastric fluid (SGF) and the second to test the digestibility in simulated intestinal fluid (SIF). SGF contains pepsin and SIF contains pancreatin, a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease. Both studies used *E. coli*-produced Cry3Bb1 variant protein, which has been previously characterised and shown to be physicochemically and functionally equivalent to the Cry3Bb1 variant protein expressed in MON863 corn. The SGF study also used Cry3Bb1 variant protein purified directly from the grain fraction of MON863 corn.

In the SGF study digestibility was measured at selected time points (0, 15, 30 seconds and 1, 2, 4, 8, 15, 30 and 60 minutes) using SDS-PAGE and visualised using colloidal blue staining. Both the corn-produced and the *E. coli*-produced Cry3Bb1 variant proteins were rapidly degraded. The corn-produced protein was degraded to a small transient peptide fragment (MW ≈ 3kDa) within 15 seconds. This peptide fragment persisted for a further 15 minutes before it was degraded to a level below the limit of detection (≤17 ng of Cry3Bb1 variant protein per lane). The *E. coli*-produced protein was also degraded to a small transient peptide fragment within 15 seconds and was degraded to a level below the limit of detection (≤10 ng of Cry3Bb1 variant protein per lane) after 2 minutes in SGF. Differences in the rate at which the transient peptide fragment degraded to below the limit of detection were attributed to the impurities (in the form of other proteins) present in the corn-produced protein extract which was significantly less pure (53.9%) than the *E. coli*-produced protein extract (97.5%).

In the SIF study, digestibility was evaluated at selected time points (0, 1, 5, 15, 30 minutes and 1, 2, 4, 8, 16 and 24 hours) using SDS-PAGE and products visualised using immunoblot analysis. The limit of detection for the study was 0.2 ng protein per lane. A total of 10 ng of protein per lane was loaded onto the gel. As expected from studies with other Cry proteins, the *E. coli*-produced Cry3Bb1 variant protein was degraded to a stable digestion product that persisted for at least 24 hours. After one minute of incubation in SIF, the 74kDa Cry3Bb1 variant protein had been degraded to two bands having approximate molecular weights of 68 and 57kDa. These molecular weights are similar to those observed previously during the protein characterisation studies, which were determined to correspond to N-terminally truncated forms of the full-length 74kDa protein. Time points greater than 1 min appeared as a single predominant band of approximately 57kDa. The intensity of this band remained essentially unchanged from the 5 min to 24-hour time points indicating it is stable to digestion by pancreatin. These results are consistent with those obtained for other Cry proteins, which when exposed to trypsin, are degraded to a stable tryptic core fragment. It is this stable core protein, which is the activated toxin in the insect gut.

Neomycin phosphotransferase II

The potential allergenicity of NPTII has been investigated on numerous previous occasions where simulated mammalian digestion studies have been submitted for evaluation as well as studies where its amino acid sequence has been compared with known allergens. None of these has revealed any potential for NPTII to be a food allergen. In addition, the safety of this protein, including its potential allergenicity, has also been considered on numerous occasions in the peer reviewed scientific literature (Flavell *et al* 1992, Nap *et al* 1992, Fuchs *et al* 1993a, Fuchs *et al* 1993b). In all instances it has been concluded that NPTII has limited potential to be a food allergen. This conclusion also applies to NPTII in MON863 corn, which is identical to the NPTII assessed for potential allergenicity on previous occasions. In addition, protein expression analyses indicate that NPTII is below the level of detection in

grain from MON863 corn, indicating the exposure to the protein from the consumption of food derived from MON863 corn would not occur.

4.5 Conclusion

MON863 corn expresses two novel proteins – Cry3Bb1 variant protein and NPTII. Both proteins are expressed at relatively low levels in most tissues of the corn plant with concentrations ranging from 10-81 µg/g fresh weight for the Cry3Bb1 variant protein and <0.076 (non-detectable)-1.4 µg/g fresh weight for NPTII. NPTII was below the limit of detection in kernels and the mean concentration of Cry3Bb1 variant protein in kernels was 70 µg/g fresh weight.

A large number of studies have been done on the Cry3Bb1 variant protein to confirm its identity and physicochemical and functional properties as well as to determine its potential toxicity and allergenicity. These studies have demonstrated that the protein expressed in MON863 corn conforms in size and amino acid sequence to that expected for the Cry3Bb1 variant protein and also exhibits the expected insecticidal activity. In relation to the potential toxicity and allergenicity of the Cry3Bb1 variant protein it is worth noting that *Bt* proteins are inherently non-toxic to mammals and have exhibited little tendency to be allergenic to humans over their long history of use. In addition, *Bt* formulations containing the Cry3Bb1 protein have been used safely since 1996 in the United States and an acute toxicity study using the Cry3Bb1 variant protein has confirmed the absence of toxicity in mice. It has also been shown that processing, involving heat treatment, renders the Cry3Bb1 variant protein non-functional (i.e. unable to exert a toxic effect in insects). Bioinformatic studies have confirmed the absence of any significant amino acid similarity with known protein toxins and allergens and digestibility studies have demonstrated that the Cry3Bb1 variant protein would be rapidly degraded in the stomach following ingestion. Taken together, the evidence indicates there is very limited potential for the Cry3Bb1 variant protein to be either toxic or allergenic to humans.

The safety of NPTII has been assessed on numerous previous occasions and is well documented in the peer reviewed scientific literature. In all instances it has been concluded that NPTII is non-toxic to humans and has limited potential as a food allergen. In addition, protein expression analyses indicate that NPTII is below the level of detection in kernels from MON863 corn, therefore dietary exposure to the NPTII protein is expected to be insignificant.

5. COMPARATIVE ANALYSES

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

components of corn that should be considered in the comparison include protein, fat, carbohydrates, amino acids, fatty acids, vitamins, minerals, and phytic acid (OECD 2002).

Study submitted:
Ridley, W.P., Nemeth, M.A., Astwood, J.D., Breeze, M.L. and Sorbet, R. (2002). Amended report for MSL-17199: Compositional analyses of forage and grain collected from corn rootworm protected maize event MON863 grown in 1998 U.S. field trials. Monsanto Company, MSL-17669.

To determine whether unexpected changes had occurred in the composition of MON863 corn as a result of the modification, and to assess its nutritional adequacy, compositional analyses were done on forage and grain samples collected from MON863 corn, its parental control line and 18 commercial corn hybrids grown under field conditions. Field trials were conducted in the United States in 1999 at four replicated sites. These sites were chosen because they provide a variety of environmental conditions representative of where MON863 corn would be grown commercially. MON863 corn and its parental control line MON846 corn were planted at all sites. As well, a total of 18 commercial corn hybrids (non-GM) were used as reference lines for this study. At each site, test lines, control lines and reference hybrids were planted as a randomised complete block design with four replications, except for the reference hybrids which had two replications.

Forage was collected from whole plants (above ground parts) at the early dent stage from each of the replications of test lines, control lines and reference hybrids. Ears were hand-harvested from all self-pollinated test, control and reference plants at normal kernel maturity, dried to a moisture level below 15%, shelled and the kernels pooled to provide grain samples. The analyses were done at Covance Laboratories, Inc., Madison, Wisconsin using standard methods. Except for moisture, all component values were converted from a fresh weight to a dry weight basis.

Compositional data was provided to Certus International for statistical analysis. Statistical analyses were conducted using a randomised complete block model analysis of variance for five sets of comparisons for each component in forage and grain: analyses for each of the four replicated trials, and for a combination of all four trials. Compositional data from the commercial reference hybrids were not included in the statistical analysis; however, a range of the reference values was determined for each component measured. Additionally, the commercial reference hybrid data was used to develop population tolerance intervals. A tolerance interval is an interval with a specified degree of confidence, which contains at least a specified proportion of an entire sampled population for the parameter measured. For each component measured, tolerance levels were calculated that are expected to contain, with 95% confidence, 99% of the values expressed in the population of commercial hybrids.

A total of 44 components were analysed in grain and 7 in forage. These were: in grain, proximate content (protein, fat, carbohydrate, ash, moisture), acid detergent fibre (ADF), neutral detergent fibre (NDF)[10], amino acids, fatty acids, vitamin E, minerals (calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium and zinc), phytic acid and trypsin inhibitor and in forage, proximate content, ADF and NDF. Carbohydrate levels in forage and grain were determined by calculation. The results of the combined site comparisons are presented in Tables 6-11. A summary of the statistically significant

[10] ADF and NDF are analyses typically used for the proximate analysis of animal feed and substitute for crude fibre analysis. They give an indication of the digestibility of the feed and are particularly important for forage analysis.

differences between MON863 and the parent control are presented in Table 12. The results from individual trial sites were also evaluated but are not presented in this report.

The results of the compositional analyses showed that the 51 components measured in MON863 corn were within the range observed for commercial corn hybrids planted at the same sites in 1999. Furthermore, all 51 components were within published literature ranges, or historical ranges for non-GM corn varieties. There were no statistically significant differences in 224 of the 255 comparisons made between MON863 corn and the control line, MON846.

Of the 31 comparisons found to be significantly different, about 13 can be attributed to random differences expected in the analysis of plant material. Differences that were not observed consistently across all five comparisons are unlikely to be of biological significance. The magnitude of the differences between MON863 corn and the control line expressed as a percent of the control values ranged from 1.38% - 15.52%. Furthermore, the range of values for those components associated with the small statistical differences were found to all fall within the 95% tolerance interval for commercial varieties planted at the same sites. This demonstrates that the levels of key nutrients and other components for MON863 corn were within the same population as expected for the non-GM commercial hybrids used in this study. Therefore, these minor differences are unlikely to be biologically meaningful, and the grain and forage from MON863 can be considered to be compositionally equivalent to that of non-GM corn.

Conclusion

The comparative analyses do not indicate any compositional differences of biological significance in the grain or forage derived from MON863 corn, compared to the non-GM control. Several minor differences in key nutrients and other constituents were noted, however the levels observed were within the range of natural variation for commercial corn hybrids and do not indicate an overall pattern of change that would warrant further investigation. On the whole, it can be concluded that grain from MON863 corn is equivalent in its composition to that from non-GM corn.

Table 6: Combined site statistical comparison of fibre and proximate content in MON863 corn and control grain

Constituent	MON863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON863 minus Control)			Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
			Mean ± S.E. (Range)	p-value	95% C.I. (Lower, Upper)			
Ash (% DW)	1.35 ± 0.12 (0.84 – 1.71)	1.41 ± 0.12 (0.89 – 1.89)	-0.064 ± 0.047 (-0.45 – 0.31)	0.196	-0.17, 0.037	0.62 – 1.53 (0.26, 2.06)	1.1 – 3.9	1.2 – 1.8
Carbohydrates (% DW)	83.30 ± 0.56 (81.83 – 85.00)	82.76 ± 0.56 (80.70 – 84.80)	0.54 ± 0.27 (-0.78 – 2.43)	0.138	-0.32, 1.40	82.51 – 87.84 (78.97, 90.36)	NA	81.7 – 86.3
ADF (% DW)	4.45 ± 0.15 (3.49 – 5.23)	4.50 ± 0.15 (3.62 – 5.89)	-0.050 ± 0.18 (-1.77 – 1.16)	0.778	-0.43, 0.33	3.65 – 6.09 (1.98, 6.62)	3.3 – 4.3	3.1 – 5.3
NDF (% DW)	11.64 ± 0.54 (9.21 – 13.47)	12.02 ± 0.54 (10.31 – 15.82)	-0.37 ± 0.61 (-4.32 – 2.30)	0.585	-2.33, 1.58	9.50 – 14.95 (6.51, 16.28)	8.3 – 11.9	9.6 – 15.3
Moisture (% FW)	10.03 ± 0.50 (8.54 – 11.20)	10.23 ± 0.50 (8.60 – 11.40)	-0.20 ± 0.13 (-0.90 – 0.26)	0.216	-0.61, 0.21	8.75 – 15.70 (5.09, 18.62)	7 – 23	9.4 – 15.8
Total fat (% DW)	3.77 ± 0.20 (3.00 – 4.56)	3.64 ± 0.20 (3.02 – 4.29)	0.13 ± 0.18 (-0.77 – 1.02)	0.520	-0.44, 0.70	2.18 – 3.86 (1.68, 4.64)	3.1 – 5.7, 2.9 – 6.1	2.4 – 4.2
Protein (% DW)	11.60 ± 0.48 (10.43 – 12.82)	12.19 ± 0.48 (10.45 – 13.80)	-0.59 ± 0.22 (-1.52 – 0.12)	0.071	-1.28, 0.097	7.95 – 13.83 (5.47, 16.57)	6.0 – 12.0, 9.7 – 16.1	9.0 – 13.6

Key:
MON863 and control mean values are for 16 replicates collected from 4 sites
S.E. = standard error of the mean
C.I. = confidence interval
Comm. = commercial; the range of sample values for commercial hybrids grown at the same field sites
T.I. = tolerance interval, specified to contain 95% of the commercial line population
Historical range for control lines refers to data collected Monsanto field trials conducted between 1993 and 1995.

Table 7: Combined site statistical comparison of amino acid levels in MON863 and control grain

Amino Acid (% total)	MON863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON863 minus Control)			Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
			Mean ± S.E. (Range)	p-value	95% C.I. (Lower, Upper)			
Alanine	7.74 ± 0.032 (7.65 – 7.85)	7.79 ± 0.032 (7.46 – 7.98)	-0.045 ± 0.031 (-0.23 – 0.24)	0.247	-0.14, 0.055	7.30 – 8.06 (6.94, 8.46)	6.4 – 9.9	7.2 – 8.8
Arginine	4.43 ± 0.062 (4.21 – 4.68)	4.33 ± 0.062 (4.09 – 4.63)	0.10 ± 0.044 (-0.16 – 0.51)	0.030	-0.0099, 0.19	3.86 – 4.83 (3.38, 5.22)	2.9 – 5.9	3.5 – 5.0
Aspartic acid	6.51 ± 0.053 (6.38 – 6.72)	6.45 ± 0.053 (6.30 – 6.67)	0.061 ± 0.021 (-0.11 – 0.23)	0.064	-0.0070, 0.13	6.05 – 7.14 (5.54, 7.65)	5.8 – 7.2	6.3 – 7.5
Cystine	2.20 ± 0.027 (1.98 – 2.40)	2.09 ± 0.027 (1.99 – 2.29)	0.11 ± 0.029 (-0.15 – 0.39)	<0.001	0.054, 0.17	1.84 – 2.35 (1.59, 2.65)	1.2 – 1.6	1.8 – 2.7
Glutamic acid	19.39 ± 0.16 (18.99 – 19.91)	19.56 ± 0.16 (18.97 – 20.26)	-0.17 ± 0.090 (-0.76 – 0.24)	0.157	-0.46, 0.12	18.31 ± 20.25 (17.55, 21.25)	12.4 – 19.6	18.6 – 22.8
Glycine	3.60 ± 0.048 (3.45 – 3.74)	3.53 ± 0.048 (3.32 – 3.72)	0.072 ± 0.030 (-0.075 – 0.31)	0.100	-0.025, 0.17	3.20 ± 4.13 (2.81, 4.46)	2.6 – 4.7	3.2 – 4.2
Histidine	2.84 ± 0.032 (2.70 – 2.95)	2.83 ± 0.032 (2.72 – 2.94)	0.011 ± 0.023 (-0.082 – 0.24)	0.665	-0.063, 0.085	2.60 – 3.20 (2.37, 3.35)	2.0 – 2.8	2.8 – 3.4
Isoleucine	3.67 ± 0.033 (3.45 – 3.89)	3.74 ± 0.033 (3.61 – 3.87)	-0.064 ± 0.033 (-0.33 – 0.15)	0.072	-0.13, 0.0065	3.47 – 3.94 (3.20, 4.17)	2.6 – 4.0	3.2 – 4.3
Leucine	13.36 ± 0.081 (12.88 – 13.65)	13.65 ± 0.081 (13.27 – 14.17)	-0.29 ± 0.084 (-0.75 – 0.13)	0.039	-0.56, -0.026	11.94 – 14.47 (11.30, 15.63)	7.8 – 15.2	12.0 – 15.8
Lysine	2.92 ± 0.061 (2.65 – 3.26)	2.88 ± 0.061 (2.67 – 3.08)	0.042 ± 0.036 (-0.19 – 0.32)	0.328	-0.073, 0.16	2.40 – 3.52 (1.87, 3.89)	2.0 – 3.8	2.6 – 3.5
Methionine	2.28 ± 0.060 (1.89 – 2.49)	2.24 ± 0.060 (1.96 – 2.58)	0.034 ± 0.035 (-0.20 – 0.25)	0.348	-0.040, 0.11	1.61 – 2.29 (1.34, 2.74)	1.0 – 2.1	1.3 – 2.6

Phenylalanine	4.99 ± 0.015 (4.93 – 5.06)	5.04 ± 0.015 (4.95 – 5.23)	-0.048 ± 0.017 (-0.17 – 0.041)	0.052	-0.096, 0.0010	4.80 – 5.35 (4.53, 5.66)	2.9 – 5.7	4.9 – 6.1
Proline	8.73 ± 0.054 (8.30 – 9.21)	8.78 ± 0.054 (8.60 – 9.05)	-0.052 ± 0.046 (-0.32 – 0.38)	0.267	-0.15, 0.045	8.57 – 9.61 (8.04, 10.35)	6.6 – 10.3	8.7 – 10.1
Serine	4.70 ± 0.11 (3.93 – 5.09)	4.67 ± 0.11 (4.20 – 4.94)	0.031 ± 0.094 (-0.77 – 0.89)	0.743	-0.17, 0.23	4.24 – 4.99 (3.76, 5.69)	4.2 – 5.5	4.9 – 6.0
Threonine	3.41 ± 0.035 (3.16 – 3.60)	3.36 ± 0.035 (3.16 – 3.49)	0.049 ± 0.024 (-0.15 – 0.23)	0.056	-0.0016, 0.099	3.19 – 3.59 (2.93, 3.83)	2.9 – 3.9	3.3 – 4.2
Tryptophan	0.66 ± 0.015 (0.60 – 0.83)	0.65 ± 0.015 (0.60 – 0.68)	0.013 ± 0.012 (-0.043 – 0.17)	0.295	-0.013, 0.039	0.54 – 0.82 (0.37, 0.90)	0.5 – 1.2	0.4 – 1.0
Tyrosine	3.63 ± 0.057 (3.33 – 3.77)	3.48 ± 0.057 (2.71 – 3.82)	0.15 ± 0.078 (-0.14 – 0.92)	0.073	-0.016, 0.32	2.60 – 3.73 (2.15, 4.65)	2.9 – 4.7	3.7 – 4.3
Valine	4.94 ± 0.043 (4.71 – 5.13)	4.94 ± 0.043 (4.64 – 5.12)	-0.0091 ± 0.043 (-0.36 – 0.50)	0.833	-0.097, 0.079	4.49 – 5.30 (4.15, 5.63)	2.1 – 5.2	4.2 – 5.3

Key:
MON863 and control mean values are for 16 replicates collected from 4 sites
S.E. = standard error of the mean
C.I. = confidence interval
Comm. = commercial; the range of sample values for commercial hybrids grown at the same field sites
T.I. = tolerance interval, specified to contain 95% of the commercial line population
Historical range for control lines refers to data collected Monsanto field trials conducted between 1993 and 1995.

Table 8 Combined site statistical comparison of fatty acid levels in MON863 and control grain

Fatty Acid (% total)	MON863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON863 minus Control)			Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
			Mean ± S.E. (Range)	p-value	95% C.I. (Lower, Upper)			
16:0 palmitic	12.01 ± 0.11 (11.61 – 12.56)	11.88 ± 0.11 (11.66 – 12.20)	0.12 ± 0.11 (-0.21 – 0.79)	0.337	-0.22, 0.47	9.07 – 12.14 (7.74, 13.87)	7 – 19	9.9 – 12.0
18:0 stearic	1.66 ± 0.083 (1.40 – 1.86)	1.66 ± 0.083 (1.33 – 1.81)	0.0044 ± 0.013 (-0.087 – 0.078)	0.738	-0.023, 0.032	1.44 – 2.40 (1.04, 2.68)	1 – 3	1.4 – 2.2
18:1 oleic	22.00 ± 0.36 (20.97 – 23.55)	21.87 ± 0.36 (21.00 – 22.53)	0.13 ± 0.12 (-0.16 – 1.05)	0.365	-0.26, 0.52	21.26 – 32.06 (13.28, 36.31)	20 – 46	20.6 – 27.5
18:2 linoleic	62.23 ± 0.38 (60.02 – 63.21)	62.47 ± 0.38 (61.55 – 63.60)	-0.23 ± 0.18 (-1.83 – 0.32)	0.293	-0.81, 0.35	54.15 – 63.64 (50.21, 70.86)	35 – 70	55.9 – 66.1
18:3 linolenic	1.20 ± 0.020 (1.13 – 1.29)	1.24 ± 0.020 (1.09 – 1.45)	-0.037 ± 0.021 (-0.30 – 0.071)	0.079	-0.080, 0.0047	0.97 – 1.36 (0.75, 1.51)	0.8 – 2	0.8 – 1.1
20:0 arachidic	0.41 ± 0.0068 (0.39 – 0.44)	0.40 ± 0.0068 (0.39 – 0.42)	0.0052 ± 0.0062 (-0.017 – 0.027)	0.460	-0.014, 0.025	0.35 – 0.45 (0.30, 0.51)	0.1 – 2	0.3 – 0.5
20:1 eicosenoic	0.30 ± 0.011 (0.28 – 0.35)	0.30 ± 0.011 (0.28 – 0.35)	0.0011 ± 0.0037 (-0.039 – 0.040)	0.783	-0.011, 0.013	0.25 – 0.39 (0.18, 0.42)	NA	0.2 – 0.3
22:0 behenic	0.18 ± 0.0068 (0.17 – 0.21)	0.18 ± 0.0068 (0.15 – 0.21)	0.0043 ± 0.0056 (-0.023 – 0.029)	0.498	-0.013, 0.222	0.089 – 0.21 (0.055, 0.30)	NA	0.1 – 0.3

Key:
MON863 and control mean values are for 16 replicates collected from 4 sites
S.E. = standard error of the mean
C.I. = confidence interval
Comm. = commercial; the range of sample values for commercial hybrids grown at the same field sites
T.I. = tolerance interval, specified to contain 95% of the commercial line population
Historical range for control lines refers to data collected Monsanto field trials conducted between 1993 and 1995.

Table 9: Combined site statistical comparison of mineral and vitamin levels in MON863 and control grain

Constituent	MON863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON863 minus Control)			Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
			Mean ± S.E. (Range)	p-value	95% C.I. (Lower, Upper)			
Calcium (% DW)	0.0052 ± 0.00041 (0.0041 – 0.0064)	0.0053 ± 0.00041 (0.0043 – 0.0089)	-0.00013 ± 0.00020 (-0.0027 – 0.00081)	0.538	-0.00056, 0.00031	0.0039 – 0.0060 (0.0022, 0.0073)	0.01 – 0.1	0.003 – 0.006
Copper (mg/kg DW)	2.26 ± 0.17 (1.72 – 3.18)	2.19 ± 0.17 (1.60 – 2.88)	0.078 ± 0.076 (-0.58 – 1.10)	0.315	-0.078, 0.23	1.03 – 2.15 (0.25, 2.70)	0.9 – 10	NA
Iron (mg/kg DW)	23.55 ± 1.16 (21.13 – 26.36)	24.18 ± 1.16 (20.57 – 28.16)	-0.63 ± 0.80 (-3.92 – 1.83)	0.490	-3.18, 1.92	16.74 – 28.69 (12.52, 35.06)	1 – 100	NA
Magnesium (% DW)	0.13 ± 0.0034 (0.12 – 0.14)	0.14 ± 0.0034 (0.12 – 0.16)	-0.0049 ± 0.0024 (-0.018 – 0.0049)	0.135	-0.013, 0.0028	0.091 – 0.14 (0.082, 0.17)	0.09 – 1.0	NA
Manganese (mg/kg DW)	5.81 ± 0.78 (3.75 – 7.40)	6.15 ± 0.78 (4.01 – 8.28)	-0.34 ± 0.16 (-0.94 – 0.58)	0.122	-0.84, 0.17	3.51 – 9.80 (0, 12.84)	0.7 – 54	NA
Phosphorus (% DW)	0.4 ± 0.0068 (0.37 – 0.45)	0.42 ± 0.0068 (0.39 – 0.46)	-0.022 ± 0.0094 (-0.070 – 0.019)	0.065	-0.045, 0.0020	0.27 – 0.41 (0.21, 0.47)	0.26 – 0.75	0.288 – 0.363
Potassium (% DW)	0.43 ± 0.0088 (0.40 – 0.48)	0.44 ± 0.0088 (0.39 – 0.48)	-0.0074 ± 0.0087 (-0.056 – 0.037)	0.457	-0.035, 0.020	0.33 – 0.43 (0.28, 0.48)	0.32 – 0.72	NA
Zinc (mg/kg DW)	22.15 ± 1.44 (17.95 – 25.25)	23.68 ± 1.44 (18.77 – 28.14)	-1.53 ± 0.69 (-4.60 – 0.90)	0.112	-3.73, 0.66	12.84 – 31.22 (6.31, 37.95)	12 – 30	NA
Vitamin E (mg/g DW)	0.011 ± 0.0012 (0.0062 – 0.014)	0.013 ± 0.0012 (0.0088 – 0.016)	-0.0015 ± 0.00047 (-0.0077 – 0.00090)	0.002	-0.0025, -0.00058	0.0041 – 0.014 (0, 0.019)	0.017 – 0.047	0.008 – 0.015

Key:
MON863 and control mean values are for 16 replicates collected from 4 sites
S.E. = standard error of the mean
C.I. = confidence interval
Comm. = commercial; the range of sample values for commercial hybrids grown at the same field sites
T.I. = tolerance interval, specified to contain 95% of the commercial line population
Historical range for control lines refers to data collected Monsanto field trials conducted between 1993 and 1995.

Table 10: Combined site statistical comparison of anti-nutrient levels in MON863 and control grain

Constituent	MON863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON863 minus Control)			Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
			Mean ± S.E. (Range)	p-value	95% C.I. (Lower, Upper)			
Phytic Acid (% DW)	1.11 ± 0.033 (0.92 – 1.28)	1.23 ± 0.033 (1.01 – 1.37)	-0.12 ± 0.034 (-0.31 – 0.19)	0.001	-0.91, -0.050	0.73 – 1.17 (0.39, 1.33)	To 0.9%	NA
Trypsin Inhibitor (TIU/mg DW)	2.30 ± 0.16 (0.56 – 3.10)	2.48 ± 0.16 (1.91 – 3.45)	-0.18 ± 0.16 (-1.70 – 0.63)	0.288	-0.53, 0.17	0.58 – 3.05 (0, 4.25)	NA	NA

Key:
MON863 and control mean values are for 16 replicates collected from 4 sites
S.E. = standard error of the mean
C.I. = confidence interval
Comm. = commercial; the range of sample values for commercial hybrids grown at the same field sites
T.I. = tolerance interval, specified to contain 95% of the commercial line population
Historical range for control lines refers to data collected Monsanto field trials conducted between 1993 and 1995.

Table 11: Combined site statistical comparison of fibre and proximate content in MON863 corn and control forage

Constituent	MON863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON863 minus Control)			Comm. Range (95% T.I. Lower, Upper)	Historical Range
			Mean ± S.E. (Range)	p-value	95% C.I. (Lower, Upper)		
Ash (% DW)	4.73 ± 0.22 (3.62 – 5.65)	5.00 ± 0.22 (3.81 – 6.27)	-0.27 ± 0.16 (-1.29 – 1.09)	0.106	-0.61, 0.066	3.74 – 5.02 (3.04, 5.58)	2.9 – 5.1
Carbohydrates (% DW)	84.24 ± 0.53 (82.29 – 86.32)	84.32 ± 0.53 (80.78 – 87.21)	-0.084 ± 0.43 (-2.70 – 2.52)	0.859	-1.47, 1.30	82.59 – 87.10 (81.22, 88.97)	84.6 – 89.1
ADF (% DW)	28.67 ± 1.66 (21.74 – 43.30)	28.41 ± 1.66 (23.39 – 32.08)	0.26 ± 2.06 (-7.90 – 14.03)	0.907	-6.29, 6.81	19.78 – 39.00 (9.33, 45.44	21.4 – 29.2
NDF (% DW)	43.25 ± 1.26 (37.97 – 49.67)	42.94 ± 1.26 (37.32 – 51.85)	0.31 ± 1.25 (-10.81 – 12.34)	0.807	-2.25, 2.87	30.30 – 47.75 (22.71, 56.02)	39.9 – 46.6
Moisture (% FW)	71.09 ± 0.46 (69.30 – 73.10)	71.68 ± 0.46 (69.80 – 74.50)	-0.58 ± 0.43 (-3.70 – 2.90)	0.269	-1.95, 0.79	67.00 – 74.10 (62.70, 77.69)	68.7 – 73.5
Total fat (% DW)	2.40 ± 0.23 (0.92 – 3.16)	2.35 ± 0.23 (1.30 – 3.33)	0.053 ± 0.15 (-0.91 – 1.14)	0.721	-0.26, 0.36	1.39 – 2.62 (1.03, 3.24)	1.4 – 2.1
Protein (% DW)	8.62 ± 0.53 (6.91 – 10.40)	8.33 ± 0.53 5.99 – 10.55)	0.30 ± 0.37 (-2.54 – 2.42)	0.478	-0.87, 1.47	6.45 – 10.14 (4.94, 11.97)	4.8 – 8.4

Key:
MON863 and control mean values are for 16 replicates collected from 4 sites
S.E. = standard error of the mean
C.I. = confidence interval
Comm. = commercial; the range of sample values for commercial hybrids grown at the same field sites
T.I. = tolerance interval, specified to contain 95% of the commercial line population
Historical range for control lines refers to data collected Monsanto field trials conducted between 1993 and 1995.

Table 12: Summary of statistically significant differences in composition between MON863 corn and parental control values

Tissue/component	Site Code	MON863 Mean	Control Mean	Mean Difference (MON863 minus Control)	Significance (p-value)	Mean Difference (% of Control Value)	MON863 (Range)	Comm. Range (95% T.I. Lower, Upper)
Forage								
Moisture	RD	70.23	71.43	-1.20	0.023	-1.68	(69.80 – 70.50)	(62.70, 77.69)
Grain								
Cystine	MN	2.18	2.03	0.15	0.012	7.39	(2.15 – 2.21)	(1.59, 2.65)
Leucine	MN	13.17	13.59	-0.42	0.013	-3.09	(12.88 – 13.42)	(11.30, 15.63)
Phenylalanine	MN	4.99	5.09	-0.093	0.038	-1.83	(4.93 – 5.06)	(4.53, 5.66)
Zinc	MN	20.51	22.79	-2.28	0.038	-10.00	(19.71 – 21.41)	(6.31, 37.95)
Total fat	MN	3.87	3.35	0.52	0.046	15.52	(3.59 – 4.06)	(1.68, 4.64)
Phytic acid	MN	1.15	1.33	-0.18	0.027	-13.53	(1.08 – 1.21)	(0.39, 1.33)
Leucine	RD	13.44	13.67	-0.23	0.023	-1.68	(13.33 – 13.63)	(11.30, 15.63)
Protein	RD	11.82	12.16	-0.34	0.039	-2.80	(11.63 – 11.97)	(5.47, 16.57)
Vitamin E	RD	0.013	0.015	-0.0023	0.011	-15.33	(0.012 – 0.014)	(0, 0.019)
18:3 linolenic	RD	1.26	1.28	-0.020	0.043	-1.56	(1.23 – 1.29)	(0.75, 1.51)
Cystine	VH	2.25	2.15	0.11	0.001	5.12	(2.22 – 2.29)	(1.59, 2.65)
20:0 Arachidic	VH	0.43	0.41	0.022	0.001	5.37	(0.43 – 0.44)	(0.30, 0.51)
Iron	VH	21.73	21.20	0.53	0.013	2.50	(21.13 – 23.05)	(12.52, 35.06)
Total fat	VH	3.08	3.42	-0.34	0.037	-9.94	(3.00 – 3.240	(1.68, 4.64)
Moisture	VH	9.86	10.37	-0.51	0.039	-4.92	(9.38 – 10.30)	(5.09, 18.62)
Aspartic acid	YK	6.44	6.36	0.088	0.040	1.38	(6.42 – 6.47)	(5.54, 7.65)
Tyrosine	YK	3.67	3.48	0.19	0.026	5.46	(3.59 – 3.74)	(2.15, 4.65)
Calcium	YK	0.0044	0.0047	-0.00023	0.035	-4.89	(0.0041 – 0.0047)	(0.0022, 0.0073)
Copper	YK	1.85	1.69	0.16	0.002	9.47	(1.72 – 2.01)	(0.25, 2.70)
Iron	YK	24.87	27.45	-2.58	0.013	-9.40	(23.99 – 25.42)	(12.52, 35.06)
Manganese	YK	7.17	7.91	-0.75	0.012	-9.48	(6.94 – 7.40)	(0, 12.84)
Phosphorus	YK	0.39	0.43	-0.036	0.037	-8.37	(0.37 – 0.41)	(0.21, 0.47)
Zinc	YK	24.20	27.16	-2.96	0.013	-10.90	(23.54 – 25.25)	(6.31, 37.95)
Carbohydrate	YK	82.56	81.28	1.28	0.046	1.57	(81.83 – 83.13)	(78.97, 90.36)
Protein	YK	12.44	13.62	-1.18	0.009	-8.66	(12.19 – 12.82)	(5.47, 16.57)

Arginine	All	4.43	4.33	0.10	0.030	2.31	(4.21 – 4.68)	(3.38, 5.220
Cystine	All	2.20	2.09	0.11	<0.001	5.26	(1.98 – 2.40)	(1.59, 2.65)
Leucine	All	13.36	13.65	-0.29	0.039	-2.12	(12.88 – 13.65)	(11.30, 15.63)
Phytic acid	All	1.11	1.23	-0.12	0.001	-9.76	(0.92 – 1.28)	(0.39, 1.33)
Vitamin E	All	0.011	0.013	-0.0015	0.002	-11.54	(0.0062 – 0.014)	(0, 0.019)

Key:
MON863 and control mean values are for 16 replicates collected from 4 sites
Comm. = commercial; the range of sample values for commercial hybrids grown at the same field sites
T.I. = tolerance interval, specified to contain 99% of the commercial line population
Mean difference calculated as follows: Mean Difference = (MON863 minus Control)/Mean Control x 100
RD = Richland, Iowa; MN = Monmouth, Illinois; VH = Van Horne, Iowa; YK = York, Nebraska

NUTRITIONAL IMPACT

In assessing the safety and suitability of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and 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.

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

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

In the case of MON863 corn, the extent of the compositional and other available data is considered to be sufficient to establish the nutritional adequacy of the food. However, a feeding study has been conducted on MON863 corn and is evaluated below as additional supporting information.

Studies submitted:

Taylor, M.L., Hartnell, G.F., Riordan, S.G., Nemeth, M.A., Cavato, T., Karunanadaa, K., George, B., Carpenter, D.M. and Astwood, J.D. (2001). Comparison of broiler performance when fed diets containing event MON863, nontransgenic parental line or commercial corn. Monsanto Company, MSL-17243.

Taylor, M.L., Astwood, J.D., Breeze, M. and Stibem C. (2001). Pesticide profile, mycotoxin and compositional analysis of corn event MON863 and control lines LH82Xa634 produced in Kihei, Hawaii in 2000. Monsanto Company, MSL-16953.

The study was done to compare the wholesomeness of MON863 corn to six non-GM commercial corn varieties in addition to the non-transformed parental corn line when fed to rapidly growing Ross x Ross broiler chicks. The rapidly growing broiler is considered to be sensitive to changes in nutrient quality in diets, and therefore is often used as a model to assess the wholesomeness of corn.

Diets were formulated on the basis of individual nutrient analyses for grain from each test, control and reference substance tested. The only sources of dietary protein used in the study were from the lines of corn used and from supplemented commercial soybean meal. Methionine and lysine were added as amino acid supplements. From days 1-20, broilers were fed a starter diet containing approximately 55% w/w corn (crude protein ranging from 19.1 – 23.9%). From days 20-42, broilers were fed a grower finisher diet containing approximately 60% w/w corn (crude protein ranging from 19.2 – 21.5%). Both feed and water were provided *ad libitum*.

A randomised complete block design was used, consisting of eight treatments corresponding to the eight corn lines tested. Treatments were assigned to pens with 80 males and 80 females per each of five blocks. All treatments were represented in each block consisting of 16 pens (8 male and 8 female) with 10 broilers/pen for a total of 80 pens and 800 broilers. For each treatment group there were 100 broilers in 10 pens, 5 pens of males (10 broilers/pen) and 5 pens of females (10 broilers/pen). At study start, two additional broilers were added to each pen to compensate for possible losses due to mortality from starve outs (broilers refusing feed) and dehydration, which normally occurs during the first few days of a chicken feeding study. At study day 7, the group size was culled to 10 broilers/pen. Broilers were weighed by pen at day 0 and day 42 and individually at study termination (day 43 for males and day 44 for females). The average body weight/pen and body weight/broiler for each treatment group by sex was calculated. The average feed efficiency/pen was calculated for the entire duration of the study by using the total feed intake during the study divided by the total body weight of the surviving broilers in the pen. This was averaged for each treatment group by sex. Adjusted feed efficiency was calculated by using the total feed intake/pen divided by the total body weight of the surviving broilers and body weight of broilers that died or were removed from the pen. At study termination, carcass measurements were taken including those for fat pads. One broiler per pen was sampled for breast and thigh meat quality assays.

A standardised randomised block analysis of variance (ANOVA) statistical model was used to analyse the data. Means were compared at the 5% level of significance. Additional statistical analyses were done to compare the fit of MON863 corn to the population of responses from the reference varieties to determine if the responses obtained from broilers fed diets containing MON863 corn were consistent with the expected variation of responses of broilers fed the other corn varieties.

Chick mortality was observed during the first 7 days of the study – mortality ranged from 0 to 7%. This mortality was randomly distributed across all treatment groups without any relationship to treatment. The remaining broilers were observed to be in good health throughout the remainder of the study based on pen observations made twice daily.

No biologically relevant differences were observed in performance parameters tested between broilers fed MON863 corn and its parental control. In addition, when individual treatment comparisons were made, broilers in general performed and had similar carcass yield and meat composition with diets containing MON863 corn, the parental control and six commercially available reference lines.

These data demonstrate that MON863 corn is equivalent to its conventional counterpart and other commercial varieties of corn in terms of its ability to support the rapid growth of broiler chicks.

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ATTACHMENT 3

SUMMARY OF PUBLIC SUBMISSIONS

A: FIRST ROUND

Submitter	Comments
1. Australian Food and Grocery Council (AFGC)	• supports the application for insect-protected MON863 corn, contingent upon a satisfactory safety assessment by FSANZ; • notes that MON863 corn has been approved for food use in the United States and Japan and considers it unfortunate that FSANZ, with its limited resources, has not negotiated "equivalence agreements" with the United States or Japan which would permit FSANZ to accept surrogate assessment of the data provided to them and safety clearance of MON863 corn; • supports FSANZ's proposed safety assessment of MON863 corn but considers it unlikely that FSANZ will come to any different conclusions from those of the overseas authorities who have approved its use; • provided FSANZ has assessed MON863 corn as safe, considers that it should be approved for use so that importers, food manufacturers and retailers can make their own choice with regard to its use; • an appropriate information and labelling regime for foods produced using gene technology, which provides consumers with independently verified factual information and appropriate label information, will ensure consumers are able to make their own informed choice.
2. Food Technology Association of Victoria Inc. (FTA Victoria)	• supports Option 2 – to approve food from insect-protected MON863 corn.
3. Department of Agriculture, Fisheries and Forestry – Australia (AFFA)	• generally supports FSANZ's assessments; • main concern relates to the lack of information on an appropriate, validated testing regime; • AFFA encompasses the Australian Quarantine and Inspection Service (AQIS) who are specifically concerned with enforcement/monitoring arrangements; • There is no reference in FSANZ's assessment reports to any need for future testing of either the corn itself or the products derived from it beyond noting that certain food fractions may require labelling if novel DNA and/or protein is present in the final food; • Inspection of foods at the border to verify labelling requirements is performed by AQIS on behalf of FSANZ, under the imported food inspection program. If FSANZ makes a future request to AQIS to test food containing or suspecting to contain novel corn with this genetic modification, it is imperative that appropriate reference material is available to enable qualitative analysis to be performed and validated testing methods established. The Application makes no reference to appropriate and validated testing methods used to detect the modified corn line in food products. These issues should be addressed if possible.

4. GE Free New Zealand in Food and Environment	• Do not support the growing or utilisation of GE crops. • States there is no evidence that these GE varieties, or their food products, are safe for human consumption in the long term. • Raises general concerns about antibiotic resistance genes, the lack of toxicity testing and long-term clinical trials, and the 'serious problems' within the biotech industry worldwide where consumers are losing confidence in GM crops and resultant foods. • States that FSANZ has failed to appreciate problems with the differences in approved varieties combing by pollen contamination and also crops being physically mixed by accident with unapproved varieties. • States that FSANZ has demonstrated an inadequate commitment to the precautionary principle, which they consider should underpin all food policy.

B: SECOND ROUND

Submitter	Comments
1. Australian Food and Grocery Council (AFGC)	• Now that FSANZ has assessed food derived from MON863 corn as safe it should be approved so that food manufacturers can make their own decision with regard to its use.
2. Food Technology Association of Victoria Inc. (FTA Victoria)	• Comments under '7.2 Impact on regulatory options' re 'segregation' are irrelevant to Option 1. It would appear that the comments re 'segregation' are applicable to Option 2. • If GM corn may be imported for non-human food use, i.e. animal feed, it is not clear how this would relate to retail costs. This situation also begs the question of the subsequent effects on consumers of foods derived from animals feed GM foods.
3. Gary Bilton (AUS)	• Is fundamentally opposed to GMOs in the food chain. • If GMOs are to be introduced, wants to see the principles of precaution applied. • Wants to see long term feeding studies and full labelling, regardless of whether novel DNA or protein is detectable. • Does not have any confidence in the scientific assessment process used by FSANZ.
4. Agriculture, Forestry and Fisheries Australia (AFFA)	• AQIS does not anticipate that the proposed variation to Standard 1.5.2 will present any major operational issues. • The proposed amendment is a routine one and AQIS does not expect it have any regulatory impact under the *Imported Food Control Act 1992*.
5. Federated Farmers of New Zealand Inc. (FFNZ)	• Supports Option 2, as proposed by FSANZ. • Provided MON863 corn derived products are proven to be safe by FSANZ, there should be no additional restrictions imposed on imports into New Zealand. • The FFNZ supports the WTO. A non-amendment to the Code would be inconsistent with New Zealand and Australian WTO obligations. • There will be no risk of contamination as only by-products of the corn will be imported into New Zealand. • For FSANZ to make an informed decision on the amendment of the Code, the risks to the health and safety of people need to be thoroughly addressed. The results and references of testing methods should be produced by the applicant for FSANZ to view and consider.

6. New Zealand Food Safety Authority (NZFSA)	• The NZFSA asked the Institute of Environmental Science & Research Limited (ESR) to prepare a report on the application. • The NZFSA report that they concur with the conclusions of the ESR report. • The ESR concluded that: - the assessment followed internationally accepted practices; - the absence of significant compositional differences, or data to suggest the potential for toxicological or allergenic risks supports the overall conclusion that no potential public health or safety concerns have been identified; - the single insertion site containing one copy of the cassette, with only minor changes resulting from the insertion, reduces the potential for unintended changes; - the compositional differences identified as statistically significant are unlikely to have biological significance; - food derived from MON863 corn is likely to have been processed so that protein is either removed or denatured and so exposure to novel protein will be minimal; - this GM crop expresses a protein which has 7 amino acids different to that of the native protein. These differences may cause structural changes and represent the key issue in this safety assessment. The acute oral toxicity and insect bioassay studies are of particular value in assessing the potential toxicity and demonstrating retention of functionality (and hence the relevance of the history of Cry protein safe use). - while the allergenicity assessment conforms to current international best practice, it does serve to highlight the value that an animal allergenicity model would provide. • The NZFSA considered that the food poses no greater risk than its conventional counterpart.
7. GE Free New Zealand in Food and Environment	• Does not support the use of any genetically modified crops either in food or in the environment. • Cites general concerns about the safety of GM foods and argues there is no evidence that MON863 corn, or any resultant food products, will prove safe for human consumption over the long term.