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

[6-08]
11 April 2008

APPLICATION A1001
FOOD DERIVED FROM INSECT-PROTECTED CORN LINE MIR162
ASSESSMENT REPORT

Executive Summary

Purpose

Food Standards Australia New Zealand (FSANZ) received an Application from Syngenta Seeds Pty Ltd (the Applicant) on 10 October 2007. The Applicant has requested an amendment to the *Australia New Zealand Food Standards Code* (the Code), specifically to Standard 1.5.2 – Food produced using Gene Technology, to permit the sale and use of food derived from a new genetically modified (GM) variety of corn, MIR162. Standard 1.5.2 requires that GM foods undergo a pre-market safety assessment before they may be sold in Australia and New Zealand.

MIR162 corn has been genetically modified to be protected against feeding damage caused by the larvae of certain insect pest species. Protection is achieved through the expression in the plant of an insecticidal protein derived from *Bacillus thuringiensis*, a common soil bacterium.

Corn line MIR162 is intended to be grown in North America. However, once commercialised, corn products imported into Australia and New Zealand could contain ingredients derived from MIR162 corn. Approval is therefore necessary before these products may enter the Australian and New Zealand markets.

The Application is being assessed under the General Procedure.

Safety Assessment

FSANZ has completed a comprehensive safety assessment of food derived from insect-protected corn line MIR162, as required under Standard 1.5.2. The assessment included consideration of (i) the genetic modification to the plant; (ii) the potential toxicity and allergenicity of the novel proteins; and (iii) the composition of MIR162 corn compared with that of conventional corn varieties.

No public health and safety concerns were identified as a result of the safety assessment.

On the basis of the available evidence, including detailed studies provided by the Applicant, food derived from insect-protected corn line MIR162 is considered as safe and wholesome as food derived from other commercial corn varieties.

Labelling

If approved, food derived from insect-protected corn line MIR162 will be required to be labelled as genetically modified if novel DNA and/or novel protein is present in the final food. Studies conducted by the Applicant show that the novel proteins are present in the grain.

Labelling addresses the objective set out in section 18(1)(b) of the *Food Standards Australia New Zealand Act 1991* (FSANZ Act); the provision of adequate information relating to food to enable consumers to make informed choices.

Impact of regulatory options

Two regulatory options were considered in the assessment: (1) no approval; or (2) approval of food derived from insect-protected corn line MIR162 based on the conclusions of the safety assessment.

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

Assessing the Application

In assessing the Application and the subsequent development of a food regulatory measure, FSANZ has had regard to the following matters as prescribed in section 29 of the FSANZ Act:

- Whether costs that would arise from an amendment to the Code approving food derived from insect-protected corn line MIR162 outweigh the direct and indirect benefits to the community, Government or industry that would arise from this food regulatory measure.

- There are no other measures that would be more cost-effective than a variation to Standard 1.5.2 that could achieve the same end.

- There are no relevant New Zealand standards.

- There are no other relevant matters.

Preferred Approach after Assessment

Amend Standard 1.5.2 – Food produced using Gene Technology, to include food derived from insect-protected corn line MIR162 in the Table to clause 2.

Reasons for Preferred Approach

An amendment to the Code approving food derived from insect-protected corn line MIR162 in Australia and New Zealand is proposed on the basis of the available scientific evidence, for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce insect-protected corn line MIR162;
- food derived from insect-protected corn line MIR162 is equivalent to food from the conventional counterpart and other commercially available corn varieties in terms of its safety for human consumption and nutritional adequacy;
- labelling of certain foods derived from insect-protected corn line MIR162 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 preferred option is option 2, an amendment to the Code;
- there are no relevant New Zealand standards; and
- there are no other measures that would be more cost-effective than a variation to Standard 1.5.2 that could achieve the same end.

Consultation

Public submissions are now invited on this Assessment Report. Comments are specifically requested on the scientific aspects of this Application, in particular, information relevant to the safety assessment of food from insect-protected corn MIR162.

As this Application is being assessed as a general procedure, there will be one round of public comment. Responses to this Assessment Report will be used to develop the Approval Report for the Application.

Invitation for Submissions

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

Written submissions are invited from interested individuals and organisations to assist FSANZ in further considering this Application/Proposal. Submissions should, where possible, address the objectives of FSANZ as set out in section 18 of the FSANZ Act. Information providing details of potential costs and benefits of the proposed change to the Code from stakeholders is highly desirable. Claims made in submissions should be supported wherever possible by referencing or including relevant studies, research findings, trials, surveys etc. Technical information should be in sufficient detail to allow independent scientific assessment.

The processes of FSANZ are open to public scrutiny, and any submissions received will ordinarily be placed on the public register of FSANZ and made available for inspection. If you wish any information contained in a submission to remain confidential to FSANZ, you should clearly identify the sensitive information, separate it from your submission and provide justification for treating it as confidential commercial material. Section 114 of the FSANZ Act requires FSANZ to treat in-confidence, trade secrets relating to food and any other information relating to food, the commercial value of which would be, or could reasonably be expected to be, destroyed or diminished by disclosure.

Submissions must be made in writing and should clearly be marked with the word 'Submission' and quote the correct project number and name. While FSANZ accepts submissions in hard copy to our offices, it is more convenient and quicker to receive submissions electronically through the FSANZ website using the Standards Development tab and then through Documents for Public Comment. Alternatively, you may email your submission directly to the Standards Management Officer at submissions@foodstandards.gov.au. There is no need to send a hard copy of your submission if you have submitted it by email or the FSANZ website. FSANZ endeavours to formally acknowledge receipt of submissions within 3 business days.

DEADLINE FOR PUBLIC SUBMISSIONS: 6pm (Canberra time) 23 May 2008

SUBMISSIONS RECEIVED AFTER THIS DEADLINE WILL NOT BE CONSIDERED

Submissions received after this date will only be considered if agreement for an extension has been given prior to this closing date. Agreement to an extension of time will only be given if extraordinary circumstances warrant an extension to the submission period. Any agreed extension will be notified on the FSANZ website and will apply to all submitters.

Questions relating to making submissions or the application process can be directed to the Standards Management Officer at standards.management@foodstandards.gov.au.

If you are unable to submit your submission electronically, hard copy submissions may be sent to one of the following addresses:

Food Standards Australia New Zealand
PO Box 7186
Canberra BC ACT 2610
AUSTRALIA
Tel (02) 6271 2222

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

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INTRODUCTION

An Application was received from Syngenta Seeds Pty Ltd on 10 October 2007 seeking an amendment to Standard 1.5.2 – Food produced using Gene Technology, in the *Australia New Zealand Food Standards Code* (the Code), to approve food derived from insect-protected corn line MIR162.

The genetic modification involved the transfer of two genes into corn. One gene is from a common soil bacterium called *Bacillus thuringiensis* and encodes an insecticidal protein (Vip3A) which protects the plant against feeding damage caused by certain insect pest larvae. The second gene, from another common bacterium called *Escherichia coli,* was used in the plant transformation process as it encodes an enzyme (PMI) which allows the transformed corn plants to grow on media containing the sugar mannose.

This Assessment includes a full scientific evaluation of food derived from MIR162 corn according to FSANZ guidelines[1], to assess its safety for human consumption. Public comment is now sought on the safety assessment and proposed recommendations prior to further consideration and completion of the Application.

1. The Issue / Problem

The Applicant has developed corn line MIR162 that is protected from feeding damage caused by certain lepidopteran insect pest larvae. Before food derived from insect-protected corn line MIR162 can enter the Australian and New Zealand food supply, it must first be assessed for safety and an amendment to the Code must be approved by the FSANZ Board, and subsequently be notified to the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council). An amendment to the Code may only be gazetted once the Ministerial Council process has been finalised.

Syngenta Seeds Pty Ltd has therefore applied to have Standard 1.5.2 amended to include food derived from corn line MIR162. The Application is at the Assessment stage.

2. Current Standard

2.1 Background

Standard 1.5.2 requires that genetically modified foods undergo a pre-market safety assessment before they may be sold in Australia and New Zealand. Foods that have been assessed under the Standard, if approved, are listed in the Table to clause 2 of the Standard.

[1] FSANZ (2007). Safety Assessment of Genetically Modified Foods – Guidance Document. http://www.foodstandards.gov.au/_srcfiles/GM%20FINAL%20Sept%2007L%20_2_.pdf

2.2 Overseas approvals

Insect-protected corn line MIR162 is intended for commercialisation in the United States and Canada. Submissions have been made to the appropriate US agencies for food, feed and environmental approvals (Environmental Protection Agency, Food and Drug Administration, Department of Agriculture). Applications will also be made in Canada for the necessary approvals (Health Canada and the Canadian Food Inspection Agency). Regulatory submissions for import approvals for MIR162 corn have been or will be made in key export markets, for example, Japan, Taiwan, Korea, Philippines and Mexico.

3. Objectives

In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives which are set out in section 18 of the FSANZ Act. These are:

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

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

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

4. Questions to be answered

Based on information provided by the Applicant on the nature of the genetic modification, the molecular characterisation, the characterisation of the novel proteins, the compositional analysis and any nutritional issues, is food derived from corn line MIR162 comparable to food derived from conventional varieties of corn in terms of its safety for human consumption?

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

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

RISK ASSESSMENT

Food from insect-protected corn line MIR162 has been evaluated according to the safety assessment guidelines prepared by FSANZ[2]. The summary and conclusions from the full safety assessment report (at **Attachment 2**) are presented below. In addition to information supplied by the Applicant, other available resource material including published scientific literature and general technical information was used for the assessment.

5. Risk Assessment Summary

5.1 Safety Assessment Process

In conducting a safety assessment of food derived from insect-protected MIR162 corn, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the corn genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic in humans.

The safety assessment applied to food from corn line MIR162 addresses only food safety and nutritional issues. It therefore does not address: environmental risks related to the environmental release of genetically modified (GM) plants used in food production; the safety of animal feed or animals fed with feed derived from GM plants; or the safety of food derived from the non-GM (conventional) plant.

5.2 Outcomes of the Safety Assessment

Detailed molecular analyses indicate that one copy of each of the *vip3Aa20* and *pmi* genes has been inserted at a single site in the plant genome and the genes are stably inherited from one generation to the next. No antibiotic resistance marker genes are present in MIR162 corn.

MIR162 expresses two novel proteins, Vip3Aa20 and PMI. The Vip3Aa20 protein is expressed at moderate levels in the corn grain, with a mean concentration of 43.6 µg/g fresh weight. The PMI protein is expressed at relatively low levels in the grain, with the mean concentration of 1.9 µg/g fresh weight.

The Vip3Aa20 protein is 99.7% identical to the native Vip3Aa1 protein, differing by two amino acids, at positions 129 and 284. The amino acid change at position 129 occurred during the plant transformation process.

[2] FSANZ (2007) Safety Assessment of Genetically Modified Foods – Guidance Document. http://www.foodstandards.gov.au/_srcfiles/GM%20FINAL%20Sept%2007L%20_2_.pdf

Apart from the unexpected amino acid change at position 129, both proteins conform in size and amino acid sequence to that expected, do not exhibit any post-translational modification including glycosylation, and also demonstrate the predicted insecticidal (Vip3A) or enzymatic (PMI) activity.

In relation to potential toxicity and allergenicity, *B. thuringiensis* has been extensively studied and has a long history of safe use as the active ingredient in a number of insecticide products for use in agriculture as well as home gardens.

In addition, bioinformatic studies with the Vip3Aa20 and PMI proteins have confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that both proteins would be rapidly degraded in the stomach following ingestion, similar to other dietary proteins. Acute oral toxicity studies in mice with both proteins have also confirmed the absence of toxicity. Taken together, the evidence indicates that both proteins are unlikely to be toxic or allergenic in humans.

Compositional analyses were done to establish the nutritional adequacy of MIR162 corn, and to compare it to conventional corn under typical cultivation conditions. No differences of biological significance were observed between MIR162 corn and its conventional counterpart. Food from insect-protected MIR162 corn is therefore considered to be compositionally equivalent to food from conventional corn varieties and its introduction into the food supply would therefore be expected to have little nutritional impact.

5.3 Conclusions

No potential public health and safety concerns have been identified in the assessment of insect-protected MIR162 corn. On the basis of the data provided in the present application, and other available information, food derived from insect-protected MIR162 corn is considered as safe and wholesome as food derived from conventional corn varieties.

RISK MANAGEMENT

6. Options

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

6.1 Option 1 – Prohibit food from corn line MIR162

Maintain the *status quo* by not amending Standard 1.5.2 of the Code to approve food derived from insect-protected corn line MIR162.

6.2 Option 2 – Approve food from corn line MIR162

Amend Standard 1.5.2 of the Code to permit the sale and use of food derived from insect-protected corn line MIR162, with or without specified conditions in the Table to clause 2 of the Standard.

7. Impact Analysis

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

7.1 Affected Parties

The affected parties may include the following:

- Consumers of food products containing corn, particularly those concerned about biotechnology.

- Industry sectors:

    - Food importers and distributors of wholesale ingredients
    - Processors and manufacturers of food products containing corn
    - Food retailers.

- Government:
    - enforcement agencies
    - national government, in terms of trade and World Trade Organization (WTO) obligations.

The cultivation of corn line MIR162 in Australia or New Zealand could have an impact on the environment, which would need to be assessed by the Office of the Gene Technology Regulator (OGTR) in Australia, and by various New Zealand government agencies including the Environmental Risk Management Authority (ERMA) and the Ministry of Agriculture and Forestry (MAF) before growing in either country could be permitted. MIR162 corn has been developed primarily for agricultural production overseas and, at this stage, the Applicant has no plans for cultivation in either Australia or New Zealand.

7.2 Benefit Cost Analysis

7.2.1 Option 1 – prohibit food from corn line MIR162

Consumers: Possible restriction in the availability of corn products if MIR162 corn is present in imported foods.

No impact on consumers wishing to avoid GM foods, as food from MIR162 corn is not currently permitted in the food supply.

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

Industry: Possible restriction on corn imports once MIR162 corn is commercialised overseas.

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

7.2.2 Option 2 – approve food from corn line MIR162

Consumers: Broader availability of imported corn products as there would be no restriction on imported corn products derived from MIR162 corn.

Potentially a wider range of imported corn products at lower prices.

Appropriate labelling would allow consumers wishing to avoid GM corn to do so.

Government: Benefit that if MIR162 corn was detected in corn imports, approval would ensure compliance of those products with the Code. This would ensure no potential for trade disruption on regulatory grounds.

Approval of MIR162 corn would ensure no conflict with WTO responsibilities.

This option could impact on monitoring resources, as certain foods derived from MIR162 corn will be required to be labelled as genetically modified.

Industry: Food manufacturers gain broader market access and increased choice in raw materials.

Importers of processed foods containing corn as an ingredient would benefit as foods derived from MIR162 corn would be compliant with the Code.

Retailers may be able to offer a broader range of corn products.

Possible cost to food industry as some food ingredients derived from MIR162 corn would be required to be labelled as genetically modified.

7.3 Comparison of Options

As food from insect-protected corn line MIR162 has been found to be as safe as food from conventional varieties of corn, Option 1 is likely to be inconsistent with Australia and New Zealand's WTO obligations. Option 1 would also offer little benefit to consumers wishing to avoid GM foods, as approval of MIR162 corn by other countries could limit supplementation of the Australian and New Zealand market with imported corn products.

As MIR162 corn has been found to be safe for human consumption and the potential benefits outweigh the potential costs, Option 2, an amendment to Standard 1.5.2 giving approval to insect-protected corn line MIR162, is therefore the preferred option.

COMMUNICATION AND CONSULTATION STRATEGY

8. Communication

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

As normally applies to all GM food assessments, this Assessment Report will be available to the public on the FSANZ website and distributed to major stakeholders. Public comment on this Assessment will be sought prior to preparation of the Approval Report.

The Applicant and individuals and organisations that make submissions on this Application will be notified at each stage of the Application. After the FSANZ Board has considered the Approval Report, if the draft variation to the Code is approved, we will notify that decision to the Ministerial Council. If the approval of food derived from insect-protected corn line MIR162 is not subject to review, the Applicant and stakeholders, including the public, will be notified of the gazettal of changes to the Code in the national press and on the website. In addition, FSANZ provides an advisory service to the jurisdictions on changes to the Code.

9. Consultation

9.1 World Trade Organization (WTO)

As members of the 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.

The draft variation to the Code would have a trade enabling effect as it would permit food derived from MIR162 corn to be imported into Australia and New Zealand and sold, where currently it is prohibited. For this reason it was determined there is no need to notify this Application as a Sanitary and Phytosanitary (SPS) measure in accordance with the WTO Agreement on the Application of SPS Measures.

CONCLUSION

10. Conclusion and Preferred Approach

Preferred Approach after Assessment

Amend Standard 1.5.2 – Food produced using Gene Technology, to include food derived from insect-protected corn line MIR162 in the Table to clause 2.

10.1 Reasons for Preferred Approach

An amendment to the Code to give approval to the sale and use of food derived from corn line MIR162 in Australia and New Zealand is proposed on the basis of the available scientific evidence, for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce insect-protected corn line MIR162;
- food derived from insect-protected corn line MIR162 is equivalent to food from the conventional counterpart and other commercially available corn varieties in terms of its safety for human consumption and nutritional adequacy;
- labelling of certain foods derived from insect-protected corn line MIR162 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 preferred option is Option 2, an amendment to the Code;
- there are no relevant New Zealand standards; and
- there are no other measures that would be more cost-effective than a variation to Standard 1.5.2 that could achieve the same end.

11. Implementation and Review

Following the consultation period for this document, an Approval Report will be completed and the draft variation will be considered for approval by the FSANZ Board. The FSANZ Board's decision will then be notified to the Ministerial Council. Following notification, the proposed draft variation to the Code is expected to come into effect on gazettal, subject to any request from the Ministerial Council for a review of FSANZ's decision.

ATTACHMENTS

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

Attachment 1

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

Subsection 87(8) of the FSANZ Act provides that standards or variations to standards are legislative instruments, but are not subject to disallowance or sunsetting

To commence: on gazettal

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

Food derived from insect-protected corn line MIR162	

Attachment 2

Safety Assessment Report

APPLICATION A1001 – FOOD DERIVED FROM INSECT-PROTECTED CORN LINE MIR162

SUMMARY AND CONCLUSIONS

Background

Insect-protected MIR162 corn has been genetically modified (GM) for protection against feeding damage caused by larvae of a number of insect species. Protection is conferred by expression in the plant of a modified *vip3Aa* gene, encoding an insecticidal Vip protein, derived from *Bacillus thuringiensis*, a common soil bacterium. The Vip proteins exert their effect on the insect by forming pores in the gut membranes of sensitive species leading to disruption of the midgut transmembrane potential and eventual insect death. MIR162 corn also expresses the *pmi* gene, encoding the enzyme phosphomannose isomerase (PMI), which serves as a selectable marker, enabling plants to grow on mannose.

MIR162 corn has been developed for commercial cultivation in North America and may therefore enter the Australian and New Zealand food supply as imported, largely processed food products.

In conducting a safety assessment of food derived from insect-protected MIR162 corn, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the corn genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic in humans.

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

History of Use

Corn is the world's third leading cereal crop, behind wheat and rice, and is grown in over 25 countries. 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 MIR162 corn may include flour, breakfast cereals, high fructose corn syrup and other starch products.

B. thuringiensis, the microorganism from which the *vip3A* gene was derived, has been extensively studied and commercially exploited for over 40 years as the active ingredient in a number of insecticide products used in agriculture as well as home gardens. *B. thuringiensis* therefore has had a long history of safe use and the Vip protein it produces is not known to be toxic to any vertebrates, including humans and other mammals.

Molecular Characterisation

Corn line MIR162 contains two novel genes. The first, *vip3Aa20,* derived from *B. thuringiensis*, encodes the insecticidal protein Vip3Aa20. The *vip3Aa20* gene is a modified version of the native *vip3Aa1* gene found in the *B. thuringiensis* strain AB88. The second gene present in MIR162 corn, *pmi*, is present as a selectable marker and encodes the enzyme phosphomannose isomerase derived from *Escherichia coli*.

Detailed molecular analyses indicate that one copy of each of the *vip3Aa20* and *pmi* genes has been inserted at a single site in the plant genome and the genes are stably inherited from one generation to the next. No antibiotic resistance marker genes are present in MIR162 corn.

Characterisation of Novel Protein

MIR162 expresses two novel proteins, Vip3Aa20 and PMI. The Vip3Aa20 protein is approximately 89 kDa and 789 amino acids in length. The Vip3Aa20 protein is 99.7% identical to the native Vip3Aa1 protein and differs by only 2 amino acids, at positions 129 and 284. The amino acid change at position 129 occurred during the plant transformation process. The phosphomannose isomerase (PMI) protein is approximately 42.8 kDa and 391 amino acids and catalyses the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. The Vip3Aa20 protein is expressed at moderate levels in the corn grain, with a mean concentration of 43.6 µg/g fresh weight. The PMI protein is expressed at relatively low levels in the grain, with the mean concentration of 1.9 µg/g fresh weight.

A large number of studies have been done to confirm the identity and physicochemical and functional properties of the expressed Vip3Aa20 and PMI proteins, as well as to determine their potential toxicity and allergenicity. Apart from the unexpected amino acid change at position 129, both proteins conform in size and amino acid sequence to that expected, do not exhibit any post-translational modification including glycosylation, and also demonstrate the predicted insecticidal (Vip3A) or enzymatic (PMI) activity.

Bioinformatic studies with the Vip3Aa20 and PMI proteins have confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that both proteins would be rapidly degraded in the stomach following ingestion, similar to other dietary proteins. Acute oral toxicity studies in mice with both proteins have also confirmed the absence of toxicity. The safety of the Vip3Aa protein is further supported by the long history of safe use of *Bt* microbial pesticides. Taken together, the evidence indicates that both proteins are unlikely to be toxic or allergenic in humans.

Compositional Analyses

Compositional analyses were done to establish the nutritional adequacy of MIR162 corn, and to compare it to a non-transgenic conventional corn under typical cultivation conditions. The components analysed were protein, fat, carbohydrate, amino acids, fatty acids, vitamins, minerals, the antinutrients phytic acid and raffinose, and the secondary metabolites furfural, ferulic acid, p-coumaric acid.

No differences of biological significance were observed between MIR162 corn and its conventional counterpart. Some minor differences in key nutrients were noted, however the levels observed were within the range of values measured for the comparator corn hybrid and other conventional corn varieties, and therefore they most likely reflect normal biological variability. Food from insect-protected MIR162 corn is therefore considered to be compositionally equivalent to food from conventional corn varieties.

Nutritional Impact

The detailed compositional studies are considered adequate to establish the nutritional adequacy of food derived from insect-protected MIR162 corn. The introduction of MIR162 corn into the food supply would therefore be expected to have little nutritional impact. The nutritional adequacy of food derived from MIR162 corn was also confirmed using a feeding study in rapidly-growing broiler chicks, which demonstrated that MIR162 corn is equivalent to its conventional counterpart and commercial 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 insect-protected MIR162 corn. On the basis of the data provided in the present application, and other available information, food derived from insect-protected MIR162 corn is considered as safe and wholesome as food derived from conventional corn varieties.

1. INTRODUCTION

Syngenta Seeds Pty Ltd has submitted an application to Food Standards Australia New Zealand (FSANZ) to vary Standard 1.5.2 – Food produced using Gene Technology – in the *Australia New Zealand Food Standards Code* (the Code) to include food from a new genetically modified (GM) corn variety. The GM corn variety is known as corn line MIR162.

Corn line MIR162 has been genetically modified to be resistant to a number of lepidopteran pests of corn, including fall armyworm (*Spodoptera frugiperda*), corn earworm/cotton bollworm (*Helicoverpa zea*), black cutworm (*Agropis ipsilon*) and western bean cutworm (*Striacosta albicosta*). Protection is conferred by the expression in the plant of the bacterially-derived *vip3Aa20* gene, which produces the insecticidal protein Vip3Aa20, a variant of the native insecticidal Vip3Aa1 protein. A selectable marker gene, *pmi*, encodes phosphomannose isomerase and allows transformed cells to utilise carbon from phosphomannose media.

Unlike Syngenta's Bt11 corn varieties, MIR162 has no insecticidal activity against European corn borer (*Ostrinia nubilalis*). The insect protection of MIR162 will be combined with Bt11 by conventional breeding. In regions where corn rootworm infestations are problematic for growers, these two traits will also be combined with Syngenta's trait, MIR604, which has been genetically modified to be resistant to Western corn rootworm (*Diabrotica vigifera vigifera*), Northern corn rootworm (*Diabrotica berberi*), and Mexican corn rootworm (*Diabrotica vigifera zeae*). Both the Bt11 and MIR604 traits have previously been assessed by FSANZ and food derived from these lines approved for human consumption.

Bt-based formulations are widely used as biopesticides on a variety of cereal and vegetable crops grown organically or under conventional agricultural conditions. Several registered Bt-based microbial pest control products contain Vip3Aa or Vip3Aa-like proteins and it is likely that small quantities of these proteins are present in the food supply.

Corn is the world's third leading cereal crop, behind wheat and rice, and is grown in over 25 countries (OECD, 2002). In 2006, worldwide production of corn was nearly 700 million tonnes, with the United States and China being the major producers (FAOSTAT, 2008). The majority of grain and forage derived from corn is used in animal feed. Corn grain is also used in industrial products, such as ethyl alcohol by fermentation and highly refined starch by wet milling.

Corn is not a major crop in Australia or New Zealand. 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 food coatings. Other corn products such as cornstarch are also imported and used by the food industry for the manufacture of dessert mixes and sauces. Corn may also be imported in finished products such as corn chips and canned corn, or dry milled goods such as cornflour.

Corn line MIR162 will be grown in North America and is not intended for cultivation in Australia or New Zealand. Therefore, if approved, food from this line may enter the Australian and New Zealand food supply as imported food products.

2. HISTORY OF USE

2.1 Donor Organisms

Bacillus thuringiensis

The source of the *vip3Aa* gene used in this GM corn is the ubiquitous soil and plant bacterium *Bacillus thuringiensis* (*Bt*) strain AB88. The *vip3Aa20* gene in MIR162 corn is a synthetic version of the native bacterial gene.

The effect of *B. thuringiensis* products on human health and the environment was the subject of a critical review by the WHO International Programme on Chemical Safety (WHO, 1999). The review concluded that '*B. thuringiensis* products are unlikely to pose any hazard to humans or other vertebrates or the great majority of non-target invertebrates provided that they are free from non-*B. thuringiensis* microorganisms and biologically active products other than the insecticidal proteins'.

Bt proteins are used widely as an insecticide in both conventional and organic agriculture. In Australia, various *Bt* insecticidal products are registered with the Australian Pesticides and Veterinary Medicines Authority (APVMA) for use on cotton, vegetables, fruits, vines, oilseeds, cereal grains, herbs, tobacco, ornamentals, forestry and turf. Numerous registered formulations contain Vip3Aa or Vip3Aa-like proteins. The very wide use of formulations containing the *Bt* insecticidal proteins indicates that people eating and handling fresh foods are commonly in contact with this protein.

Insecticidal products using *Bt* were first commercialised in France in the late 1930s (Nester *et al*., 2002) and were first registered for use in the United States by the Environment Protection Agency (EPA) in 1961 (EPA, 1998).

The EPA thus has a vast historical toxicological database for *B. thuringiensis*, which indicates that no adverse health effects have been demonstrated in mammals in any infectivity/ pathogenicity/ toxicity study (McClintock *et al*., 1995; EPA, 1998; Betz *et al*., 2000). This confirms the long history of safe use of *Bt* formulations in general, and the safety of *B. thuringiensis* as a donor organism.

Escherichia coli

The bacterium *Escherichia coli* is the source of the *pmi* gene in MIR162. *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 are normally 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 distinct from the strains of *E. coli* (the K-12 strains) that are used routinely in laboratory manipulations. The K-12 strains of *E. coli* have a long history of safe use and are commonly used as protein production systems in many commercial, including pharmaceutical and food ingredient, applications (Bogosian and Kane, 1991).

Agrobacterium tumefaciens

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

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

2.2 Host Organism

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). In 2006, worldwide production of corn was nearly 700 million tonnes, with the United States and China being the major producers (FAOSTAT, 2008).

The majority of grain and forage derived from corn is used as animal feed, however corn also has a long history of safe use as food for human consumption. The grain can be 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 corn germ can be processed to obtain corn oil and numerous other 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 (CFIA, 1994). The commercial production of corn now utilises controlled cross-pollination of two inbred lines (using conventional techniques) to combine desired genetic traits and produce hybrid varieties known to be superior to open-pollinated varieties in terms of their agronomic characteristics. This inbred-hybrid concept and resulting yield response is the basis of the modern corn seed industry and hybrid corn varieties are used in most developed countries for consistency of performance and production.

3. MOLECULAR CHARACTERISATION

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

Study submitted:
Long, N. (2007) Molecular characterization of the transgenic DNA in event MIR162 Maize. Syngenta Seeds Biotechnology Report No. SSB-119-07.

3.1 Method used in the genetic modification

Corn line MIR162 was produced by *Agrobacterium*-mediated transformation of *Zea mays* line NP2500 x NP2499. The *Agrobacterium*-mediated DNA transformation system is the basis of natural plasmid-induced crown-gall formation in many plants and is well understood (Zambryski, 1992). The genes of interest were inserted into the plasmid between DNA sequences known as the Left and Right Borders (LB and RB). These border sequences were isolated from the Ti plasmid of *Agrobacterium* and normally delimit the DNA sequence (T-DNA) transferred into the plant.

Immature embryos were co-cultivated with *A. tumefaciens* strain LB4404 carrying the transformation plasmid pNOV1300. Between the right and left borders, this plasmid contains the *vip3Aa* and *pmi* genes and regulatory elements as shown in Figure 1 and Table 1. The *vip3Aa19* gene in vector pNOV1300 was modified from the native *vip3Aa1* gene in *B. thuringiensis* strain AB88 to accommodate the preferred codon usage in corn. The Vip3Aa19 protein differs from the original Vip3Aa1 protein by a single amino acid: lysine at position 284 is changed to glutamine.

Embryos producing embryogenic callus were transferred to cell culture medium containing mannose, with the *pmi* gene used as a selectable marker. Regenerated plantlets were tested for the presence of both the *pmi* and *vip3Aa* genes, and absence of the spectinomycin (*spec*) antibiotic resistance gene by PCR analysis. Plants meeting these criteria were transferred to the greenhouse for propagation. These steps are summarised in Figure 2.

Table 1: Description of genetic elements in transformation vector pNOV1300

Genetic Element	Location in pNOV1300 (bp)	Size (bp)	Function
Active Ingredient Cassette			
ZmUbiInt	200 - 2192	1993	Promoter region from *Z. mays* polyubiquitin gene which contains the first intron (Entrez™ Accession Number S94464; (NCBI, 2006). Provides constitutive expression in monocots.
vip3Aa19	2214 - 4583	2370	A modified version of the native *vip3Aa1* gene found in the *Bacillus thuringiensis* strain AB88. The *vip3Aa19* gene in vector pNOV1300 was modified to accommodate the preferred codon usage in corn. The *vip3A19* gene (Entrez™ Accession Number DQ539887; (NCBI 2006) encodes a Vip3Aa19 protein that differs from the Vip3Aa1 protein encoded by the *vip3Aa1* gene by a single amino acid at position 284. The *vip3Aa1* gene encodes lysine at position 284 and the *vip3Aa19* gene encodes glutamine. A transformation induced change in the *vip3Aa19* coding sequence resulted in two single nucleotide differences for the insert gene, designated *vip3Aa20* (Entrez Accession number DQ539888;(NCBI 2006). The Vip3Aa19 protein differs by a single amino acid from the Vip3Aa20 protein. Vip3Aa proteins confer resistance to several lepidopteran insect pests.
iPEPC9	4600 - 4707	108	Intron #9 from the phosphoenolpyruvate carboxylase gene (Entrez Accession Number X15239; (NCBI 2006) from *Z. mays*.
35S Termin-ator	4710 - 4779	70	Terminator sequence from the 35S RNA from the cauliflower mosaic virus genome (Similar to Entrez™ Accession Number AF140604; (NCBI 2006). Its function is to provide a polyadenylation sequence.
Selectable Marker Cassette			
ZmUbilInt	4798 - 6790	1993	Promoter region from *Z. mays* polyubiquitin gene which contains the first intron (Entrez™ Accession Number S94464; (NCBI 2006). Provides constitutive expression in monocots.
pmi	6803 - 7978	1176	*E. coli pmi* gene encoding the enzyme phosphomannose isomerase (PMI) (Entrez™ Accession Number M15380; (NCBI 2006); this gene is also known as *manA*. Catalyzes the isomerisation of mannose-6-phosphate to fructose-6-phosphate. Used as a selectable marker during transformation.
NOS Termin-ator	8039 - 8291	253	Terminator sequence from the nopaline synthase gene of *A. tumefaciens* (Entrez™ Accession Number V00087; (NCBI 2006). Its function is to provide a polyadenylation site.
Vector Backbone Components			
LB (left border)	8362 - 8386	25	Left border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (EntrezTM Accession Number J01825; (NCBI 2006). Short direct repeat that flanks the TDNA and is

Genetic Element	Location in pNOV1300 (bp)	Size (bp)	Function
			required for the transfer of the T-DNA into the plant cell (Zambryski *et al*., 1982).
spec	9562-10350	789	Spectinomycin adenylyltransferase, *aadA* gene from *E. coli* Tn7 (EntrezTM Accession Number X03043 (NCBI, 2006)). Confers resistance to erythromycin, streptomycin, and spectinomycin; used as a bacterial selectable marker (Fling *et al*., 1985).
ColE1ori	11549-12355	807	Origin of replication that permits replication of plasmid in *E. coli*. (Similar to EntrezTM Accession Number V00268 (NCBI, 2006 (Itoh and Tomizawa, 1979).
cos	12736-13167	432	The sequence that is cut to produce the cohesive, single-stranded extensions located at the ends of the linear DNA molecules of certain bacteriophages, such as lambda (Sanger *et al*., 1982).
RB (right border)	1 - 25	25	Right border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (EntrezTM Accession Number J01826 (NCBI, 2006)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang *et al*., 1984).

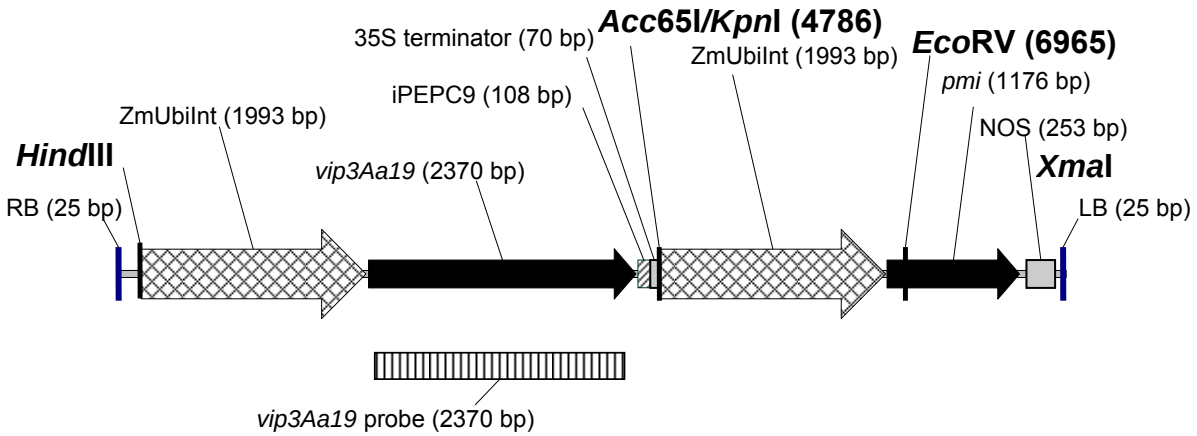


Figure 1: Genes and regulatory elements inserted in MIR162

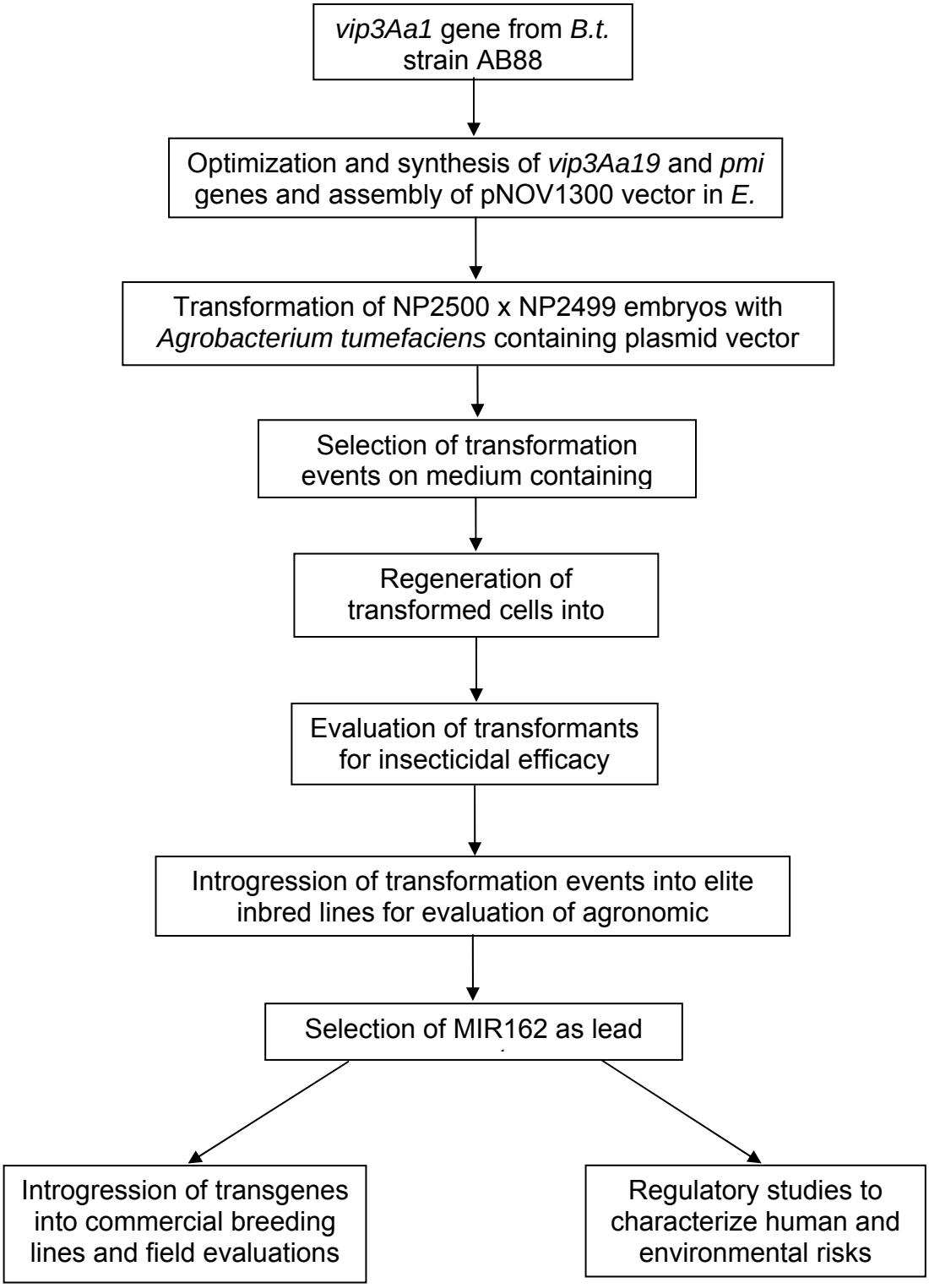


Figure 2: Steps in development of corn line MIR162

3.2 Function and regulation of novel genes

vip3Aa20 gene

The Vip3Aa proteins confer resistance to several lepidopteran insect pests. Corn line MIR162 expresses a modified version of the native Vip3Aa1 protein found in the *B. thuringiensis* strain AB88. The Vip3Aa19 protein encoded by the *vip3Aa19* gene in vector pNOV1300 differs from the Vip3Aa1 protein in *B. thuringiensis* strain AB88 by a single amino acid: lysine at position 284 is changed to glutamine (K284Q). Transformation induced changes in the *vip3Aa19* coding sequence resulted in two nucleotide differences for the inserted gene, designated *vip3Aa20* (Entrez Accession number DQ539888; NCBI, 2006). One of these nucleotide changes resulted in a single amino acid change: methionine at position 129 of Vip3Aa19 has been substituted by isoleucine (M129I). The other change was a silent mutation, not resulting in an amino acid change.

The regulatory elements are described in Table 1. The *vip3Aa20* gene is regulated by the promoter and first intron from the *Zea mays* polyubiquitin gene, which confers constitutive expression, and the 35S terminator from the cauliflower mosaic virus.

pmi gene

The *pmi* gene represents the *manA* gene from *E. coli* and encodes the enzyme phosphomannose isomerase (PMI). It was used as a selectable marker gene during the transformation process. Mannose, a hexose sugar, is taken up by plants and converted to mannose-6-phosphate by hexokinase. This product cannot be further utilised in plants as they lack the PMI enzyme. The accumulation of mannose-6-phosphate inhibits phosphoglucose isomerase, causing a block in glycolysis. It also depletes cells of orthophosphate required for the production of ATP. Therefore, while mannose has no direct toxicity on plant cells, it causes growth inhibition (Negrotto *et al.,* 2000). This does not occur in plants transformed with the *pmi* gene as they can utilise mannose as a source of carbon.

The *pmi* gene is regulated by the same polyubiquitin promoter (ZmUbiInt) as the *vip3Aa20* gene and the NOS terminator from *A. tumefaciens*.

No other genes were transferred to corn line MIR162.

3.3 Characterisation of the genes in the plant

Insert DNA sequence analysis

To characterise the integrity of the inserted T-DNA, the sequence of the entire DNA insert was determined. The sequence of the insert was compared to the DNA sequence of the transforming plasmid (pNOV1300). In total, 8314 bp of T-DNA had become inserted into the corn genome. During the transformation process the T-DNA insert was truncated at both the left border (entire LB plus 32 bp of non-coding sequence) and the right border (the entire RB plus two bp of non-coding sequence).

While T-DNA border sequences are known to play a critical role in T-DNA insertion into the genome, this result is not unexpected since insertions are often imperfect, particularly at the Left T-DNA border (Tinland B. and Hohn B., 1995). Two base pair changes were noted in the MIR162 insert, both within the *vip3Aa19* gene. The first change results in a predicted single amino acid change at position 129 of Vip3Aa19 from methionine to isoleucine (M129I). The second nucleotide change was silent and did not result in a predicted amino acid change. As the *vip3Aa* gene in MIR162 is slightly different from the *vip3Aa19* gene encoded on pNOV1300, it was designated *vip3Aa20* (Entrez Accession number DQ539888; NCBI, 2006). The coding sequences of *pmi*, the ZmUbiInt promoters, the 35S terminator and the NOS terminator in MIR162 are identical to those in the pNOV1300 transformation plasmid.

Insert and copy number

Southern blot analysis was used to determine the insert and copy number of the *vip3Aa20* and *pmi* genes and to confirm the absence of DNA sequence from outside the T-DNA borders of the transformation vector.

Southern blot analyses of pooled leaf tissue from plants in MIR162 backcross generation four (BC4F1) and negative segregants demonstrate that insertion event MIR162 occurred as an integration of a single intact T-DNA from plasmid pNOV1300. Probes corresponding to *vip3Aa19*, *pmi*, the ZmUbiInt promoter and the NOS terminator produced the expected hybridisation patterns. Southern analysis using the pNOV1300 backbone sequence as probe confirmed that plasmid backbone DNA is not present in corn line MIR162.

Flanking regions and putative Open Reading Frame (ORF) analysis

Corn genomic DNA on either side of the MIR162 T-DNA insert was sequenced. The DNA sequence of 1000 bp of genomic DNA flanking the 5' end and the 3' end of the MIR162 insert was determined.

Analysis of the sequence flanking the 5' region of the insert revealed significant homology with Dissociation1 (Ds1)-related transposable elements. Ds1 is a non-autonomous transposable element that requires an active Activator (Ac) element to become mobile. The homology between the 5' flanking sequence and the Ds1-related elements is over 500 bp from the insert sequence. Previous studies have shown that the excision of Ds elements can extend beyond the element, but the largest amount of surrounding DNA that has been shown to be deleted is 36 bp.

The genomic sequence flanking the 3' region of the T-DNA insert shows homology with a sequence defined as a cyclophilin gene, but the region of homology lies outside of the cyclophilin coding region.

The junction regions between the insert and genomic DNA were further analysed for their potential to be involved in the production of chimeric proteins. The production of unexpected chimeric proteins as a result of transgene insertion is of particular relevance to food safety.

In cases where there is 100% molecular identity between the plasmid T-DNA and inserted DNA in the plant, and all regulatory elements including termination and polyadenylation signals are intact, there is little likelihood of unintended formation of gene fragments that are transcriptionally active or likely to produce a chimeric protein. In the case of corn line MIR162, the transformation event has not resulted in any additions, deletions, rearrangements or partial insertions of the gene of interest, or its regulatory elements, as determined by the Southern blot and direct DNA sequencing of the entire insert region. In addition, analysis of all six potential reading frames at the junctions of both the 5' and 3' end of the DNA insert with the corn genome did not detect the presence of any functional novel open reading frames.

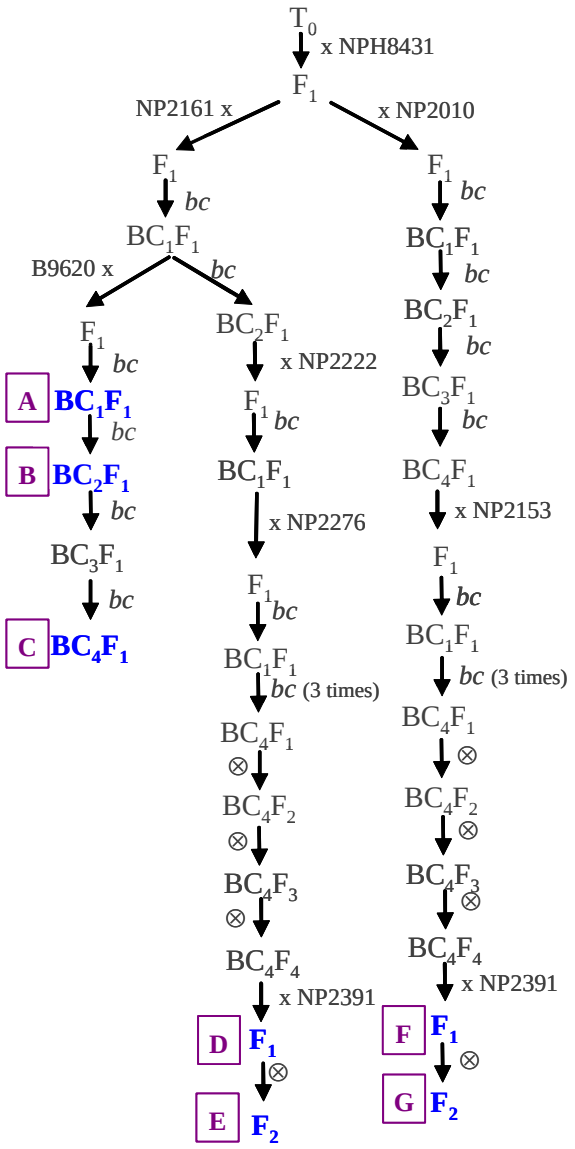
Conclusion

Molecular analyses have been performed on corn line MIR162 to characterise the novel genes present in the genome. Results indicate that there is one insertion site consisting of the entire T-DNA from plasmid pNOV1300. Sequence analysis showed that two single nucleotide changes had occurred within the insert, both within the *vip3Aa* gene. One of these resulted in a predicted amino acid change from methionine to isoleucine at position 129. The T-DNA insert in MIR162 has not disrupted any known endogenous corn genes, nor resulted in the creation of any unexpected open reading frames.

3.4 Development and breeding of corn line MIR162

A number of corn lines were generated following transformation with vector pNOV1300. The progeny of these primary transformants (T_0 plants) were field tested for both resistance to insect feeding damage and agronomic performance. Following introgression into multiple elite lines of corn and further evaluation of agronomic performance and insecticidal efficacy, event MIR162 was selected for further development and regulatory trials. These steps in the development of MIR162 are shown schematically in Figure 2.

A pedigree chart showing the further breeding of event MIR162 is shown in Figure 3. The plant test materials used in studies to support the food safety assessment are also indicated. Finished hybrids, produced from converted MIR162 inbreds, were used as test materials for compositional analysis and protein quantification. Parental control hybrids with a genetic background matched to that of the MIR162 hybrids were utilised in all studies so that the effect of the transformation event could be assessed.



T_0 – original transformant

x – cross

bc – backcross

⊗ - self-pollination

Molecular characterisation & Mendelian inheritance	A
Molecular characterisation & Mendelian inheritance	B
Molecular characterisation & Mendelian inheritance	C
Protein characterisation	C
Composition analysis (Forage and grain)	D&E
Protein Quantification – Hybrid 1	D&E
Protein Quantification – Hybrid 2	F&G
42-day broiler chicken	G

Figure 3: Pedigree diagram for MIR162 and test materials

3.5 Stability of the genetic changes

A number of analyses were done to demonstrate the stability of the genetic changes in corn line MIR162. Southern blot analysis over multiple generations was done to determine the stability of the inserted DNA. Segregation analysis over multiple generations was done to determine the heritability and stability of the *vip3Aa20* and *pmi* genes and Vip3Aa20 and PMI proteins.

Generational stability

To determine whether the insert in corn line MIR162 is stable over a number of generations, Southern blot analysis was conducted on samples from three backcross generations – $BC1F_1$, $BC2F_1$ and $BC4F_1$ (see Figure 3). Genomic DNA from MIR162 and a negative control from the $BC4F_1$ generation was digested with *Acc*65I, a restriction enzyme that digests once in the DNA insert but not within the region of the *vip3Aa19*-specific probe (Figure 1). Southern analysis identified the same single hybridisation band in each of these generations when probed with DNA specific to the *vip3Aa19* gene, as expected. No hybridisation was detected in the negative control genomic DNA. The consistent and predicted hybridisation pattern in several generations of MIR162 indicates that the insert is stably integrated into the corn genome.

Segregation analysis

Segregation analysis was performed on MIR162 plants over three generations to assess whether the DNA insert is inherited in a stable and predictable manner. PCR analyses were conducted on individual plants from three generations of MIR162 to determine the inheritance ratios of both the *vip3Aa20* and the *pmi* genes. For each generation tested, the expected inheritance frequency was 1:1 positive to negative for both traits, as a result of back-crossing a hemizygous transgenic plant to a non-transgenic recurrent parent. The expected and observed ratios of positive and negative plants were analysed by Chi square analysis to determine if the trait is segregating in a Mendelian fashion. The expected and observed segregation frequencies of the *vip3Aa20* and *pmi* genes are shown in Tables 2 and 3.

Table 2: Observed vs. expected genotype for *vip3Aa* for multiple MIR162 generations as determined by PCR analysis

Trait	$BC1F_1$		$BC2F_1$		$BC4F_1$	
	O*	**E***	**O***	**E***	**O***	**E***
Positive	21	20.5	45	48.5	148	143.5
Negative	20	20.5	52	48.5	139	143.5
Total	41	41.0	97	97.0	287	287.0
X^2 value	0.000		0.371		0.223	

* O = Observed values and E = Expected values

X^2= sum $[|(\text{Observed-expected})|- 0.5]^2/\text{expected}$

Table 3: Observed vs. expected genotype for *pmi* for multiple MIR162 generations as determined by PCR analysis.

Trait	$BC1F_1$		$BC2F_1$		$BC4F_1$	
	O*	**E***	**O***	**E***	**O***	**E***
Positive	21	20.5	45	48.5	148	143.5
Negative	20	20.5	52	48.5	139	143.5
Total	41	41.0	97	97.0	287	287.0
X^2 value	0.000		0.371		0.223	

* O = Observed values and E = Expected values

X^2= sum [|(Observed-expected)|- 0.5]^2/expected

No significant differences between observed and expected segregation frequencies were found. The critical Chi square value to reject the hypothesis of 1:1 segregation at the 5% level is 3.84. The Chi square values obtained are less than 3.84 for each gene in all generations tested. This indicates that the insert in MIR162 is segregating in a predictable manner according to Mendelian principles.

Stability of protein expression

Study submitted:
Pence, K. (2006) Stability of Vip3Aa20 and phosphomannose isomerise (PMI) protein expression across multiple generations of maize (corn) derived from transformation event MIR162. Syngenta Seeds Biotechnology Report No. SSB-002-06.

The stability of Vip3Aa20 and PMI protein expression was evaluated over the same three generations of corn line MIR162. Individual plants derived from the three backcross generations $BC1F_1$, $BC2F_1$ and $BC4F_1$ (see Figure 3) were genotyped by PCR as described in the preceding section. Leaf tissue from five plants hemizygous for the transgenes in each generation, along with tissue from one near-isogenic, non-transgenic plant, was collected and analysed by enzyme-linked immuno-sorbent assay (ELISA). The mean Vip3Aa20 and PMI concentrations in leaves measured in the three backcross generations are given in Table 4. The concentrations of Vip3Aa20 and PMI are consistent across generations. Vip3Aa20 and PMI were either not detectable or not quantifiable in the non-transgenic control samples analysed.

Table 4: Vip3Aa20 and PMI Concentrations in Leaf Tissue from Multiple Generations of corn line MIR162

Generation	Mean µg Vip3Aa20/gdw ± SD (range)	Mean µg PMI/gdw ± SD (range)
BC1F1	77.94 ± 4.32 (72.83 – 84.18)	10.47 ± 0.54 (9.95 – 11.16)
BC2F1	74.33 ± 4.07 (68.26 – 78.33)	10.59 ± 0.65 (9.86 – 11.16)
BC4F1	78.80 ± 5.42 (74.61 – 87.50)	10.34 ± 1.23 (8.80 – 11.93)

N = 5 (samples analysed). Values have not been corrected for extraction efficiency.

Stability of insect protection phenotype

Studies submitted:
Huber, S.A., White, J., Mroczkiewicz, S. and Ward, D.P. (2007) Insecticidal Efficacy Field Evaluations with MIR162 Maize Hybrids in 2005 and 2006. Syngenta Seeds Biotechnology Report No. SSB-522-07.

White, J., Sagers, J., Meehan, M. and Meghji, M. (2007) Fall Armyworm Tolerance of a MIR162 Maize Hybrid: 2006 Field Trial Results. Syngenta Seeds Biotechnology Report No. SSB-503-07.

White, J., Meehan, M. and Meghji, M. (2007) Corn Earworm Tolerance of a MIR162 Maize Hybrid: 2006 Field Trial Results. Syngenta Seeds Biotechnology Report No. SSB-502-07.

Field trials were conducted to assess the insecticidal efficacy of MIR162 corn in comparison to a conventional corn hybrid control and a conventional corn hybrid treated with a commercial insecticide standard. The results of trials conducted at multiple locations which represented the major corn growing regions of the continental United States demonstrate that MIR162 provides significant crop protection against feeding damage caused by black cutworm (*Agropis ipsilon*), fall armyworm (*Spodoptera frugiperda*), corn earworm (*Helicoverpa zea*), southwestern corn borer (*Diatraea grandiosella*), beet armyworm (*Spodoptera exigua*), common stalk borer (*Pepaipema nebris*) and western bean cutworm (*Striacosta albicosta*).

Conclusion

The results of the segregation analysis are consistent with a single site of insertion for both the *vip3Aa20* and the *pmi* gene expression cassette and confirm the results of the molecular characterisation. The studies indicate that the T-DNA insert is stably integrated into the corn genome in corn line MIR162 and is inherited as expected over several generations. The level of expression of both the Vip3Aa20 and PMI proteins is stable across multiple backcross generations of corn line MIR162. The Vip3Aa20 protein in MIR162 corn has insecticidal activity against a range of lepidopteran insects.

3.6 Antibiotic resistance marker genes

No antibiotic marker genes are present in insect-protected corn line MIR162. The molecular characterisation shows that the region outside the T-DNA of plasmid pNOV1300 was not integrated into the corn genome during transformation. Consequently, the spectinomycin resistance gene, which was used as a bacterial selectable marker gene, is not present in insect-protected corn line MIR162. The absence of the bacterial marker gene in the plant was confirmed by Southern hybridisation analysis using a probe corresponding to the backbone of the transformation vector, pNOV1300, as described in section 3.3.

4. CHARACTERISATION OF NOVEL PROTEINS

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects, although a small number have the potential to impair health, e.g., because they are allergens or anti-nutrients.

As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutritional and allergenic effects. To effectively identify any potential hazards requires knowledge of the characteristics, concentration and localisation of all novel proteins expressed in the organism as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the novel protein is expressed as expected, including whether any post-translational modifications have occurred.

Corn line MIR162 contains two novel proteins: Vip3Aa20 and PMI. A number of different analyses were done to determine the identity, physiochemical properties, *in planta* expression, bioactivity and potential toxicity and allergenicity of the two proteins. Because the expression of proteins *in planta* is usually too low to allow purification of sufficient quantities for safety assessment studies, a bacterial expression system was used to generate large quantities of the Vip3Aa20 and PMI proteins. The Vip3Aa20 and PMI proteins produced in *E. coli* were engineered so their amino acid sequence matched that of the plant-produced Vip3Aa20 and PMI proteins. The equivalence of the bacterial-produced proteins to the plant-produced proteins was determined as part of the protein characterisation.

4.1 Biochemical function and phenotypic effects

Vip3Aa20

The Vip3Aa20 protein in corn line MIR162 has insecticidal activity against several lepidopteran pests of corn. The *vip3Aa20* gene in corn line MIR162 is a variant of the *vip3Aa* gene present in *B. thuringiensis* (I) strain AB88. The *vip3Aa20* gene encodes a Vip3Aa20 protein of approximately 89 kDa and 789 amino acids in length. The Vip3Aa20 protein is 99.7% identical to the native Vip3Aa1 protein and differs by only 2 amino acids, at positions 129 and 284. The Applicant states that both these substitutions are conservative and do not significantly impact insecticidal activity.

The Vip (vegetative insecticidal protein) proteins in *Bt* are produced during vegetative bacterial growth and are secreted as soluble proteins into the extracellular environment. *Bt* cultures continue to produce Vip protein during the stationary phase of development and sporulation. This contrasts with the other insecticidal *Bt* crystal (Cry) proteins that are produced during sporulation and spontaneously form small crystals that are contained within cytoplasmic inclusions.

Following ingestion of Vip3A by target insects, the protein is activated by insect gut proteases and binds to the membranes of midgut epithelial cells. Subsequent pore formation in the cell membranes leads to disruption of the midgut transmembrane potential and eventual insect death.

Both Vip and Cry proteins are proteolytically activated to insecticidal toxic cores in the lepidopteran gut and form pores in the gut membranes of sensitive species. However, Vip3Aa has been shown to have significantly different receptor binding properties and pore forming properties to Cry proteins, indicating that Vip and Cry proteins have different targets and specific modes of action. The mode of action of Vip3Aa proteins is highly specific to insects (Lee *et al*., 2003; Lee *et al*., 2006).

Phosphomannose isomerase

The phosphomannose isomerase (PMI) protein in MIR162 is a protein of approximately 42.8 kDa and 391 amino acids that catalyses the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. The *pmi* gene, which expresses PMI in MIR162, is derived from *E. coli*. The lack of PMI in many plant species allows it to be utilised as a selectable marker for transformation. Plant cells that have been transformed with the *E. coli manA* (*pmi*) gene are able to survive and grow on media containing mannose as the only or primary energy source (Miles and Guest, 1984). Plants lacking this enzyme are unable to grow on culture media containing mannose.

Mannose is converted to mannose 6-phosphate by hexokinase in the plant. In the absence of PMI, this accumulates, inhibiting the enzyme phosphoglucose isomerase and blocking glycolysis. In addition, depletion of the pyrophosphate required for ATP production and transcriptional repression of genes involved in photosynthesis and glyoxylate cycle occurs (Privalle, 2002; Freeze, 2002).

4.2 Protein characterisation

A range of analytical techniques was used to determine the identity as well as the physicochemical and functional properties of the plant-produced Vip3Aa20 and PMI proteins isolated from MIR162 corn and to compare them to *E. coli*-produced proteins. These techniques included Western blot analysis, peptide mass mapping analysis, insect bioactivity assays, enzyme activity assays and glycosylation analysis.

The *E. coli*-produced proteins were used as reference standards for determination of protein concentration and immunoblot analysis using polyclonal antibody. These proteins were also used as reference standards to evaluate equivalence between plant- and *E. coli*-produced proteins for molecular weight and functional activity assays, as a reference and a positive control in immunoblot analyses using anti-Vip3Aa20 and anti-PMI antibodies, and as a reference and negative control in glycosylation analysis.

Vip3Aa20

Studies submitted:
Graser, G and Stacy, C. (2006) Characterization of the Vip3A Protein Expressed in Event MIR162-Derived Maize (Corn) and Comparison with Microbially Produced and Plant-Derived Vip3A Test Substances. Syngenta Seeds Biotechnology Report No. SSB-017-06.

Graser, G and Stacy, C. (2006) Characterization of Microbially-Produced Vip3A Test Substance MIR162VIP3A-0106 and Comparison with Vip3A Expressed in Event MIR162-Derived Maize (Corn). Syngenta Seeds Biotechnology Report No. SSB-023-06.

Vip3Aa20 expressed in MIR162 is nearly identical in amino acid sequence to that encoded by the native *vip3Aa1* gene from *B. thuringiensis*, with the exception of two amino acids: lysine-284 has been substituted by glutamine, and methionine-129 has been substituted by isoleucine. The Vip3Aa19 protein encoded by the synthetic *vip3Aa19* gene (modified to accommodate the preferred codon usage in corn) on transformation plasmid pNOV1300 differs from the native Vip3Aa1 protein encoded by the native *vip3Aa1* gene from *Bt* strain AB88 by a single amino acid at position 284.

The second amino acid difference between Vip3Aa1 and Vip3Aa20, at position 129, is the result of a transformation induced change in the *vip3Aa19* coding sequence that was incorporated in the MIR162 corn genome. These differences in the Vip3A proteins are shown in Table 5.

The Vip3Aa20 protein was purified from a homogenate of leaf tissue from MIR162 corn hybrids using a combination of ammonium sulphate fractionation and dialysis, and subsequent immunoaffinity chromatography. A negative control substance was prepared from leaf material derived from a corresponding negative segregant corn. The Vip3Aa20 protein prepared by expressing a synthetic *vip3Aa20* gene in an *E. coli* over-expression system is predicted to be identical in amino acid sequence to Vip3Aa20 expressed in MIR162 corn. *E. coli* produced Vip3Aa20 was purified with anion exchange chromatography and dialysis.

Table 5: Comparison of the Amino Acid Sequence Differences of Vip3Aa Proteins Examined in this Study

Protein		**Amino acid at position 129**	**Amino acid at position 284**	**Source**	**Vip3Aa Test Samples**
Vip3Aa1	native Vip3A in *Bt* strain AB88	**M**	**K**	*B. thuringiensis*	
Vip3Aa19	Codon optimised version on pNOV1300	**M**	**Q**	Recombinant *E. coli*	
Vip3Aa20	MIR162 corn	**I**	**Q**	MIR162 corn	**LPMIR162-0105 and 0106; IAPMIR162-0105 and 0106**
Vip3Aa20		**I**	**Q**	Recombinant *E. coli*	**MIR162VIP3A-0106**

Protein identity

The identity of the plant- and microbially-produced Vip3Aa20 was confirmed by Western blot analysis using anti-Vip3A antibody, and peptide mass mapping analysis. The identity of the microbially produced Vip3Aa20 was also confirmed by N-terminal amino acid sequence analysis:

(i) Western blot using anti-Vip3A antibody – the plant-produced Vip3Aa20 protein and the *E. coli*-produced reference standard were subjected to SDS-PAGE on the same gel. After blotting, goat anti-Vip3Aa immunoaffinity-purified polyclonal antibodies and donkey anti-goat IgG linked to alkaline phosphatase were used to detect the presence of immunoreactive Vip3Aa. The major immunoreactive bands corresponded to the predicted molecular weight of ~89 kDa in both the plant-produced and *E. coli*-produced samples. The bands of lower molecular weight are partial Vip3A fragments, which the Applicant states are likely to represent proteolytic degradation products;

(ii) Peptide mass mapping analysis – Vip3Aa20 protein isolated from MIR162 and recombinant *E. coli* was reduced, alkylated with iodoacetamide and digested with trypsin. The mass analysis of the Vip3Aa20-derived peptides was performed on a quadrupole time-of-flight mass spectrometer. The detected peptide masses were compared to a protein database. The ability to identify a protein using this method is dependent on matching a sufficient number of observed tryptic mass fragments to expected (theoretical) mass fragments. In general, a protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen *et al*., 1997). The tryptic mass fragments identified from the plant- and microbially derived Vip3Aa20 that matched the expected tryptic peptides generated *in silico* covered ~40% and 45%, respectively, of the predicted total Vip3Aa20 amino acid sequence. The identified peptides were representative of regions throughout the sequence of Vip3Aa20, including peptides close to the N- and C- termini.

(iii) N-terminal amino acid sequence analysis – Vip3Aa20 protein isolated from recombinant *E. coli* was immobilized on a membrane and subjected to N-terminal amino acid sequence analysis using Edman-based chemistry. The analysis confirmed the predicted amino acid sequence for the first twelve amino acids. The N-terminal sequence of the plant derived protein could not be determined due to technical difficulties. The Applicant states this is most likely due to N-terminal blockage of the protein.

Insect bioassays

The insecticidal activity of the plant- and *E. coli*-produced Vip3Aa20 protein was determined using an insect bioassay with freshly hatched first-instar fall armyworm (*S. frugiperda*). The bioassay included a range of concentrations of Vip3Aa20 protein overlayed on insect diet. Each treatment included 24 larvae that were allowed to feed for around nine days, when mortality was assessed.

The plant- and microbially-derived Vip3Aa20 showed comparable responses with the highest dose producing up to 100% and 88% mortality respectively, with LC_{50} values that were not significantly different by t-test (Table 6). No mortality was observed in negative controls using insect diet treated with water or buffer. These results indicate that the plant- and *E. coli*-produced Vip3Aa20 proteins can be regarded as equivalent in terms of their insecticidal activity.

Table 6: Comparative Bioactivity of plant and microbially-derived Vip3Aa20 in Diet Surface Bioassay with First-Instar *S. frugiperda*

Test Substance	**LC_{50} [ng Vip3Aa20/cm^2] (95% Confidence Intervals)**
Plant derived Vip3Aa20 MIR162VIP3A-0106	225 ng/cm^2 (155 - 289)
E. coli derived Vip3Aa20 LPMIR162-0106	318 ng/cm^2 (232 - 451)

Glycosylation analysis

To assess whether post-translational glycosylation of the plant-produced Vip3Aa20 protein occurred, the purified protein sample was subjected to glycosylation analysis. Creatinase, a non-glycosylated protein was included as a negative control. Transferrin, known to be a glycosylated protein, was used as a positive control. The test and control protein samples were separated by SDS-PAGE and protein-bound glycan moieties were labelled with digoxigenin and detected with an anti-digoxigenin antibody coupled to alkaline phosphatase. The transferrin protein produced a clearly visible band of approximately the expected molecular weight upon DIG Glycan analysis. No signal was detected for either the creatinase control or the plant-produced Vip3Aa20 protein. As prokaryotic organisms lack the capacity for protein glycosylation, the *E. coli*-produced Vip3Aa20 protein is expected to act as a negative control. This was confirmed in a similar, subsequent experiment.

These results indicate that the plant-produced Vip3Aa20 protein is not glycosylated and is equivalent to the *E. coli*-produced Vip3Aa20 protein in terms of its lack of glycosylation.

Conclusion

A large number of studies have been done on the Vip3Aa20 protein to confirm its identity and physicochemical and functional properties as well as to determine its equivalence to *E. coli*-produced Vip3Aa20 protein. Vip3Aa20 produced by MIR162 corn and recombinant *E. coli* were both shown to have the predicted molecular weight of ~89 kDa and both immunologically cross-reacted with the same anti-Vip3A antibody in Western blot analysis. Peptide mass mapping analysis provided additional evidence of the identity and equivalence of Vip3Aa20 expressed in MIR162 corn and in recombinant *E. coli*. Vip3Aa20 protein from both sources had comparable bioactivity against sensitive lepidopteran larvae. Analysis of protein glycosylation supports the conclusion that Vip3Aa20 protein produced by MIR162 corn and recombinant *E. coli* is not glycosylated.

The studies support the conclusion that Vip3Aa20 is expressed as expected in MIR162 corn, and further, that the Vip3Aa20 produced by recombinant *E. coli* is a suitable surrogate for the plant produced protein.

PMI

Study submitted:
Stacy, C. and Graser, G. (2006) Characterization of Phosphomannose Isomerase (PMI) Produced in Event MIR162 Maize and Comparison to PMI as Contained in Test Substance PMI-0198. Syngenta Seeds Biotechnology Report No. SSB-037-06.

The size, immunoreactivity and specific enzymatic activity of PMI extracted from MIR162 corn leaf tissue was compared to PMI purified from recombinant *E. coli.* PMI protein was extracted from a homogenate of leaf tissue from greenhouse- grown MIR162 corn hybrids A negative control substance was prepared from leaf material from near-isogenic, non-transgenic corn hybrids.

The PMI protein isolated from recombinant *E. coli* from an over-expression system is predicted to be identical in amino acid sequence to PMI expressed in MIR162 corn, with the exception of an additional, non-functional 16 amino acids at the N-terminus (comprising a 13 amino acid T7-Tag® to facilitate purification and 3 amino acids arising from the polylinker). *E. coli* produced PMI was purified by extraction and differential ammonium sulphate concentration, followed by chromatography and ion exchange chromatography.

Protein identity

The molecular weight and immunoreactivity of PMI produced by MIR162 corn was determined by Western blot analysis. The plant-produced PMI protein and the *E. coli*-produced reference standard were subjected to SDS-PAGE on the same gel. After blotting, goat anti-PMI polyclonal antibodies, raised against recombinant *E. coli* produced PMI, and donkey anti-goat IgG linked to alkaline phosphatase were used to bind to the primary antibody and visualized by development with an alkaline phosphatase substrate solution. The Western blot was examined for the presence of immunoreactive PMI. A single immunoreactive PMI band corresponding to the predicted molecular weight of ~42.8 kDa was observed in the MIR162-derived plant extract. The microbially expressed PMI protein showed one major band with a slightly lower mobility than the plant expressed PMI protein, which is consistent with its slightly higher predicted molecular weight of ~44.4 kDa, resulting from the addition of 16 amino acids at the N-terminus. A second minor immunoreactive band of ~90 kDa was also observed for *E. coli* derived PMI. The Applicant states that, as this protein cross-reacts with the anti-PMI antibody and shows the mobility of the molecular weight of two PMI proteins, it likely represents a dimer of the PMI protein.

Enzymatic Activity

The enzymatic activity of plant- and microbially-derived PMI was measured in triplicate in a coupled enzyme activity assay, as shown diagrammatically below.

PMI **PGI** **G6PDH**
Mannose 6-P → Fructose 6-P → Glucose 6-P + NADP → 6-Gluconolactone + NADPH
(PMI = Phosphomannose isomerase; PGI = Phosphoglucose isomerase;
G6PDH = Glucose 6-phosphate dehydrogenase)

Briefly, protein extract was added to an assay mixture containing NADP, mannose-6-hosphate, PGI and G6PDH. PMI activity was measured by monitoring the formation of NADPH at 340 nm on a spectrophotometer. The specific activity of PMI was determined using the known molar extinction coefficient of NADPH. The concentration of PMI added to the assay was determined, with one unit of PMI defined as 1 µmol of NADP reduced to NADPH per minute.

The mean specific activity for PMI extracted from MIR162 corn was 55.5 ± 4.18 U/mg PMI, while for microbially-produced PMI it was 33.2 ± 2.37 U/mg PMI. The Applicant concludes that, considering the expected levels of variability in the measurements, the PMI extracted from MIR162 corn has similar activity to PMI from recombinant *E. coli*.

Glycosylation potential

The Applicant states that it is unlikely that PMI expressed in MIR162 corn is post-translationally glycosylated. The PMI protein contains no consensus amino acid sequences for N-glycosylation, although O-glycosylation could theoretically occur at the serine or threonine residue present in the protein (Privalle, 2002). However, PMI is not expected to be post-translationally glycosylated in MIR162 corn, because its expression is not targeted to a cellular glycosylation pathway. Mass spectrometric analysis of human PMI indicates that this protein is also not post-translationally modified (Freeze, 2002). In addition, as the Western blot analysis shows that the plant-derived PMI has a clear higher mobility (indicating a lower molecular weight) than microbially-produced PMI (which has 16 additional amino acids at the N-terminus and therefore a higher molecular weight); a massive glycosylation (e.g. more than 10 gluc units) of the PMI in MIR162 corn can therefore be excluded.

Conclusion

Studies conducted on the PMI protein have demonstrated that the protein expressed in MIR162 corn conforms in size and immunoreactivity to that expected and also exhibits the expected enzymatic activity. The *E. coli*-produced protein was also shown to be equivalent to the plant produced proteins in terms of its size and immunoreactivity as well as having comparable enzymatic activity. The *E. coli*-produced proteins are therefore suitable as surrogates for the plant-produced protein for safety assessment purposes.

4.3 Protein expression analysis

Study submitted:
Hill, K. (2006) Quantification of Vip3Aa20 and phophomannose isomerise (PMI) in tissues of maize derived from transformation event MIR162. Syngenta Seeds Biotechnology Report No. SSB-020-06.

The expression levels of the two novel proteins (Vip3Aa20 and PMI) in corn line MIR162 were determined by ELISA. Plants from two MIR162 hybrid lines (referred to as MIR162-A and MIR162-B) were analysed. The hybrid plants were hemizygous for event MIR162 and are representative of the corn varieties that would (upon regulatory approval) be grown commercially. Two corresponding near-isogenic, non-transgenic control hybrids were also analysed.

Ten plants per transgenic genotype and two plants from each control genotype were harvested at each of four growth stages: V9-V12 (~8 weeks after planting), anthesis (pollen shed, 10-11 weeks), seed maturity (18-20 weeks) and senescence (23-24 weeks) from the two hybrids grown at two field locations (Illinois and Nebraska). Protein was extracted from samples of leaves, roots, kernels, silk, pollen, whole plant and silage and analysed quantitatively for Vip3Aa20 and PMI by ELISA using goat and rabbit polyclonal antibodies. ELISA values were corrected for extraction efficiency (estimated to be >78% and >75% for Vip3Aa20 and PMI respectively, for the various plant tissues analysed).

Vip3Aa20

The concentrations of Vip3Aa20 were generally similar between the two MIR162 hybrids for each tissue type at each stage sampled. The mean concentrations across the two locations for Vip3Aa20 protein, determined on a dry weight basis, are given in Table 7. Vip3Aa20 concentrations in the near-isogenic, non-transgenic negative control samples were either below the limit of detection or below the limit of quantification.

Table 7: Vip3Aa20 concentrations on a dry-weight basis in MIR162 plants (corrected for extraction efficiency)

	Mean µg Vip3Aa20/g DW Across Locations (range)			
Tissue	**V9-V12**	**Anthesis**	**Seed Maturity**	**Senescence**
Leaves	97.26 (76.12 – 119.12)	107.74 (97.10 – 118.80)	121.79 (77.25 – 159.66)	21.31 (12.93 – 30.28)
Roots	31.80 (28.10 – 35.65)	28.34 (26.30 – 30.20)	20.29 (9.87 – 27.48)	21.66 (11.58 – 32.13)
Pith	N/A	31.71 (29.43 – 36.18)	58.21 (52.74 – 63.68)	N/A
Kernels	N/A	N/A	43.56 (40.47 – 50.50)	34.24 (30.90 – 37.67)
Silk	N/A	97.40 (60.54 – 149.00)	N/A	N/A
Pollen	N/A	47.13 (41.45 – 53.52)	N/A	N/A
Whole Plants	91.53 (88.68 – 96.51)	67.61 (61.68 – 72.63)	49.04 (34.84 – 63.14)	34.30 (21.12 – 55.17)

N/A = Not analysed at this stage.

PMI

The concentrations of PMI were generally similar between the MIR162 hybrids for each tissue type at each time point. The mean concentrations across the two locations for PMI protein, determined on a dry weight basis, are given in Table 8. PMI concentrations in the near-isogenic, non-transgenic negative control samples were below the limit of detection.

Table 8: PMI concentrations on a dry-weight basis in MIR162 plants (corrected for extraction efficiency)

	Meaṇ µg PMI/g DW Across Locations (range)			
Tissue	**V9-V12**	**Anthesis**	**Seed Maturity**	**Senescence**
Leaves	11.12 (8.26 – 16.76)	9.75 (6.92 – 14.68)	5.77 (4.57 – 7.55)	<0.26 (<LOD – <LOQ)
Roots	4.32 (3.17 – 7.08)	3.49 (2.51 – 5.22)	1.99 (1.08 – 3.09)	1.51 (0.47 – 2.53)
Pith	N/A	2.01 (1.53 – 2.40)	2.75 (2.36 – 3.19)	N/A
Kernels	N/A	N/A	1.93 (1.33 – 2.54)	0.75 (0.54 –0.97)
Silk	N/A	20.70 (12.60 – 27.16)	N/A	N/A
Pollen	N/A	5.29 (3.82 – 7.62)	N/A	N/A
Whole Plants	8.74 (7.49 – 9.75)	7.10 (6.32 – 7.61)	3.76 (2.16 – 5.37)	2.36 (1.85 – 3.09)

Means preceded by '<' (less than) indicate that the estimated LOQ or LOD was used to represent the samples when calculating the average.
N/A = Not analysed at this stage.

Conclusion

Kernels from MIR162 corn are the most likely tissue to be used as food, either as grain or grain by-products. Both novel proteins are expressed in the grain of MIR162 corn. The average Vip3Aa20 level in mature kernels from MIR162 corn was 43.56 µg/g DW. The average PMI level in mature MIR162 corn kernels was 1.93 µg/g DW. Assuming corn grain contains 10% total protein by weight, Vip3Aa20 represents less than 0.004% of total protein, and PMI represents less than 0.0002% of total protein by weight.

4.4 Potential toxicity of novel proteins

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

4.4.1 History of use

Vip3Aa20

Study submitted:
Graser, G and Song, S. (2006) Analysis of Vip3A or Vip3A-like proteins in six different commercial microbial *Bacillus thuringiensis* products. Syngenta Seeds Biotechnology Report No. SSB-036-06.

B. thuringiensis, the microorganism from which the *vip3A* gene was derived, has been extensively studied and commercially exploited for over 40 years as the active ingredient in a number of insecticide products used in agriculture as well as home gardens. *B. thuringiensis* therefore has had a long history of safe use. Analysis of six of the commercially available *Bt*-based insecticides demonstrated that Vip3Aa or Vip3Aa-like proteins were present in all six *Bt* products, as analysed by ELISA. The products utilizing *Bt* subsp. *kurstaki* contained sufficient immunoreactive material to be detected by Western blot analysis, while levels in products containing *Bt* subsp *aizawi* had lower levels of detectable Vip3Aa-like proteins.

Products based on both *Bt* subsp. *kurstaki* and *aizawi* are available in Australia and New Zealand, for use both on commercial agricultural crops (including vegetables, fruits, vines and herbs) and for home garden use. Since *Bt* products have been used to control lepidopteran larvae on a wide variety of crops, including fresh market products, it is likely that small quantities of Vip3Aa of Vip3Aa-like proteins are present in the non-GM food supply.

PMI

As PMI proteins are ubiquitous in nature, including being present in food plants and animals, it is likely that small amounts of PMI proteins from various sources have always been present in food. PMI proteins have been found in food plant species such as Brassica species (Chen *et al*., 1989), and in seeds of soybeans and other legumes (Lee and Matheson, 1984). Genes encoding putative PMI proteins have been characterised from a number of other food organisms, including yeast and pigs (Proudfoot *et al*., 1994). The presence of PMI proteins in a variety of food sources supports a history of safe use of PMI proteins.

4.4.2 Similarities with known protein toxins

Vip3Aa20

Study submitted:
Harper, B. (2007) Vip3Aa20: Assessment of amino acid sequence homology with known toxins. Syngenta Seeds Biotechnology Report No. SSB-116-07.

Bioinformatic analysis was done to assess the Vip3Aa20 protein for any amino acid sequence similarity with known protein toxins. The Vip3Aa20 sequence (789 amino acids, Entrez Accession No. ABG20429) was systematically compared with the latest posting of the National Center for Biotechnology Information (NCBI) Entrez Protein Database (NCBI, 2007) using the BLASTP program (Altschul *et al*., 1997). The NCBI Entrez protein database is a public database containing over 5 million sequences, representing a good source to identify any potential protein toxin homologies.

The BLASTP program is frequently used for searching protein sequences for sequence similarities. The BLAST algorithm searches for short stretches or domains of sequence similarity by performing local alignments. This detects more similarities that would be found using the entire query sequence length.

The analysis was designed to identify any proteins in the database that showed significant amino acid sequence identity to Vip3Aa20, indicating they may be closely related to Vip3Aa20, and whether any proteins with significant sequence identity to Vip3Aa20 are known to be toxins, indicating possible implications for the toxic potential of Vip3Aa20. All database sequences with an Expect value (E-value) of 10 or lower were identified by default by the BLASTP program. The E-value reflects the degree of amino acid similarity between a pair of sequences and can be used to evaluate the significance of the alignment. Comparisons between highly homologous proteins yield E-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. Typically, alignments between two sequences will need to have an E-value of less than 1×10^{-5} for them to be considered to have significant homology.

A threshold E-value was calculated below which similarity to the query sequence (Vip3Aa20) is considered to be statistically significant and not a random result. This was done by randomly shuffling the primary Vip3Aa20 sequence to create five different 789 amino acid sequences and using these as target sequences. The shuffled sequences all have the same overall amino acid composition as Vip3Aa20 but are unlikely to have amino acid sequence similarity with either Vip3Aa20 or proteins in the NCBI Entrez protein database. Analysis of these target sequences provides an estimate of the background incidence of random hits that can be expected for any sequence with the same amino acid composition as Vip3Aa20. The search results using the five shuffled versions of Vip3Aa20 sequence identified hits with E-values that ranged from 8×10^{-8} to 8.0. Therefore, a score of 8×10^{-8} was set as the threshold, and scores below this were considered to represent proteins with significant amino acid sequence similarity and were those proteins were evaluated for biological significance.

The analysis returned 34 entries with E-values below 8×10^{-8}. All identified proteins were identified as vegetative insecticidal proteins, of which 31 were from *B. thuringiensis.* The analyses did not demonstrate any significant similarity between the Vip3Aa20 protein and other proteins that may potentially be toxic to humans or other animals.

PMI

Study submitted:
Harper, B. (2007) Phosphomannose isomerise protein (Entrez accession number AA24109): Assessment of amino acid sequence homology with known toxins. Syngenta Seeds Biotechnology Report No. SSB-117-07.

To determine whether the PMI protein sequence has any significant homology with known protein toxins, the PMI sequence (391 amino acids) was systematically compared to the latest posting of the National Centre for Biotechnology Information (NCBI) Entrez Protein Database (NCBI, 2007) containing all the publicly available protein sequences, as described in the preceding section.

Using five randomly shuffled versions of the PMI sequence (as described above for Vip3Aa20) the appropriate cut-off E-value was determined to be 0.51 and amino acid sequences with E values lower than this were considered to be significantly similar and were evaluated for biological significance.

The database search returned 332 entries with E-values below 0.51. Of these entries, 271 were identified as known or putative phosphomannose isomerise enzymes from 178 species (E-values from 0.0 to 0.39)

The PMI query sequence showed no significant sequence similarity to any proteins identified as, or known to be, toxins.

4.4.3 Digestibility

See Section 4.5.

4.4.4 Acute oral toxicity study

Proteins which cause toxicity act via acute mechanisms and generally at very low doses (Sjoblad *et al.*, 1992). Therefore, when a protein demonstrates no acute oral toxicity at a high dose level using a standard laboratory mammalian test species, this supports the determination that the protein will be non-toxic to humans and other mammals, and will not present a hazard under any realistic exposure scenario, including long-term exposures.

Acute oral toxicity studies using mice were conducted to examine the potential toxicity of the Vip3Aa20 and PMI proteins. As it is very difficult to extract and purify sufficient quantities of the subject protein from transgenic corn plants for the acute oral toxicity studies, it has become standard practice to instead use equivalent proteins that have been produced using bacterial expression systems. For these studies, *E. coli*-produced Vip3Aa20 and PMI proteins were used as the test substances. The equivalence of the *E. coli*- and MIR162 corn-produced proteins was established using a range of methods including Western blot analysis, peptide mass mapping analysis, bioactivity assays and glycosylation analysis (see Section 4.2).

<u>Vip3Aa20</u>

Study submitted:
Draper, C. (2007) MIR162 VIP3A-0106 single dose oral toxicity study in mice. Central Toxicology Laboratory Study No. AM7543-REG. Macclesfield, Cheshire, UK.

Test material	Vip3Aa20 protein preparation produced in *E. coli* (MIR162VIP3A-0106, 84% purity)
Vehicle	Corn oil
Test Species	Alpk:AP$_f$CD-1 mice (five males and five females per group)
Dose	1250 mg Vip3Aa20 protein/kg body weight (1488 mg test substance/kg bw) dosed orally by gavage
Control	vehicle only

The mice received 1250 mg/kg bw Vip3Aa20 as two fractions (each 2.5 mL) dosed 2 hours apart on a single day. This was the highest dose that could be physically administered to the animals, because of limitations of solubility and viscosity. Mice were observed for two weeks. Clinical observations, body weights and food consumption were measured daily throughout the study. At the end of the scheduled study period (day 15), the animals were killed and examined post mortem. Cardiac blood samples were taken for clinical pathology (haematology and blood clinical chemistry). Selected organs (adrenal glands, brain, epididymides (males), heart, ovaries (females), kidneys, liver, spleen and testes (males)) were weighed. Specified tissues were examined in situ and processed for subsequent histopathology examination.

There were no mortalities and no test substance-related clinical signs in either males or females. Diarrhoea was observed in both the control and treated groups in both sexes on days 1 and 2 and was considered to be due to the use of corn oil as a vehicle/control substance.

The body weights of treated females were significantly higher on days 2 and 3, by 3% and 2% respectively compared to controls. Body weights were not statistically significantly higher on any of the other 12 study days. The slight increases on days 2 and 3 are not considered to be adverse and are considered to be sporadic differences incidental to treatment.

There were no obvious differences in food consumption between the control and treated groups in either sex.

There were no statistically significant differences in haematology parameters between treated and control animals.

There were no statistically significant differences in blood clinical chemistry parameters between treated and control females, whereas in males the group mean plasma aspartate aminotransferase level was statistically significantly lower in the treated group compared to the control (51.0 vs. 77.8). However, the group mean value for the test group was within the historical control range of means (43.8-59.6) while the group mean value for the control group was higher than seen in the historical database. Although the difference is statistically significant, it is considered to be unrelated to treatment. No statistically significant differences in other blood clinical chemistry parameters or organ pathology were observed.

There were no statistically significant differences in organ weights between the treated and control animals.

One treated male was observed to have pelvic dilation of the kidney. This is considered to be spontaneous in nature and unrelated to treatment. This finding has been seen previously in control animals of this strain. One control and one treated male were observed to have hydronephrosis of the kidney. This microscopic finding is synonymous with the macroscopic observation of pelvic dilation of the kidney. Hydronephrosis has previously been seen in control animals of this strain and is considered to be unrelated to treatment.

In summary, the administration of 1250 mg Vip3Aa20/kg bw to mice produced no adverse effects on clinical condition, body weight, food consumption, clinical pathology, organ weights, macroscopic or microscopic pathology that were considered to be related to Vip3Aa20.

PMI

Study submitted:
Kuhn, J.O. (1999) Phosphomannose isomerise (sample PMI-098): Acute oral toxicity study in mice. Stillmeadow Inc Study No. 4708-98.

Test material	Phosphomannose isomerase preparation from *E. coli* (60% phosphomannose isomerase enzyme)
Vehicle	0.5% w/v aqueous carboxymethyl cellulose
Test Species	HSD:ICR albino mice (seven males and six females)
Dose	5050 mg/kg bw (equivalent to 3080 mg/kg bw PMI protein) in two gavage doses, 1 hour apart.
Control	vehicle only

The mice received a single dose of 3080 mg/kg bw PMI and were observed for two weeks. Parameters evaluated included body weights and detailed clinical observations. At the end of the study all animals were killed and examined post mortem. Brain, liver, kidneys and spleen were weighed.

One male in the control group and two in the test group died shortly after dosing or were in distress after dosing and subsequently died. Necropsy revealed perforated oesophagi in these animals, a sign of gavage error and not test-substance related. One replacement animal was available for each group and dosed in the same manner on day 0. There was no test article-related mortality during the study.

No clinical signs of toxicity were observed in either group. There were no test-substance related effects on body weight, organ weights or gross pathology.

Under the conditions of this study, the acute oral LD_{50} of the PMI protein in mice is greater than 3080 mg /kg bw.

4.4.5 Conclusion

The data from the bioinformatics analyses and acute oral toxicity studies, together with evidence of a history of presence of the novel proteins in food, indicate that neither of the proteins is likely to be toxic to mammals, including humans.

4.5 Potential allergenicity of novel proteins

While the majority of food allergens are proteins, only a small fraction of the many proteins in foods are allergenic, therefore the likelihood of a novel protein being allergenic is small. However, as the use of gene technology can result in additional protein diversity being added to the food supply, the potential allergenicity of any novel protein present in a GM food is considered as part of the safety assessment.

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

4.5.1 Source of novel proteins

Vip3Aa20

The Vip3Aa20 protein is derived from *B. thuringiensis* strain AB88. *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 (Nester *et al*., 2002).

PMI

The gene encoding PMI was initially isolated from *E. coli*, which has no history of allergenicity.

4.5.2 Similarity to known allergens

A comparison of the amino acid sequence of the introduced proteins to known protein allergens is one of the steps in a multilevel decision tree to assess allergenic potential (Metcalfe *et al.*, 1996).

Vip3Aa20

Study submitted:
Harper, B. (2007) Vip3Aa20: Assessment of amino acid sequence homology with known allergens. Syngenta Seeds Biotechnology Report No. SSB-102-07.

To determine whether Vip3Aa20 has significant sequence identity to proteins known or suspected to be allergens, the amino acid sequence of Vip3Aa20 (Entrez Accession Number ABG20429) was compared to the Syngenta Biotechnology, Inc. (SBI) Allergen Database (version 5.0, 2006). The SBI Allergen Database, containing 1735 non-redundant entries, was compiled from entries in public protein databases identified as allergens or putative allergens. Additional amino acid sequences of proteins identified as allergens or putative allergens in the scientific literature were also included in the database.

Two approaches were used to identify potential similarity between Vip3Aa20 and known or potential allergens. In the first approach, sequential 80-amino acid peptides of the Vip3Aa20 protein sequence were compared to the protein sequences in the SBI Allergen Database using the FASTA search algorithm to assess structural similarity.

Any matches of greater than 35% amino acid identity were identified as having significant identity to the allergen sequence. Related protein sequences are considered to be potentially cross-reactive if linear identity is 35% or greater in a segment of 80 or more amino acids (FAO/WHO, 2001). No comparisons met this criterion, leading to the conclusion that Vip3Aa20 does not have significant sequence homology with any known allergenic protein.

In the second approach, the Vip3Aa20 protein sequence was also screened for any matches of eight contiguous amino acids with any allergen sequences in the SBI Allergen Database. This screens for short, local regions of amino acid identity that might indicate the presence of common T-cell binding epitopes. There were no matches of eight contiguous amino acids, representing putative IgE binding epitopes, between Vip3Aa20 and any entry in the SBI Allergen Database.

PMI

Study submitted:
Harper, B. (2007) Phosphomannose isomerise (Entrez accession number AA24109): Assessment of amino acid sequence homology with known allergens. Syngenta Seeds Biotechnology Report No. SSB-101-07, including Report by C. Hilger (2004) titled 'Evaluation of Syngenta Protein Samples POI1 and POI2 for Reactivity with Serum IgE from a Petient Allergic to Frog Alpha-Parvalbumin from Rana Species'.

To determine whether PMI has any significant homology with allergenic proteins, the PMI protein sequence was systematically compared to the SBI Allergen database (version 5.0, 2006).

Overall similarity was examined by comparing sequential 80-amino acid sequences covering the entire PMI protein sequence to the allergen sequences using the FASTA search algorithm. Any 80-amino acid peptide having greater than 35% amino acid identity was defined as having significant similarity to the allergen sequence (FAO/WHO, 2001). There was no significant similarity between any of the sequential PMI 80-amino acid peptides and any entries in the database, indicating that PMI does not share overall sequence homology with any known allergenic protein.

The PMI sequence was also screened for matches of eight or more contiguous amino acids to identify any short local regions of identity that might indicate the presence of common IgE binding epitopes. There was one region of eight identical amino acids between PMI and the known allergen α-parvalbumin from *Rana species* CH2001 (Entrez Accession Number CAC83047) (Hilger *et al*., 2002).

One case of severe food-induced anaphylaxis in a single individual who consumed Indonesian frogs legs has been shown to be due to the protein α-parvalbumin from *Rana species* (Hilger *et al*., 2002). The same protein from *Rana esculenta* (the common edible frog) elicited no response in serum from the same individual.

To determine if IgE antibodies present in this patient's serum recognised PMI, Syngenta sent a sample of PMI to Dr Hilger's laboratory for cross-reactivity analysis (Hilger, 2004). The PMI sample used in the serum screen analysis was the bacterially derived PMI that had been shown to be a suitable surrogate for plant-derived PMI protein (see Section 4.2).

No cross reactivity between the human serum IgE and PMI occurred. This indicates that the allergic patient's serum IgE does not recognise any portion of the PMI protein as an allergenic epitope. Therefore, the short stretch of sequence similarity between PMI and *Rana species* α-parvalbumin is not biologically relevant and has no implications for the potential allergenicity of PMI.

4.5.3 Digestibility

Resistance to hydrolysis by digestive proteases has been observed in several food allergens (Astwood and Fuchs, 1996), therefore a correlation exists between resistance to digestion by pepsin and allergenic potential. As a consequence, one of the criteria for assessing potential allergenicity is to determine the stability of novel proteins in conditions mimicking human digestion. Proteins that are rapidly degraded in such conditions are considered less likely to be involved in eliciting an allergic response. A pepsin digestibility assay was conducted to determine the digestive stability of the Vip3Aa20 and PMI proteins.

In addition to the pepsin protocol using simulated gastric fluid (SGF), a second digestibility study was done using simulated intestinal fluid (SIF) containing pancreatin, which is a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease. The relevance of the SIF study however is limited because ordinarily an ingested protein would first be exposed to pepsin-mediated hydrolysis in the acidic environment of the stomach before being subject to further digestion in the small intestine.

<u>Vip3Aa20</u>

Simulated gastric fluid study

Study submitted:
Stacy, C. (2007) *In vitro* digestibility of Vip3Aa20 under simulated mammalian gastric conditions. Syngenta Seeds Biotechnology Report No. SSB-038-06.

The *in vitro* digestibility of Vip3Aa20 protein in SGF containing pepsin was evaluated by SDS-PAGE and Western blot analysis. Vip3Aa20 protein affinity purified from MIR162 corn was assessed, as was Vip3Aa20 derived from recombinant *E. coli*.

Digestibility of the protein in SGF was measured by taking samples at selected time points (0, 1, 2, 5, 10, 20, 30 and 60 minutes) and subjecting these to SDS-PAGE. Proteins were visualized by staining the gel or by transferring the protein to a membrane for Western blot analysis. No intact microbially-derived Vip3Aa20 protein was detectable by Western blot after one minute exposure to SGF. No intact plant-derived Vip3Aa20 was evident after two minutes exposure to SGF, as assessed by Western blot.

The plant-derived Vip3Aa20 preparation showed an additional band of ~60kDa after one minute incubation with SGF, but this band was not visible after 2 minutes digestion. It likely represents a degradation product of Vip3Aa20. Faint, immunoreactive, low molecular weight bands (~8 kDa) were observed following one minute incubation in SGF.

These bands, that likely represent proteolytic degradation products of Vip3Aa20, gradually decreased in intensity following longer incubations with SGF.

Simulated intestinal fluid study

Study submitted:
Stacy, C. (2007) *In vitro* digestibility of Vip3Aa20 (MIR162VIP3A-0106) under simulated mammalian intestinal conditions. Syngenta Seeds Biotechnology Report No. SSB-002-07.

The digestibility of Vip3Aa20 protein in SIF containing pancreatin was assessed using Western blot analysis.

Digestibility of Vip3Aa20 in SIF was measured by incubating microbially-produced Vip3Aa20 with SIF, taking samples at selected time points (0, 5, 15, 30 minutes, 1, 2, 3, 6, 24 and 48 hours), subjecting these to SDS-PAGE and also Western blot analysis using immunoaffinity-purified polyclonal antibodies. The full length Vip3Aa20 was rapidly digested, with no intact protein detectable after 5 minutes of incubation in SIF. A proteolytic fragment of approximately 62 kDa was observed at the 5 minute time point. After 15 minutes digestion a second proteolytic fragment of ~55 kDa was also visible on the Western blot. The intensity of these bands slowly decreased following longer digestion times, but both bands were still detectable after 48 hours digestion. The intensity of these bands is low relative to the initial, intact Vip3Aa20 band, indicating that the majority of Vip3Aa20 is rapidly digested in SIF.

PMI

Study submitted:
Privalle, L. (1999) *In vitro* digestibility of PMI protein under simulated mammalian gastric and intestinal conditions. Novartis Seeds Biotechnology Report No. NSB-002-99.

PMI was digested in simulated gastric fluid containing pepsin and in simulated intestinal fluid containing pancreatin. Samples were examined by SDS-PAGE. PMI was degraded rapidly by pepsin: no PMI was detected by SDS-PAGE upon immediate sampling of the reaction mix (0 seconds). When the pepsin was diluted to 0.0001X of the standard concentration, no PMI remained after 10 minutes of incubation. Similarly, no PMI enzymatic activity was detectable after 10 minutes under these conditions.

PMI was degraded by pancreatin in simulated intestinal fluid after two minutes. In the unlikely event that PMI survived digestion by pepsin, it would be digested in the mammalian intestinal environment by pancreatin.

4.5.4 Thermolability

Vip3Aa20

Study submitted:
Stacy, C. (2007) Effect of temperature on the stability of Vip3Aa20 protein. Syngenta Seeds Biotechnology Report No. SSB-039-06.

The effect of temperature on Vip3Aa20 was determined by incubation for 30 minutes at a range of temperatures (4ºC, 25ºC, 37ºC, 65ºC, and 95ºC) followed by an insect bioassay against fall armyworm (*S. frugiperda*) larvae. Vip3Aa20 exposed to different temperatures were incorporated at increasing concentrations into insect diet and assessed for insecticidal activity using freshly hatched larvae. No reduction in Vip3Aa20 bioactivity was observed following incubation at 25ºC or 37ºC compared to 4ºC. Incubation at 65ºC and 95ºC resulted in very low mortality, indicating a loss of bioactivity. The data support the conclusion that Vip3Aa20 is unstable upon heating at temperatures of 65ºC and above.

PMI

Study submitted:
Hill, K. (2003) Effects of temperature on the stability of phosphomannose isomerise. Syngenta Seeds Biotechnology Report No. SSB-013-03.

The heat stability of PMI was evaluated. Loss of enzyme activity was used to determine the instability of the protein after exposure to various temperatures (4, 25, 37, 55, 65 and 95ºC) for 30 minutes. Incubation at ambient temperature (25ºC), or 37ºC for 30 minutes had little effect on enzyme activity. At 55 ºC, approximately half the enzymatic activity was lost. Incubation at 65ºC and 95ºC essentially inactivated the protein.

4.5.5 Conclusion

A range of information and data have been provided to assess the potential allergenicity of the two novel proteins in MIR162 corn. Both the Vip3Aa20 and PMI proteins are sourced from bacteria with a history of human exposure and no association with allergenicity. Bioinformatic analysis indicates that Vip3Aa20 does not share any significant sequence similarity with known or suspected allergens. The PMI protein sequence was found to share a region of sequence identity of eight contiguous identical amino acids with a known allergen, the protein α-parvalbumin from *Rana species*. However, further analysis using serum from the single individual known to have demonstrated IgE-mediated allergy to this specific α-parvalbumin did not indicate any cross-reactivity with the PMI protein. Both the Vip3Aa20 and PMI proteins are rapidly degraded in simulated mammalian gastric fluid and both are labile upon heating to temperatures of 65ºC and above. The weight-of-evidence indicates that the two novel proteins in MIR162 corn are unlikely to allergenic.

4.6 Conclusion

MIR162 corn expresses two novel proteins, Vip3Aa20 and PMI. Vip3Aa20 is expressed at moderate levels in the grain, with a mean concentration of 44 µg/g dry weight. PMI is expressed at relatively low levels in the grain, with a mean concentration of 2 µg/g dry weight.

A large number of studies have been done with the Vip3Aa20 and PMI proteins to confirm their identity and physicochemical and functional properties as well as to determine their potential toxicity and allergenicity. These studies have demonstrated that both proteins conform in size and amino acid sequence to that expected.

Vip3Aa20 does not exhibit any post-translational modification including glycosylation, and also demonstrate the expected insecticidal activity. PMI exhibited the expected enzymatic activity.

Bioinformatic studies with Vip3Aa20 have confirmed the absence of any significant amino acid sequence similarity to known protein toxins or allergens. Although PMI has a short region of sequence identity with a known allergen, subsequent analysis using human serum showed this to be of no biological relevance. Digestibility studies have demonstrated that both proteins would be rapidly degraded in the stomach following ingestion, similar to other dietary proteins. Acute oral toxicity studies in mice with both proteins have also confirmed the absence of toxicity in animals. The safety of the Vip3Aa protein is further supported by the long history of safe use of *Bt* microbial pesticides. Taken together, the evidence indicates that both proteins are unlikely to be toxic or allergenic to humans.

5. COMPOSITIONAL ANALYSES

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

The classic approach to the compositional analysis of GM food is a targeted one; rather than analysing every single constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question (FAO, 1996). The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes).

In the case of corn, the key components that should be considered in the comparison include protein, fat, carbohydrate, amino acids, fatty acids, vitamins, minerals, and the anti-nutrient phytic acid (OECD, 2002).

Most crops exhibit considerable variability in their nutrient composition. Environmental factors and the genotype of the plant have an enormous impact on composition. Variation in these nutrient parameters is a natural phenomenon and is considered to be normal. Therefore, in addition to a comparison of the composition of the GM food to a closely related non-GM control, further comparison is made to the range of natural variation found in the conventional (non-GM) food crop.

5.1 Study design and conduct

To determine whether unexpected changes have occurred in the composition of MIR162 corn as a result of the modification, and to assess its nutritional adequacy, compositional analyses were done on forage and grain samples collected from MIR162 transgenic hybrid grown under field conditions with a non-transgenic, near-isogenic hybrid as the conventional control.

The MIR162 corn hybrid used for this study is a hybrid between the BC_4F_4 generation (MIR162 progenitor backcrossed 4x to inbred line NP2276 then self-fertilised 4x) and a conventional inbred corn line, NP2391 (see Section 3.4). The MIR162 hybrid is designated NP2276(MIR162)/NP2391. The line used as the comparator for this study is a conventional NP2276/NP2391 hybrid, which has a genetic background representative of the MIR162 hybrid lines, but without the transferred genes.

Field trials were conducted in the United States in 2005 at six replicated sites. The field sites were located in regions of the U.S. that represent the agricultural regions where the hybrid varieties would typically be cultivated[3]. At each location, one hybrid pair, composed of the MIR162 hybrid and the corresponding non-transgenic hybrid, was grown in a randomized complete block design, with three replicates for each genotype. All the corn plants were grown according to local agronomic practices, including treatment of both hybrids with conventional pesticides as needed. Plants were self-pollinated by hand and the developing ears were bagged to avoid cross-pollination.

Selection of analytes for measurement in forage and grain was based on recommendations of the OECD (OECD, 2002) for comparative assessment of new varieties of corn. Forage and grain samples were analysed for 65 different components (7 in forage, and 56 in grain). Compositional analysis of the forage samples included proximates (ash, fat, moisture, protein and carbohydrates), acid detergent fibre (ADF), neutral detergent fibre (NDF) and minerals (calcium and phosphorus). For the grain samples, compositional analysis included proximates, ADF, NDF, total dietary fibre, amino acids, fatty acids, vitamins (A, B_1, B_2, B_6, E, niacin and folic acid), anti-nutrients (phytic acid, raffinose and trypsin inhibitor), secondary plant metabolites (furfural, ferulic acid and p-coumaric acid), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, selenium, sodium and zinc) and inositol. All compositional analyses were conducted using methods published and approved by AOAC International, or other specified industry-standard analytical methods.

Following compositional analysis, the results were statistically analysed using analysis of variance across locations for the hybrid pair. For each component analysed for MIR162 corn, the result was compared with that from the conventional counterpart. The statistical significance of the difference between MIR162 and the control mean values was determined using a standard F-test. An F-test probability of <5% indicates that the difference between the genotypes was statistically significant at the 5% level.

[3] The sites used were: Stanton, Minnesota; Janesville, Wisconsin; Alleman, Iowa; Seward, Nebraska; Bloomington, Illinois; Bondville, Illinois.

An F-test was also used to assess the significance of a potential location x genotype interaction. A significant outcome (F-test probability <5%) indicates that the effect of genotype was not consistent across all locations, and undermines the validity of the comparison of genotypes across locations. In these cases, the average analyte levels measured at each location were compared to values reported in the literature. For all analytes measured, the average levels across locations were compared with the range of natural variation, as reported in the International Life Sciences Institute (ILSI) Crop Composition Database (ILSI, 2006) and the OECD consensus document (OECD, 2002).

The results of the comparisons of MIR162 and the conventional counterpart are presented in Tables 9 – 17.

Table 9: Proximate composition of forage from a MIR162 hybrid and a non-transgenic hybrid

		Moisture	Protein	Total Fat	Ash	Carbohydrates	ADF	NDF
		(% FW)	(% DW)	(% DW)	(% DW)	(% DW)	(% DW)	(% DW)
MIR162	average	71.2	7.2	1.5	4.1	87.3	28.2	43.2
	range	66.2-77.2	3.1-10.1	0.8-1.9	3.1-5.8	82.9-90.7	23.6-34.2	35.1-56.1
Non-transgenic	average	70.5	7.3	1.6	4.0	87.1	28.8	38.8
	range	64.1-75.8	3.4-8.9	0.4-2.3	2.9-5.7	83.6-91.7	23.3-34.8	32.1-46.9
Percent difference from non-transgenic hybrid[1]		0.99	-0.91	-5.71	1.11	0.13	-2.10	11.57
F-test Probability for Genotype[2]		0.184	0.711	0.421	0.796	0.694	0.635	***0.010***
F-test Probability for Location x Genotype Interaction		0.730	0.645	0.100	0.948	0.973	0.687	0.286
ILSI (2006)	average	70.2	7.78	2.039	4.628	85.6	27.00	41.51
	range	49.1 - 81.3	3.14 - 11.57	0.296 - 4.570	1.527 - 9.638	76.4 - 92.1	16.13 - 47.39	20.29 - 63.71
	N	945	945	921	945	945	945	945
OECD (2002)	range	62 - 78	4.7 - 9.2	1.5 - 3.2	2.9 - 5.7		25.6 - 34	40 - 48.2

[1] ((MIR162-non-transgenic)/non-transgenic)*100.
[2] Statistically significant F-test probability (<0.05) indicated in bold and italics type.

Table 10: Calcium and phosphorus composition of forage from a MIR162 hybrid and a non-transgenic hybrid

		Calcium	Phosphorus
		(mg/kg DW)	(mg/kg DW)
MIR162	average	2106	1997
	range	1720-2930	1270-2240
Non-transgenic	average	2039	2079
	range	1440-2620	1760-2560
Percent difference from non-transgenic hybrid[2]		3.24	-3.93
F-test Probability for Genotype[3]		0.484	0.153
F-test Probability for Location x Genotype Interaction		0.944	0.066
	average	2028.6	2066.1
ILSI (2006)	range	713.9 - 5767.9	936.2 - 3704.1
	N	481	481
OECD (2002)	range	0.15 - 0.31% DW	0.20 - 0.27% DW

[1] For conversion, 1% = 10,000 mg/kg.
[2] ((MIR162-non-transgenic)/non-transgenic)*100.
[3] Statistically significant F-test probability (<0.05) indicated in bold and italics type.

Table 11: Proximate composition of grain from a MIR162 hybrid and a non-transgenic hybrid

		Moisture[1]	**Protein**	**Fat**	**Ash**	**Carbohydrates**	**ADF**	**NDF**	**TDF**	**Starch**
		(% FW)	(% DW)	(% DW)	(% DW)	(% DW)	(% DW)	(% DW)	(% DW)	(% DW)
MIR162	average	10.3	9.8	3.8	1.4	85.0	5.0	11.7	16.8	63.1
	range	9.5-11.5	7.5-11.2	3.3-4.6	1.1-1.6	83.2-87.1	3.3-7.0	10.1-13.0	14.1-19.4	54.8-68.1
Non-transgenic	average	10.5	9.6	3.8	1.3	85.3	4.6	11.1	16.3	64.9
	range	9.4-12.0	7.1-11.0	3.0-4.4	1.1-1.5	83.3-88.1	3.3-6.2	9.5-12.8	14.3-17.8	60.6-69.2
Percent difference from non-transgenic hybrid[2]		-2.33	1.97	0.29	9.33	-0.36	7.70	5.32	3.18	-2.82
F-test Probability for Genotype[3]			0.103	0.904	***0.012***	0.062	0.154	***0.016***	0.092	***0.015***
F-test Probability for Location x Genotype Interaction			0.832	0.538	0.381	0.696	0.309	0.703	0.653	0.455
	average	11.3	10.30	3.555	1.439	84.6	4.05	11.23	16.43	57.7
ILSI (2006)	range	6.1 - 40.5	6.15 - 17.26	1.742 - 5.823	0.616 - 6.282	77.4 - 89.5	1.82 - 11.34	5.59 - 22.64	8.85 - 35.31	26.5 - 73.8
	N	1434	1434	1174	1410	1410	1350	1349	397	168
OECD (2002)	range	7.0 - 23	6 - 12.7	3.1 - 5.8	1.1 - 3.9	82.2 - 82.9	3.0 - 4.3	8.3 - 11.9	11.1	

[1] Moisture levels in grain not subject to analysis of variance as grain was mechanically dried after harvest.
[2] ((MIR162-non-transgenic)/non-transgenic)*100.
[3] Statistically significant F-test probability (<0.05) indicated in bold and italics type.

Table 12: Mineral composition of grain from a MIR162 hybrid and a non-transgenic hybrid

		Calcium	Copper	Iron	Magnesium	Manganese	Phosphorus	Potassium	Selenium[2,3]	Sodium[2,3]	Zinc
		(mg/kg DW)	(mg/kg DW)	(mg/kg DW)	(mg/kg DW)	(mg/kg DW)	(mg/kg DW)	(mg/kg DW)	(mg/kg DW)	(mg/kg DW)	(mg/kg DW)
MIR162	average	38.1	1.3	20.2	125	6.3	3173	3352	<LOQ - 0.414	<LOQ - <LOQ	21.7
	range	29.4-47.2	0.96-1.95	17.3-22.9	1090-1480	4.14-7.97	2810-3550	3160-3710	N/A	N/A	18.8-24.3
Non-transgenic	average	35.3	1.2	19.2	1218	6.1	3073	3357	<LOQ - 0.531	<LOQ - <LOQ	21.5
	range	25.7-44.0	1.00-1.58	15.7-22.5	960-1470	4.59-8.01	2710-3400	2950-3660	N/A	N/A	19.2-23.8
Percent difference from non-transgenic hybrid[4]		7.95	9.63	5.05	2.78	4.33	6.25	-0.15	N/A	N/A	1.01
F-test Probability for Genotype[5]		***0.002***	0.073	***0.011***	0.102	0.078	***0.030***	0.916			0.606
F-test Probability for Location x Genotype Interaction		0.148	0.360	0.685	0.288	0.697	0.136	0.291			0.326
	average	46.4	1.75	21.81	1193.8	6.18	3273.5	3842	0.2	31.75	21.6
ILSI[6] (2006)	range	12.7 - 208.4	0.73 - 18.5	10.42 - 49.07	594.0 - 1940.0	1.69 - 14.30	1470.0 - 5330.0	1810.0 - 6030.0	0.05 - 0.75	0.17 - 731.54	6.5 - 37.2
	N	1344	1249	1255	1257	1256	1349	1257	89	223	1257
OECD (2002)	range	3 -100	0.09 - 1.0	0.1 - 10	82 - 1000		234 - 750	320 - 720	0.001 - 0.1	0 - 150	1.2 - 3.0
		mg/100g DW	mg/100g DW	mg/100g DW	mg/100g DW		mg/100g DW	mg/100g DW	mg/100g DW	mg/100g DW	mg/100g DW

[1] For conversion, 1 mg/100g = 10 mg/kg.
[2] Where some values were <LOQ, statistical comparison was not possible so only the range is shown.
[3] LOQ for selenium 0.056-0.057 mg/kg DW, LOQ for sodium 110-114 mg/kg DW.
[4] ((MIR162-non-transgenic)/non-transgenic)*100.
[5] Statistically significant F-test probabilities (<0.05) indicated in bold and italics type.
[6] Below LOQ values are not included.

Table 13: Vitamin analysis of grain from a MIR162 hybrid and a non-transgenic hybrid

		Vitamin A[2]	Vitamin B1	Vitamin B2	Vitamin B3	Vitamin B6	Vitamin B9	Vitamin E
		ß-Carotene	**Thiamine**	**Riboflavin**	**Niacin**	**Pyridoxine**	**Folic Acid**	**a-Tocopherol**
		(mg/100g DW)	(mg/100g DW)	(mg/100g DW)	(mg/100g DW)	(mg/100g DW)	(mg/100g DW)	(mg/g DW)
MIR162	average	0.277	0.393	0.190	2.37	0.565	0.028	0.01
	range	0.241-0.316	0.358-0.433	0.112-0.238	2.11-2.83	0.434-0.694	0.021-0.034	0.97-1.54
Non-transgenic	average	0.294	0.392	0.180	2.47	0.605	0.028	0.01
	range	0.244-0.358	0.339-0.443	0.144-0.226	2.03-3.15	0.486-0.738	0.024-0.033	1.10-1.54
Percent difference from non-transgenic hybrid[3]		-5.78	0.03	5.69	-4.12	-6.55	-1.63	-5.46
F-test Probability for Genotype[4]		***0.001***	0.983	0.314	0.093	***0.002***	0.471	***0.016***
F-test Probability for Location x Genotype Interaction[4]		***0.048***	0.952	0.278	0.639	0.765	***0.036***	0.917
	average	0.684	0.530	0.125	2.376	0.644	0.0651	0.0103
ILSI (2006)	range	0.019 - 4.681	0.126 - 4.000	0.050 - 0.236	1.037 - 4.694	0.368 - 1.132	0.0147 - 0.1464	0.0015 - 0.0687
	N	276	894	704	415	415	895	863
OECD (2002)	range		2.3 - 8.6	0.25 - 5.6	9.3 - 70	4.6 - 9.6		
			mg/kg DW	mg/kg DW	mg/kg DW	mg/kg DW		

[1] For conversion, 1 mg/100g = 10 mg/kg.
[2] For direct comparison to literature values, original units of RE/g DW were converted to units of mg/100 g DW based on 1 RE = 6 µg beta-carotene. Institute for Laboratory Animal Research (1995).
[3] ((MIR162-non-transgenic)/non-transgenic)*100.
[4] Statistically significant F-test probabilities (<0.05) indicated in bold and italics type.

Table 14: Location means of ß-carotene and folic acid in grain from a MIR162 hybrid and a non-transgenic hybrid

		Vitamin A[1]	Vitamin B9
		ß-Carotene	Folic Acid
		(mg/100g DW)	(mg/100g DW)
Location 1	MIR162	0.283	0.027
	Non-transgenic	0.296	0.028
Location 2	MIR162	0.304	0.032
	Non-transgenic	0.352	0.029
Location 3	MIR162	0.272	0.027
	Non-transgenic	0.280	0.024
Location 5	MIR162	0.266	0.029
	Non-transgenic	0.286	0.032
Location 6	MIR162	0.292	0.024
	Non-transgenic	0.291	0.025
Location 8	MIR162	0.243	0.025
	Non-transgenic	0.260	0.029
	average	0.684	0.0651
ILSI (2006)	range	0.019 - 4.681	0.0147 - 0.1464
	N	276	895

[1] For direct comparison to literature values, original units of RE/g DW were converted to units of mg/100 g DW based on 1 RE = 6 µg beta-carotene.

Institute for Laboratory Animal Research (1995).

Table 15: Amino acid composition of grain from a MIR162 hybrid and a non-transgenic hybrid

		Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
		(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)
MIR162	average	6.66	3.55	5.21	19.54	9.12	3.84	7.70	2.31	4.81
	range	5.29-7.72	2.83-4.06	3.95-6.06	14.0-23.3	6.79-10.8	3.26-4.27	5.59-9.17	1.96-2.62	3.78-5.61
Non-transgenic	average	6.54	3.47	5.11	19.16	8.96	3.79	7.55	2.29	4.74
	range	4.85-7.45	2.64-3.96	3.68-5.84	13.2-22.5	6.51-10.3	3.13-4.10	5.24-8.89	1.96-2.65	3.52-5.37
Percent difference from non-transgenic hybrid[2]		1.82	2.31	1.92	2.00	1.80	1.39	1.94	0.92	1.42
F-test Probability for Genotype		0.171	0.059	0.149	0.129	0.119	0.269	0.135	0.545	0.247
F-test Probability for Location x Genotype Interaction		0.500	0.699	0.307	0.443	0.233	0.265	0.557	0.301	0.329
	average	6.88	3.75	5.12	20.09	9.51	3.85	7.90	2.21	4.90
ILSI (2006)	range	3.35 - 12.08	2.24 - 6.66	2.35 - 7.69	9.65 - 35.36	4.62 - 16.32	1.84 - 5.39	4.39 - 13.93	1.25 - 5.14	2.66 - 8.55
	N	1350	1350	1350	1350	1350	1350	1350	1350	1350
OECD (2002)	range	0.48 - 0.85	0.27 - 0.58	0.35 - 0.91	1.25 - 2.58	0.63 - 1.36	0.26 - 0.49	0.56 - 1.04	0.08 - 0.32	0.21 - 0.85
		% DW	% DW	% DW	% DW	% DW	% DW	% DW	% DW	%DW

[1] For conversion, 1% = 10 mg/g.
[2] ((MIR162-non-transgenic)/non-transgenic)*100.

Table 15 continued: Amino acid composition of grain from a MIR162 hybrid and a non-transgenic hybrid

		Met	Ile	Leu	Tyr[2]	Phe	Lys	His	Arg	Trp
		(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)
MIR162	average	2.15	3.38	12.85	3.42	5.09	3.05	2.87	4.77	0.570
	range	1.76-2.54	2.55-4.00	8.86-15.6	2.58-4.09	3.70-6.04	2.52-3.44	2.28-3.26	3.89-5.30	0.453-0.645
Non-transgenic	average	2.10	3.31	12.57	3.35	4.99	2.96	2.85	4.68	0.562
	range	1.71-2.42	2.35-3.85	8.28-15.1	2.35-3.86	3.43-5.84	2.47-3.29	2.20-3.14	3.64-5.27	0.479-0.636
Percent difference from non-transgenic hybrid[3]		2.84	2.23	2.26	2.02	2.05	2.96	0.78	1.96	1.43
F-test Probability for Genotype		0.177	0.113	0.115	0.201	0.128	0.095	0.438	0.276	0.349
F-test Probability for Location x Genotype Interaction		0.459	0.372	0.561	0.694	0.483	0.256	0.195	0.157	0.090
	average	2.09	3.68	13.41	3.36	5.25	3.15	2.96	4.33	0.627
ILSI (2006)	range	1.24 - 4.68	1.79 - 6.92	6.42 - 24.92	1.03 - 6.42	2.44 - 9.30	1.72 - 6.68	1.37 - 4.34	1.19 - 6.39	0.271 - 2.150
	N	1350	1350	1350	1350	1350	1350	1350	1350	1350
OECD (2002)	range	0.1 - 0.46	0.22 - 0.71	0.79 - 2.41	0.12 - 0.79	0.29 - 0.64	0.05 - 0.55	0.15 - 0.38	0.22 - 0.64	0.04 - 0.13
		% DW	% DW	% DW	% DW	% DW	% DW	% DW	% DW	%DW

[1] For conversion, 1% = 10 mg/g.
[2] One non-transgenic value was an outlier and was excluded from analysis.
[3] ((MIR162-non-transgenic)/non-transgenic)*100.

Table 16: Fatty acid[1] composition of grain from a MIR162 hybrid and a non-transgenic hybrid

		16:0 Palmitic	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic
		(% of total FA)	(% of total FA)	(% of total FA)	(% of total FA)	(% of total FA)
MIR162	average	12.78	1.84	25.49	56.99	1.81
	range	12.25-13.09	1.56-1.99	22.67-26.57	55.86-59.74	1.72-1.89
Non-transgenic	average	12.69	1.88	25.22	57.36	1.75
	range	12.29-13.12	1.625-2.07	23.38-26.77	56.26-59.47	1.64-1.86
Percent difference from non-transgenic hybrid[2]		0.73	-1.91	1.07	-0.65	3.21
F-test Probability for Genotype[3]		0.275	0.051	0.057	***0.026***	***<0.001***
F-test Probability for Location x Genotype Interaction		0.554	0.934	0.154	0.444	0.163
	average	11.50	1.82	25.8	57.60	1.20
ILSI (2006)	range	7.94 - 20.71	1.02 - 3.40	17.4 - 40.2	36.2 - 66.5	0.57 - 2.25
	N	1344	1344	1344	1344	1344
OECD (2002)	range	0.29 - 0.79% DW	0.04 - 0.17% DW	0.70 - 1.39% DW	0.67 - 2.81% DW	0.03 - 0.10% DW

[1] Five most abundant fatty acids (FA) in maize grain.
[2] ((MIR162-non-transgenic)/non-transgenic)*100.
[3] Statistically significant F-test probability (<0.05) indicated in bold and italics type.

Table 17: Secondary metabolite and anti-nutrient analysis of grain from a MIR162 hybrid and a non-transgenic, near isogenic hybrid

		Ferulic Acid	*p-Coumaric Acid*	Inositol	Phytic Acid	Trypsin Inhibitor	Furfural[2,3]	Raffinose[2,3]
		(mg/kg DW)	(mg/kg DW)	(ppm)	(% DW)	(TIU/mg DW)	(mg/kg DW)	(% DW)
MIR162	average	2682	179	2957	0.745	2.82	<LOQ - <LOQ	<LOQ - 0.116
	range	2490-2980	148-202	2410-3530	0.621-0.871	2.27-3.72	N/A	N/A
Non-transgenic	average	2453	157	2792	0.727	2.92	<LOQ - <LOQ	<LOQ - 0.137
	range	2010-2760	137-179	2180-3610	0.593-0.919	2.38-3.48	N/A	N/A
Percent difference from non-transgenic hybrid[4]		9.31	14.44	5.91	2.38	-3.56	N/A	N/A
F-test Probability for Genotype[5]		***<0.001***	***<0.001***	0.196	0.334	0.416	N/A	N/A
F-test Probability for Location x Genotype Interaction		0.436	0.073	0.459	0.589	0.671	N/A	N/A
	average	2201.1	218.4	1331.5	0.745	2.73	3.697	0.132
ILSI[6] (2006)	range	291.9 - 3885.8	53.4 - 576.2	89.0 - 3765.4	0.111 - 1.570	1.09 - 7.18	3.000 - 6.340	0.020 - 0.320
	N	817	817	504	1196	696	14	701
OECD (2002)	range	0.02 - 0.3% DW	0.003 - 0.03% DW		0.45 - 1.0		<0.01 ppm	0.21 - 0.31

[1] For conversion of ferulic acid, 0.02% DW = 200 mg/kg; of *p* -coumeric acid, 0.003% DW = 30 mg/kg; of furfural, 1ppm = 1 mg/kg.
[2] Where some values were <LOQ, statistical comparison was not possible so only the range is shown.
[3] LOQ for furfural 0.55-0.57 mg/kg DW, LOQ for raffinose 0.11-0.15% DW.
[4] ((MIR162-non-transgenic)/non-transgenic)*100.
[5] Statistically significant F-test probability (<0.05) indicated in bold and italics type.
[6] Below LOQ values are not included.

5.2 Forage

Compositional analysis of the forage samples included proximates, ADF, NDF and the minerals calcium and phosphorus. The forage analyses showed a single statistically significant difference for NDF between the MIR162 and non-transgenic hybrids. However, the mean value for NDF from the MIR162 hybrid was within the range of values observed for the non-transgenic hybrid. Also, the mean values for all analytes, including NDF, were within the range of natural variation reported in the literature (OECD, 2002; ILSI 2006).

5.3 Grain

Nutrients

Proximates

Results of the proximate analysis are shown in Table 11. Statistically significant differences between MIR162 and the non-transgenic comparator were observed for ash, NDF and starch. These differences were small (9.33, 5.32 and 2.82% respectively) and the mean values were within the range of values observed for the non-transgenic hybrid. Additionally, the mean values for all proximates were within the ranges reported in the literature (OECD, 2002; ILSI 2006). The differences, while statistically significant, are not considered to be biologically meaningful or nutritionally significant.

Minerals

Results of the mineral analysis are shown in Table 12. Statistically significant differences were noted for the minerals calcium, iron and phosphorus. These differences were small (7.95, 5.05 and 6.25% respectively). The mean values were within the ranges observed for the non-transgenic control line and within the ranges reported in the literature (OECD, 2002; ILSI 2006). The differences are not considered to be biologically significant.

Vitamins

Vitamin analyses are shown in Table 13. Statistically significant differences between MIR162 and the control line were observed in the levels of Vitamin A (β-carotene), Vitamin B_6 (Pyridoxine and Vitamin E (α-tocopherol). The differences observed were small (5.78, 6.55 and 5.46%) with the mean values observed falling within the ranges of values observed for the non-transgenic control. Additionally, the mean values for all vitamins fell within the ranges reported in the literature (OECD, 2002; ILSI 2006).

For Vitamin A (β-carotene) and Vitamin B_9 (folic acid) a statistically significant genotype-by-location interaction was noted. This suggests that the effect of genotype was not consistent across locations and that the comparison of genotypes averaged across locations may not be valid Therefore, individual location means for Vitamin A and B_9 were assessed, and are shown in Table 14. The Vitamin A and B_9 levels at all locations were within the ranges reported in the literature (OECD, 2002; ILSI 2006).

Amino acids

Results of the amino acid analyses are shown in Table 15. No significant differences were noted for any of the 18 amino acids measured and all mean values were within the ranges reported in the literature (OECD, 2002; ILSI 2006).

Fatty Acids

The five most abundant fatty acids in corn grain were analysed. The results are presented in Table 16. Small but statistically significant differences were noted for linoleic (18:2) and linolenic (18:3) acid; 0.65 and 3.21% respectively. The mean values observed for these fatty acids in MIR162 corn were within the ranges of values observed for the non-transgenic control and literature ranges (OECD, 2002; ILSI 2006).

Anti-nutrients and secondary plant metabolites

Corn contains a number of anti-nutrients: phytic acid, raffinose, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and trypsin and chymotrypsin inhibitor. Only phytic acid and raffinose are considered to be biologically relevant (OECD, 2002). DIMBOA is present at highly variable levels in corn hybrids and little evidence is available on either its toxicity or anti-nutritional effects and corn contains only low levels of trypsin and chymotrypsin inhibitor, neither of which is considered nutritionally significant.

Phytic acid is considered an important anti-nutrient for animals, especially non-ruminants, since it can significantly reduce the bioavailability of phosphorus in the plant. Feed formulators add the enzyme phytase to pig and poultry diets to improve the utilisation of phosphorus. Raffinose is a non-digestible oligosaccharide and is considered an anti-nutrient because of its gastrointestinal effects (flatulence).

Secondary metabolites are defined as those natural products which do not function directly in the primary biochemical activities which support the growth, development and reproduction of the organism in which they occur. Secondary plant metabolites are neither nutrients nor anti-nutrients but are sometimes analysed as further indicators of the absence of unintended effects of the genetic modification on metabolism(OECD, 2002). Characteristic metabolites in corn are furfural and the phenolic acids, ferulic acid and p-coumaric acid.

There are no generally recognised anti-nutrients in corn at levels considered to be harmful, but for the purposes of comparative assessment, the OECD has recommended considering analytical data for the content of the anti-nutrients phytic acid and raffinose, and the secondary metabolites furfural, ferulic acid, p-coumaric acid. These selected secondary metabolites and anti-nutrients were included in the compositional analysis of MIR162 corn grain and are shown in Table 17. In addition, data on the levels of the anti-nutrient trypsin inhibitor and inositol in MIR162 corn grain are provided.

The furfural levels in all transgenic and non-transgenic corn samples were below the limits of quantitation (LOQ). For raffinose, 16 of 18 MIR162 samples, and 12 of 18 control samples were <LOQ. Analytes with values <LOQ are not suitable for statistical analysis but quantifiable levels of raffinose in both the MIR162 and control hybrids were within ranges reported in the literature.

The levels of the secondary metabolites ferulic acid and p-coumaric acid in MIR162 corn grain were significantly different from levels in the control grain. However, the mean values for both metabolities were within the ranges of values observed for the non-transgenic control, and within the ranges reported in the literature (OECD, 2002; ILSI 2006). Based on these results, the levels of anti-nutrients and secondary plant metabolites in MIR162 corn are comparable to those found in conventional corn.

5.4 Conclusion

The compositional analyses do not indicate any compositional differences of biological significance in forage or grain from transgenic corn line MIR162, compared to the non-GM control. Several minor differences in key nutrients and other constituents were noted, however, the mean levels observed were within the range of values observed for the non-transgenic comparator and literature ranges. Therefore, all values noted for MIR162 corn are considered to be within the range of natural variation for corn and suggest than no unintended changes in composition have occurred in MIR162 corn. On the whole, it can be concluded that MIR162 corn is equivalent in composition to the non-transgenic comparator and conventional corn.

6. NUTRITIONAL IMPACT

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

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

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

In this case, MIR162 corn is the result of a simple genetic modification to confer insect protection with no intention to significantly alter nutritional parameters in the food. In addition, extensive compositional analyses have been undertaken to demonstrate the nutritional adequacy of MIR162 corn and these indicate it is equivalent in composition to grain from conventional corn hybrids.

The Applicant has however submitted a feeding study comparing the nutritional performance of MIR162 corn with a non-transgenic near-isogenic control corn and a commercially available conventional corn. This study is evaluated below as additional supporting information.

6.1 Feeding study in broiler chickens

Study submitted:
Brake, J.T. (2007) Evaluation of Event MIR162 Transgenic maize in broiler chickens. Syngenta Seeds Biotechnology Report No. SSB-507-07.

The purpose of the study was to evaluate whether standard poultry diets prepared with MIR162 corn grain had any effect on male of female broiler chicken survival, growth and feed conversion (an indicator of how efficiently a bird converts feed to live body weight). The study was conducted using rapidly growing broiler chicks, which are sensitive to changes in nutrient quality in their diets, and therefore are often used as a model to assess the wholesomeness of corn.

Poultry diets were prepared with three lots of corn grain: grain from MIR162 corn, a non-transgenic, near-isogenic control corn, and a commercially available lot of corn grown in North Carolina. Diets were formulated on the basis of individual nutrient analyses of grain to meet standard nutritional recommendations for poultry.

Diets were fed to groups of 90 male and 90 female birds for 44 consecutive days. Parameters evaluated included survival, body weight, feed conversion and carcass yield.

Broiler chickens fed diets prepared with MIR162 corn grain did not show any adverse effects compared to chickens fed diets prepared either with the control or commercial grain. There were no significant differences between the three diets for rates of growth, final body weights, feed conversion, survival rates or carcass yield.

The results support the conclusion that MIR162 corn is nutritionally comparable to conventional corn.

7. REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25(17):3389-3402.

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

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

Bogosian, G. and Kane, J.F. (1991) Fate of recombinant *Escherichia coli* K-12 strains in the environment. In: Neidleman S. and Laskin, A. eds. *Advances in Applied Microbiology*. Vol 37, 87-131. Academic Press, San Diego.

CFIA (1994) *The Biology of Zea mays L. (Corn/Maize)*. Canadian Food Inspection Agency, Ottawa.

Chen, B.Y., Heneen, W.K. and Simonsen, V. (1989) Comparative and genetic studies of isozymes in resynthesized and cultivated *Brassica napus* L., *B. campestris* L. and *B. alboglabra* Bailey. *TAG Theoretical and Applied Genetics* 77(5):673-679.

EPA (1998) *RED Facts: Bacillus thuringiensis*. Report No. EPA 738-F-98-001., Environmental Protection Agency.

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

FAO/WHO (2001) *Evaluation of Allergenicity of Genetically Modified Foods. Report of the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 22 – 25 January 2001.* Food and Agriculture Organization of the United Nations, Rome.

FAOSTAT. (2008) Online database of the Food and Agriculture Organization of the United Nations. http://faostat.fao.org/.

Fling, M.E., Kopf, J. and Richards, C. (1985) Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase. *Nucleic Acids Res.* 13(19):7095-7106.

Freeze, H.H. (2002) Phosphomannose isomerase. In: Taniguchi, N., Honke, K., and Fukuda, M. eds. *Handbook of glycosyltransferases*. 1 ed, Springer-Verlag, Tokyo and New York, pp595-599.

Hilger, C., Grigioni, F., Thill, L., Mertens, L. and Hentges, F. (2002) Severe IgE-mediated anaphylaxis following consumption of fried frog legs: definition of alpha-parvalbumin as the allergen in cause. *Allergy* 57(11):1053-1058.

ILSI (2006) *International Life Sciences Institute Crop Composition Database Version 3.0*. http://www.cropcomposition.org.

Itoh, T. and Tomizawa, J. (1979) Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H, and DNA polymerase I. *Cold Spring Harb.Symp.Quant.Biol* 43 Pt 1:409-417.

Jensen, O.N., Podtelejnikov, A.V. and Mann, M. (1997) Identification of the components of simple protein mixtures by high-accuracy peptide mass mapping and database searching. *Anal.Chem* 69(23):4741-4750.

Lee, B.T. and Matheson, N.K. (1984) Phosphomannoisomerase and phosphoglucoisomerase in seeds of *Cassia coluteoides* and some other legumes that synthesize galactomannan. *Phytochemistry* 23(5):983-987.

Lee, M.K., Miles, P. and Chen, J.S. (2006) Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. *Biochem.Biophys.Res Commun* 339(4):1043-1047.

Lee, M.K., Walters, F.S., Hart, H., Palekar, N. and Chen, J.S. (2003) The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab delta-endotoxin. *Appl Environ.Microbiol* 69(8):4648-4657.

McClintock, J.T., Schaffer, C.R. and Sjoblad, R.D. (1995) A comparative review of the mammalian toxicity of *Bacillus thuringiensis*-based pesticides. *Pestic.Sci.* 45:95-105.

Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L. and Fuchs, R.L. (1996) Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit Rev Food Sci Nutr* 36 Suppl:S165-S186.

Miles, J.S. and Guest, J.R. (1984) Nucleotide sequence and transcriptional start point of the phosphomannose isomerase gene (manA) of *Escherichia coli*. *Gene* 32(1-2):41-48.

NCBI (2006) *National Center for Biotechnology Information. Entrez Nucleotide Database, including GenBank*. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide.

Negrotto, D., Jolley, M., Beer, S., Wenck, A.R. and Hansen, G. (2000) The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. *Plant Cell Reports* 19:798-803.

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

OECD (2002) *Consensus document on compositional considerations for new varieties of maize (Zea mays): Key food and feed nutrients, antinutrients and secondary plant metabolites*. Series on the safety of novel foods and feeds, No. 6. Organisation for Economic Co-operation and Development, Paris.

OECD (2003) *Considerations for the safety assessment of animal feedstuffs derived from genetically modified plants*. Series on the Safety of Novel Foods and Feeds, No. 9. Organisation for Economic Cooperation and Development, Paris.

Privalle, L.S. (2002) Phosphomannose isomerase, a novel plant selection system: Potential allergenicity assessment. In: Fu, T.J. and Gendel, S.M. eds. *Genetically Engineered Foods: Assessing potential allergenicity*. Ann. N.Y. Acad Sci, pp129-138.

Proudfoot, A.E., Payton, M.A. and Wells, T.N. (1994) Purification and characterization of fungal and mammalian phosphomannose isomerases. *J Protein Chem* 13(7):619-627.

Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Petersen, G.B. (1982) Nucleotide sequence of bacteriophage lambda DNA. *J Mol Biol* 162(4):729-773.

Sjoblad, R.D., McClintock, J.T. and Engler, R. (1992) Toxicological considerations for protein components of biological pesticide products. *Regul.Toxicol.Pharmacol.* 15(1):3-9.

Tinland B. and Hohn B. (1995) Recombination between prokaryotic and eukaryotic DNA: integration of *Agrobacterium tumefaciens* T-DNA into the plant genome. *Genet Eng* 17:209-229.

Wang, K., Herrera-Estrella, L., VAN, M.M. and Zambryski, P. (1984) Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from agrobacterium to the plant genome. *Cell* 38(2):455-462.

White, P.J. and Pollak, L.M. (1995) Corn as a food source in the United States: Part II. Processes, products, composition, and nutritive values. In: *Cereal Foods World 40*. pp756-762.

WHO. (1999) Microbial pest control agent *Bacillus thuringiensis*. Environmental Health Criteria. Vol 217, World Health Organization, Geneva.

Zambryski, P., Depicker, A., Kruger, K. and Goodman, H.M. (1982) Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. *J Mol Appl Genet.* 1(4):361-370.

Zambryski, P.C. (1992) Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annual review of plant physiology and plant molecular biology* 43:465-490.