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

5-06
9 August 2006

DRAFT ASSESSMENT REPORT

APPLICATION A575

FOOD DERIVED FROM GLYPHOSATE-TOLERANT LUCERNE J101 AND J163

DEADLINE FOR PUBLIC SUBMISSIONS: 6pm (Canberra time) 20 September 2006
SUBMISSIONS RECEIVED AFTER THIS DEADLINE
WILL NOT BE CONSIDERED
(See 'Invitation for Public Submissions' for details)

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

Executive Summary

An Application has been received from Monsanto Australia Limited to amend the *Australia New Zealand Food Standards Code* (the Code) to approve food derived from genetically modified (GM) herbicide-tolerant lucerne lines J101 and J163. Standard 1.5.2 – Food Produced Using Gene Technology – requires that GM foods undergo a pre-market safety assessment before they may be sold in Australia and New Zealand.

Lucerne lines J101 and J163 have been genetically modified to be tolerant to the herbicide glyphosate. The GM lucerne is intended principally for animal feed and is not intended for cultivation in either Australia or New Zealand. As there are some minor food uses of lucerne (primarily as alfalfa sprouts and in teas), FSANZ has undertaken a safety assessment of glyphosate-tolerant lucerne J101 and J163. If approved, food from glyphosate-tolerant lucerne J101 and J163 may enter Australia and New Zealand as imported products.

The herbicide tolerance trait introduced into glyphosate-tolerant lucerne J101 and J163 is conferred by expression in the plant of an enzyme, CP4 EPSPS, derived from a common soil bacterium. No marker genes are present in glyphosate-tolerant lucerne J101 and J163.

Safety assessment

FSANZ has completed a comprehensive safety assessment of food derived from glyphosate-tolerant lucerne J101 and J163, as required under Standard 1.5.2 in the Code. The assessment included consideration of (i) the genetic modification to the plant; (ii) the potential toxicity and allergenicity of the novel protein; and (iii) the composition of glyphosate-tolerant lucerne J101 and J163 compared with that of conventional lucerne.

The assessment of this Application identified no public health and safety concerns. On the basis of the available evidence, including detailed studies provided by the Applicant, food derived from glyphosate-tolerant lucerne J101 and J163 is considered as safe and wholesome as food derived from other commercial lucerne varieties.

Labelling

Foods derived from glyphosate-tolerant lucerne J101 and J163 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 protein is present in the harvested plant.

Labelling addresses the requirement of section 10(1)(b) of the Act; provision of adequate information relating to food to enable consumers to make informed choices.

Impact of regulatory options

Two regulatory options were considered in the assessment: (1) no approval; or (2) approval of food derived from glyphosate-tolerant lucerne J101 and J163 based on the conclusions of the safety assessment.

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

Purpose

The Applicant seeks amendment to Standard 1.5.2 - Food Produced Using Gene Technology, to include food derived from glyphosate-tolerant lucerne J101 and J163 in the Table to clause 2.

Preferred Approach

Amend Standard 1.5.2 - Food Produced Using Gene Technology, to include food derived from glyphosate-tolerant lucerne J101 and J163 in the Table to clause 2.

Reasons for Preferred Approach

An amendment to the Code approving food derived from glyphosate-tolerant lucerne J101 and J163 in Australia and New Zealand is recommended on the basis of the available scientific evidence, for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce glyphosate-tolerant lucerne J101 and J163;

- food derived from glyphosate-tolerant lucerne J101 and J163 is equivalent to food from other commercially available lucerne varieties in terms of its safety for human consumption and nutritional adequacy;

- labelling of certain food fractions derived from glyphosate-tolerant lucerne J101 and J163 will be required if novel DNA and/or protein is present in the final food; and

- a regulation impact assessment process has been undertaken that also fulfils the requirement in New Zealand for an assessment of compliance costs. The assessment concluded that the most appropriate option is option 2, an amendment to the Code.

Consultation

The Initial Assessment was advertised for public comment between 22 March 2006 and 3 May 2006. A total of nine submissions were received during this period and a summary of these is attached to this report.

FSANZ has taken the submitters' comments into account in preparing the draft assessment of this application. Specific issues relating to glyphosate-tolerant lucerne J101 and J163 have been addressed in the report.

Public submissions will be invited on this Draft Assessment Report.

CONTENTS

INVITATION FOR PUBLIC SUBMISSIONS

FSANZ invites public comment on this Draft Assessment Report based on regulation impact principles and the draft variation to the Code 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 preparing the Draft Assessment of this Application. Submissions should, where possible, address the objectives of FSANZ as set out in section 10 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 and provide justification for treating it as commercial-in-confidence. Section 39 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. 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
www.foodstandards.gov.au

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

Submissions need to be received by FSANZ by 6pm (Canberra time) 20 September 2006.

Submissions received after this date will not be considered, unless 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.

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. Questions relating to making submissions or the application process can be directed to the Standards Management Officer at the above address or by emailing slo@foodstandards.gov.au.

Assessment reports are available for viewing and downloading from the FSANZ website. Alternatively, requests for paper copies of reports or other general inquiries can be directed to FSANZ's Information Officer at either of the above addresses or by emailing info@foodstandards.gov.au.

INTRODUCTION

An Application was received from Monsanto Australia Limited on 1 February 2006 seeking approval for food derived from glyphosate-tolerant lucerne J101 and J163 under Standard 1.5.2 - Food Produced Using Gene Technology - in the Code.

A Draft Assessment of the Application has been completed, including a comprehensive safety assessment, and public comment is now being sought prior to Final Assessment of the Application.

1. Background

The genetic modification in glyphosate-tolerant lucerne J101 and J163 involves the introduction of the *cp4 epsps* gene derived from *Agrobacterium* sp. strain CP4. The *cp4 epsps* gene codes for an enzyme, 5-enolpyruvyl-3-shikimate phosphate synthase (EPSPS), which confers tolerance to the herbicide glyphosate. The EPSPS enzyme is present in all plants, bacteria and fungi and is essential for aromatic amino acid biosynthesis. The normal mode of action of glyphosate is to inhibit the endogenous plant EPSPS, thus blocking the synthesis of aromatic amino acids in cells which subsequently leads to the death of the plant. In contrast to the plant EPSPS, the bacterial EPSPS is able to function in the presence of glyphosate, therefore expression of CP4 EPSPS in the plant allows continued production of aromatic amino acids in the presence of the herbicide.

The development of glyphosate-tolerant lucerne enables the use of glyphosate-based herbicides to provide effective weed control during forage and seed production. As weed infestations are a major limiting factor in the production of high-quality forage, superior weed control is expected to improve forage quality and allow higher yields. Availability of weed control at early, pre-plant, pre-emergence, and post-emergence timings will allow greater success in stand establishment and longer stand life.

Lucerne is a premium forage for feeding to dairy cattle and horses and is also a valuable feed for beef cattle, sheep and other livestock. Glyphosate-tolerant lucerne is intended to be used primarily as animal feed. However, lucerne also has minor food uses.

In Australia and New Zealand, lucerne that is used for human food is referred to as alfalfa. There is a long history of food use of alfalfa, primarily as sprouted seeds and in alfalfa teas. In some countries, tender alfalfa shoots are used as a vegetable. Alfalfa would be expected to be consumed in minor quantities and on an occasional basis.

Glyphosate-tolerant lucerne is not intended to be grown in Australia or New Zealand at this time and therefore it is unlikely that any foods or feeds derived from lucerne J101 and J163 will be introduced into the Australian or New Zealand food supply.

1.1 Current Standard

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

1.2 Overseas approvals

Glyphosate-tolerant lucerne J101 and J163 has been approved for food and feed use and environmental release in the United States (USFDA, USDA-Animal and Plant Health Inspection Service), Canada (Health Canada and the Canadian Food Inspection Agency) and Japan (Japanese Ministry of Health, Labour and Welfare) in 2005. Submissions have also been made to Mexico and Taiwan.

The US Environmental Protection Agency previously has reviewed and established an exemption from the requirement of a tolerance for CP4 EPSPS and the genetic material necessary for the production of this protein in or on all raw agricultural commodities.

2. The Issue / Problem

Monsanto Company and Forage Genetics International have developed a new variety of herbicide-tolerant lucerne, referred to as lucerne J101 and J163, for agronomic purposes. Glyphosate-tolerant lucerne J101 and J163 are intended primarily for use as animal feed in the United States, where it is known as Roundup Ready alfalfa. There is no intention to introduce glyphosate-tolerant lucerne J101 and J163 into Australia or New Zealand at this time. In addition, there will be channelling of the product in the US and so only a low probability exists that any foods or feeds derived from glyphosate-tolerant lucerne J101 and J163 will be introduced into the Australia or New Zealand food supply.

However, as there are some food uses of lucerne (primarily as alfalfa sprouts and in teas) there is a possibility that glyphosate-tolerant lucerne J101 and J163 may enter the Australian and New Zealand food supply. Monsanto Australia Limited has therefore applied to FSANZ for approval of food derived from glyphosate-tolerant lucerne J101 and J163.

Before food derived from a GM product can enter the food supply in Australia and New Zealand, it must first be assessed for safety and an amendment to the Code must be approved by the FSANZ Board, and subsequently 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.

Although the Applicant states that there is no intention to grow glyphosate-tolerant lucerne J101 and J163 in Australia or New Zealand, or to import it into either country as animal feed, the amendment to Standard 1.5.2 sought by the Applicant would allow the use of glyphosate-tolerant lucerne J101 and J163 as food.

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 10 of the FSANZ Act. These are:

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

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

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

The key objectives of this assessment of glyphosate-tolerant lucerne J101 and J163 are therefore the protection of public health and safety and the provision of adequate information to consumers. In fulfilling these objectives, FSANZ will also have regard for the need for standards to be based on a risk analysis using the best available scientific evidence, and the benefits of an efficient and internationally competitive food industry.

4. Key Assessment Question

Is food derived from glyphosate-tolerant lucerne J101 and J163 as safe for human consumption as food from conventional lucerne varieties?

RISK ASSESSMENT

5. Risk Assessment Summary

Glyphosate-tolerant lucerne J101 and J163 have been assessed for safety according to the guidelines prepared by FSANZ[1]. 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.

In conducting a safety assessment of food derived from glyphosate-tolerant lucerne J101 and J163, a number of criteria were addressed including:

(i) characterisation of the transferred genes, their origin, function and stability;
(ii) changes at the level of DNA, protein and in the whole food;
(iii) compositional analyses, and an evaluation of intended and unintended changes; and
(iv) potential for the newly expressed proteins to be either allergenic or toxic in humans.

Detailed molecular and genetic analyses of glyphosate-tolerant lucerne J101 and J163 indicate that the transferred genes are stably integrated into the plant genome as single copies at different insertion sites, and are inherited in subsequent generations according to predicted patterns of inheritance. There was no transfer of bacterial antibiotic resistance marker genes in this modification.

[1] FSANZ (2005) Guidelines for the Safety Assessment of Genetically Modified Foods.

The novel EPSPS protein is expressed at moderate levels in glyphosate-tolerant lucerne J101 and J163 plants. The level of CP4 EPSPS in sprouted alfalfa seeds was only slightly higher than in lucerne forage. The EPSPS protein present in glyphosate-tolerant lucerne J101 and J163 has been assessed previously for safety. These assessments have shown that CP4 EPSPS administered directly to animals at high doses is not toxic, and the evidence indicates no potential for this protein to be allergenic to humans.

Compositional analyses of both forage and sprouts did not reveal any meaningful differences between glyphosate-tolerant lucerne J101 and J163 and its non-GM counterpart. The use of glyphosate-tolerant lucerne J101 and J163 for food would be expected to have minimal nutritional impact.

Overall, no potential public health and safety concerns have been identified in the comprehensive assessment of glyphosate-tolerant lucerne J101 and J163. On the basis of the data provided in the present application, and other available information, food derived from glyphosate-tolerant lucerne J101 and J163 is considered as safe and wholesome as food derived from other lucerne varieties.

RISK MANAGEMENT

6. Options

6.1 Option 1 – prohibit food from glyphosate-tolerant lucerne J101 and J163

Maintain the *status quo* by not amending the Code to approve the sale and use of food derived from glyphosate-tolerant lucerne J101 and J163.

6.2 Option 2 – approve food from glyphosate-tolerant lucerne J101 and J163

Amend the Code to permit the sale and use of food derived from glyphosate-tolerant lucerne J101 and J163, with or without listing special conditions of use in the Table to clause 2 of Standard 1.5.2.

7. Impact Analysis

7.1 Affected Parties

1. Consumers, particularly those who have concerns about biotechnology;

2. Food importers and distributors of wholesale ingredients;

3. The manufacturing and retail sectors of the food industry; and

4. Government generally, where a regulatory decision may impact on trade or WTO obligations, and enforcement agencies in particular who will need to ensure that any approved products are correctly labelled.

There is no current intention to grow glyphosate-tolerant lucerne J101 and J163 in Australia or New Zealand. Should this be decided in the future, any environmental impact would require assessment 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 Fisheries (MAF) before cultivation in these countries could be permitted. Importation of non-viable lucerne into New Zealand would not require approval by ERMA.

7.2 Benefit Cost Analysis

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

Following public consultation on the Initial Assessment, FSANZ has identified the following potential costs and benefits of the two regulatory options:

7.2.1 Option 1 – prohibit food derived from glyphosate-tolerant lucerne J101 and J163

Consumers: Benefit to consumers if there are potential public health and safety issues.

No impact on consumers wishing to avoid GM foods, as food from glyphosate-tolerant lucerne J101 and J163 is not currently permitted in the food supply.

Government: No immediate impact.

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

Industry: No immediate impact.

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

7.2.2 Option 2 – approve food derived from glyphosate-tolerant lucerne J101 and J163

Consumers: No direct impact.

The amount of glyphosate-tolerant lucerne J101 and J163 entering the food supply is likely to be low so the cost to consumers wishing to avoid GM food by a potential restriction of choice of products, or increased prices for non-GM food is likely to be low.

Government: No direct impact.

Benefit that if glyphosate-tolerant lucerne J101 and J163 were to inadvertently enter the human food supply, this Application will ensure food imports containing glyphosate-tolerant lucerne J101 and J163 comply with the Code. This would ensure that there is no potential for trade disruption on regulatory grounds.

This decision could impact on monitoring resources, as certain foods derived from glyphosate-tolerant lucerne J101 and J163 will be required to be labelled as genetically modified.

Industry: No direct impact.

Possible cost to food industry as some food ingredients derived from glyphosate-tolerant lucerne J101 and J163 would be required to be labelled as genetically modified.

7.3 Comparison of Options

As food from glyphosate-tolerant lucerne J101 and J163 has been found to be as safe as food from other varieties of lucerne, option 1 is likely to be inconsistent with Australia and New Zealand's WTO obligations. This option would also offer very little benefit to those consumers wishing to avoid GM foods, as foods derived from lucerne (e.g. alfalfa sprouts) are generally produced locally and so would not be GM.

Option 2 is the preferred option as glyphosate-tolerant lucerne J101 and J163 has been found to be safe for human consumption

The proposed amendment to Standard 1.5.2 of the Code, giving approval to food from glyphosate-tolerant lucerne J101 and J163, is therefore considered appropriate.

COMMUNICATION

8. Communication and Consultation Strategy

FSANZ developed a communication strategy to allay ongoing concerns raised by specific groups and the general public regarding food derived from genetically modified organisms. Part of this strategy was the publication, in June 2000, of an information booklet titled *GM foods and the consumer*. Due to the success of this booklet in providing information on safety issues, FSANZ has recently launched a revised and updated version of this booklet, now titled *GM Foods*[2]. The booklet aims to communicate recent developments in the safety evaluation of GM foods to interested members of the community.

As normally applies to all GM food assessments, the Draft Assessment Report for Application A575 will be available to the public on the FSANZ website and distributed to major stakeholders. Public comment on this Draft Assessment will be sought prior to preparation of the Final Assessment of the Application.

[2] http://www.foodstandards.gov.au/_srcfiles/GM%20Foods_text_pp_final.pdf

9. Consultation

9.1 Public Consultation

The Initial Assessment was advertised for public comment between 22 March 2006 and 3 May 2006.

Nine submissions were received during this period and a summary of these is included in **Attachment 3** to this Report.

FSANZ has taken the submitters' comments into account in preparing the draft assessment of this application. Specific issues relating to glyphosate-tolerant lucerne J101 and J163 have been addressed in the report. The major issues raised are discussed here.

9.1.1 Enforcement costs

The Queensland Environmental Health Unit considers that rising costs associated with the enforcement of the Standard for GM foods must be addressed by demanding that future GM food approvals be contingent upon the applicants providing detection methodology that is freely available to all regulatory agencies to minimise cost increases associated with monitoring and to promote national consistency in enforcement of the Standard. A national repository for suitable reference material, provided by each Applicant as a condition of approval, should also be established.

9.1.1.1 Response

Although detection methods for GM plant lines can be event-specific, certain analytical material is suitable for detecting a number of approved GM lines with the same introduced genetic trait, and routinely can distinguish a GM from a non-GM source when genetic material is present. For example, analytical methods and reagents necessary for detection of the *cp4 epsps* gene are common to the majority of approved glyphosate-tolerant plant lines. The usefulness of such material in detecting GM from non-GM varieties depends on the level of detail required for the investigation, as the number of introduced genetic traits is relatively small compared to the number of individually approved GM lines.

Labelling requirements under Standard 1.5.2 – Food Produced Using Gene Technology call for food manufacturers to seek and maintain documentation relating to the GM status of individual ingredients used in their products. In approving the expanded labelling requirements for GM foods in 2000, Health Ministers indicated that the purpose of the paper trail was to reduce the reliance on laboratory testing of foods as the sole tool for enforcement of the Standard.

Costs associated with the enforcement by jurisdictions of any new food regulatory measure are considered by FSANZ in the Regulatory Impact Statement (RIS) and are not unique to GM foods. Australia and New Zealand's current system of food regulation provides for the discussion of such issues by the Implementation Sub-Committee (ISC). Inevitably, enforcement costs would be expected to rise over time as a result of the need to regulate an ever-increasing number of new food additives, processing aids and novel technologies in the Code.

9.1.2 Survival of cp4 epsps transgenes in the intestine

GE Free New Zealand raised the concern that the *cp4 epsps* transgenes could be transferred to human gut microflora.

9.1.2.1 Response

In the study by Netherwood *et al*. (2004)[3], the fate of the *epsps* gene from GM soy was traced in seven ileostomy patients. In the subjects with intact gastrointestinal tracts, none of the endogenous bacteria in the faeces were found to contain the *epsps* gene from the GM soy. This indicates that either the *epsps*-containing bacterium in the small bowel of the ileostomists did not survive passage through the human colon or that in intact digestive systems gene transfer from plant material to the intestinal microflora does not occur at the same frequency as in the ileostomists. Furthermore, no intact novel DNA was found in the faeces of volunteers with intact gastrointestinal tracts. The authors conclude that the data presented in this study support the view that GM foods do not represent a significant risk to human health through gene transfer to either the intestinal epithelium or the microflora within the human intestine.

9.1.3 Unintended changes in CP4 EPSPS protein in glyphosate-tolerant lucerne J101 and J163

GE Free New Zealand expressed concern about the potential for unintended changes in protein structure and that FSANZ only assessed the original bacterial protein. Ivan Jeray (private submission) does not support approval as GM ingredients have not been proven safe. Both submissions cite the example of CSIRO GM field peas which have been claimed to cause an allergic reaction in mice.

9.1.3.1 Response

CSIRO developed GM field peas that are protected against attack from pea weevils by expressing the alpha-amylase inhibitor from common bean. Further studies revealed that a structurally modified form of alpha-amylase inhibitor protein was unexpectedly produced in the GM peas. The modified form of the alpha-amylase inhibitor appears to have altered immunogenicity in mice compared to the native form of the protein produced in beans. Following the publication of these findings (Prescott et al., 2005)4, CSIRO announced it would discontinue work on the GM peas.

The CSIRO GM field peas were still in the development phase and no application was made to FSANZ for a safety assessment of the GM pea line. The animal model used for the CSIRO study has not been validated to predict human immune or allergic responses therefore the relevance of the findings is unclear at this stage. At present, no validated animals models are available that can be used to predict the allergenic potential of novel proteins.

---

[3] Netherwood, T., Martin-Orue, S.M., O'Donnell, A.G., Gockling, S., Graham, J., Mathers, J.C. and Gilbert, H.J. (2004) Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. *Nat.Biotechnol.* 22(2):204-209.

[4] Prescott, V.E., Campbell, P.M., Moore, A., Mattes, J., Rothenberg, M.E., Foster, P.S., Higgins, T.J. and Hogan, S.P. (2005) Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity. *J.Agric.Food Chem.* 53(23):9023-9030.

It is important to note however that the modified form of the alpha-amylase inhibitor protein would have been readily identified by the types of protein characterization studies that are routinely undertaken with all novel proteins and submitted to FSANZ for assessment. Such a finding would have automatically triggered further testing of the protein.

In the safety assessment of glyphosate-tolerant lucerne J101 and J163, a panel of analytical tests was used to characterise the plant-produced CP4 EPSPS proteins. No modified or abnormal forms of the novel protein were identified or observed in these studies.

9.2 World Trade Organization (WTO)

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

Guidelines for assessing the safety of GM foods have been developed by the Codex Alimentarius Commission and have the status of standards for WTO purposes. The proposed amendment to the Code to allow food derived from glyphosate-tolerant lucerne J101 and J163 may be of interest to other WTO member nations because it pertains to the safety of GM food and is likely to have a liberalising effect on international trade.

For these reasons, notification will be recommended to the agencies responsible in accordance with Australia's and New Zealand's obligations under the WTO Sanitary and Phytosanitary Measure (SPS) Agreements. This will enable other WTO member countries to comment on proposed changes to standards where they may have a significant impact on them.

CONCLUSION

10. Conclusion and Preferred Option

An amendment to the Code to give approval to the sale and use of food derived from glyphosate-tolerant lucerne J101 and J163 in Australia and New Zealand is proposed on the basis that food derived from glyphosate-tolerant lucerne J101 and J163 is as safe for human consumption as food from non-GM lucerne varieties.

11. Implementation

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

ATTACHMENTS

1. Draft variation to the *Australia New Zealand Food Standards Code*
2. Draft Safety Assessment Report for glyphosate-tolerant lucerne J101 and J163
3. Summary of first round public submissions

ATTACHMENT 1

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

To commence: on gazettal

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

Food derived from glyphosate-tolerant lucerne J101 and J163	

ATTACHMENT 2

DRAFT SAFETY ASSESSMENT

APPLICATION A575: FOOD DERIVED FROM GLYPHOSATE-TOLERANT LUCERNE J101 AND J163

SUMMARY AND CONCLUSIONS

Background

Glyphosate-tolerant lucerne J101 and J163 has been genetically modified for tolerance to the broad-spectrum herbicide glyphosate. Glyphosate-tolerant lucerne J101 and J163 was developed primarily as animal feed for cultivation in the United States and is not intended for cultivation in Australia or New Zealand. There is a low probability that food or feed derived from glyphosate-tolerant lucerne J101 and J163 will be introduced into the Australian or New Zealand food supply.

In conducting a safety assessment of food derived from glyphosate-tolerant lucerne J101 and J163, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the lucerne 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.

History of Use

Lucerne (*Medicago sativa* L.), also known as alfalfa, is a perennial legume that was one of the first forages to be domesticated and is now the world's most important forage crop. In addition to its use in grazing systems, lucerne is primarily used for hay, silage and dried forage. As well as being recognised as a premium forage because of its high content of readily digestible protein and carbohydrate, lucerne is favoured in crop rotation because of its ability to fix atmospheric nitrogen, thus improving soil quality for subsequent crops.

There are some food uses of lucerne, primarily as alfalfa sprouts and in herbal teas. In some countries, tender alfalfa shoots are used as a vegetable.

Description of the Genetic Modification

Glyphosate-tolerant lucerne was generated through the transfer of the *cp4 epsps* gene to a lucerne clone, R2336. Two different *cp4 epsps* insertion events, known as J101 and J163, were selected and combined through a conventional breeding process. Glyphosate-tolerant lucerne plants may contain transformation event J101, J163 or both J101 and J163.

The *cp4 epsps* gene is derived from the soil bacterium *Agrobacterium sp*. strain CP4 which encodes a version of the enzyme 5-enolpyruvyl-3-shikimatephosphate synthase (CP4 EPSPS). Unlike the plant's own EPSPS, CP4 EPSPS continues to function in the biochemical pathway producing aromatic amino acids in a plant that has been sprayed with glyphosate. There was no transfer of bacterial antibiotic resistance marker genes in this modification.

Detailed molecular and genetic analyses of glyphosate-tolerant lucerne J101 and J163 indicate that the transferred genes are stably integrated into the plant genome as single copies at different insertion sites, and are inherited in subsequent generations according to predicted patterns of inheritance.

Characterisation of Novel Protein

The mature CP4 EPSPS in glyphosate-tolerant lucerne J101 and J163 is identical to the bacterial enzyme of 455 amino acids and is targeted to the plant chloroplast, the site of synthesis of essential aromatic compounds.

The novel protein is expressed at moderate levels in glyphosate-tolerant lucerne J101 and J163 plants. The mean level of CP4 EPSPS in forage was 257 µg/g fresh weight for J101 and 270 µg/g fresh weight for J163. The mean level of the CP4 EPSPS protein in lucerne population J101 x J163, across two seasons and from multiple cuttings, was 252 µg/g fresh weight. The level of CP4 EPSPS in sprouted alfalfa seeds was only slightly higher than in lucerne forage.

Potential toxicity and allergenicity

The novel protein present in glyphosate-tolerant lucerne J101 and J163 has been assessed previously for safety; the CP4 EPSPS protein is present in approved lines of canola, cotton, soybean, potato and corn. Previous assessments have shown that CP4 EPSPS administered directly to animals at a high dose is not toxic, and the evidence indicates no potential for this protein to be allergenic in humans. Given its widespread use in approved glyphosate-tolerant crops, CP4 EPSPS now has a history of safe use in food over 10 years.

Comparative Analyses

Compositional studies were conducted to establish the nutritional adequacy of glyphosate-tolerant lucerne J101 and J163 compared to the non-GM control population and conventionally produced commercial lucerne varieties. The constituents measured were proximates (crude protein, fat, ash and moisture), acid detergent fibre (ADF), neutral detergent fibre (NDF), minerals, amino acids, carbohydrates (by calculation), the anti-nutrient lignin and the phytoestrogen coumestrol.

In general, no differences of biological significance were observed between glyphosate-tolerant lucerne J101 and J163 and its non-GM counterpart. In some cases, a statistically significant difference was observed in comparison to the control. This is expected for 1 in 20 comparisons when significance is set at 95%. In those cases where values were different, the population mean was compared to a 99% tolerance interval derived from the mean values from populations of 12 unique commercial lucerne varieties. In all cases, the glyphosate-tolerant lucerne population mean value fell within the tolerance interval and is considered to be within the variance of a population of commercial lucerne varieties and so is unlikely to be biologically meaningful. Forage from glyphosate-tolerant lucerne J101 and J163 is therefore considered to be compositionally equivalent to forage from the control and commercially available lucerne varieties.

Levels of folic acid and vitamin C in alfalfa sprouts were comparable to the non-GM control and commercial lucerne varieties.

Nutritional Impact

The detailed compositional studies are considered adequate to establish the nutritional adequacy of forage derived from glyphosate-tolerant lucerne J101 and J163. It is expected that alfalfa comprises only a small proportion of the average diet. The introduction of glyphosate-tolerant lucerne J101 and J163 into the food supply would be expected to have minimal nutritional impact.

Conclusion

No potential public health and safety concerns have been identified in the comprehensive assessment of glyphosate-tolerant lucerne J101 and J163. On the basis of the data provided in the present application, and other available information, food derived from glyphosate-tolerant lucerne J101 and J163 is considered as safe and wholesome as food derived from other lucerne varieties.

1. INTRODUCTION

Monsanto Australia Ltd is seeking approval in Australia and New Zealand for herbicide-tolerant lucerne J101 and J163 under Standard 1.5.2 – Food Produced Using Gene Technology in the *Food Standards Code*. Lucerne lines J101 and J163 have been genetically modified for tolerance to the broad-spectrum herbicide glyphosate.

Two distinct lines of glyphosate-tolerant lucerne, J101 and J163, have been developed using the same transgene (*cp4 epsps*). Each line has a separate insertion event at different, independently segregating loci. These two events were then combined through conventional breeding methods to optimise the number of plants carrying at least one copy of the gene conferring herbicide tolerance. Glyphosate-tolerant lucerne populations will contain a mixture of plants containing transformation event J101, J163 and both J101 x J163. This variation in copy number is due to the genetics of lucerne breeding and because all lucerne varieties are composed of a heterogeneous group of individuals.

Lucerne is a perennial, autotetraploid plant with a set of eight chromosomes and four copies of each chromosome set. Most lucerne plants will not successfully self-pollinate (Viands *et al*., 1988) and are adversely affected by inbreeding (Rumbaugh *et al*., 1988). Because of this, lucerne varieties are comprised of populations of lucerne breeding lines. Individual plants within each variety are not genotypically identical. In order to achieve the high trait purity required for lucerne varieties (>90% of plants must be glyphosate-tolerant) while minimising inbreeding depression, a Forage Genetics International (FGI) propriety conventional breeding method (patent pending) has been developed. The method relies on glyphosate-tolerant plants carrying different, independently segregating transgenic events. The two independent events are subsequently combined via traditional F1 crossing between two non-related plants that each contains one of the independent events resulting in populations with more than 95% trait purity.

The glyphosate tolerance trait in lucerne lines J101 and J163 is due to the expression of the bacterial enzyme 5-enolpyruvyl-3-shikimate phosphate synthase (EPSPS) from *Agrobacterium sp*. strain CP4. The EPSPS enzyme is present in all plants, bacteria and fungi and is essential for aromatic amino acid biosynthesis. The normal mode of action of glyphosate is to bind to the endogenous plant EPSPS, blocking its enzymatic activity, and resulting in a lack of aromatic amino acids in cells which subsequently leads to the death of the plant. The bacterial EPSPS enzyme has a lower binding affinity for glyphosate, and therefore expression of CP4 EPSPS in the plant allows continued production of aromatic amino acids in the presence of the herbicide.

Glyphosate-tolerant lucerne will enable the use of herbicides to provide effective weed control during forage and seed production. As weed infestations are a major limiting factor in the production of high-quality forage, superior weed control is expected to improve forage quality and allow higher yields. Availability of weed control at early, pre-plant, pre-emergence, and post-emergence timings will allow greater success in stand establishment and longer stand life.

Lucerne is a premium forage for feeding to dairy cattle and horses and is also a valuable feed for beef cattle, sheep and other livestock. Lucerne was one of the first forages to be domesticated and is now widely distributed in temperate zones, including USA, southern Canada, Europe, China, southern Latin America and South Africa (OECD, 2005).

Lucerne is rich in protein, vitamins and minerals. In symbiosis with the soil bacterium *Rhizobium meliloti*, lucerne is able to fix nitrogen, thus improving soil quality. Estimates of nitrogen fixation by lucerne root nodules are higher than for other temperate forage legumes.

In Australia and New Zealand, food uses of lucerne are referred to as alfalfa. There is a long history of food use of alfalfa, primarily as sprouted seeds and in alfalfa teas. Alfalfa would be expected to be consumed in minor quantities and on an occasional basis.

2. HISTORY OF USE

2.1 Donor Organisms

Agrobacterium sp. strain CP4 produces a naturally glyphosate-tolerant EPSPS enzyme and was therefore chosen as a suitable gene donor for the herbicide tolerance trait (Padgette *et al*., 1996). The bacterial isolate CP4 was identified in the American Type Culture Collection as an *Agrobacterium* species. *Agrobacterium* species are known soil-borne plant pathogens but are not pathogenic to humans or other animals.

2.2 Host organism

Lucerne (*Medicago sativa* L.), also known as alfalfa, is the world's leading forage crop and is probably the first forage crop to have been domesticated. More than 33 million hectares of alfalfa are cultivated throughout the world (OECD, 2005).

Lucerne varieties are primarily bred for forage yield and quality, longevity of stands and adaptation to a geographic area. Lucerne is a perennial, autotetraploid plant with four sets of eight chromosomes. Because lucerne is an out-crossing plant with a high degree of inbreeding depression, lucerne varieties are comprised of populations of lucerne breeding lines. A typical lucerne variety may have ten to 200 parent plants that were randomly inter-crossed (open pollination) in isolation to form the breeder generation seed. A lucerne variety is maintained through multiple seed generations via open pollination of the breeder seed progeny in isolation from other lucerne varieties or pollen sources. Individual plants within each variety are phenotypically and genotypically unique. Because lucerne varieties segregate, within a defined range, for most traits, they are usually described in terms of mean or % trait expression. For example, lucerne variety registration requires that pest resistance of a variety is described as the mean percent of plants that express that trait.

Lucerne has a long history of use as animal forage and feed, both in grazing systems and as hay processed from cut and dried swards. Like other forage legumes, the high content of readily digestible protein and carbohydrate that makes them valuable as ruminant feed can also predispose animals to bloat, a potentially serious condition that can result in death.

Primary bloat is the over-distension of the rumen caused by the accumulation of fermentation gases in a stable protein foam. This stable froth forms a layer on top of the ruminal contents and prevents gas bubbles from rising to the top and dispersing their contents. Death is a result of several factors, including the depressive effect of rumen distension on the heart and lungs. The main risk factor in pasture bloat is the rapid ingestion of immature legumes in pre-flowering stages. Bloat is a common problem in all areas in which temperate legumes are used as ruminant feed. Forage legumes are generally grown in combination with grasses to reduce the incidence of bloat.

While relevant from an animal feed perspective, such issues are of no consequence for human food safety because alfalfa comprises a small proportion of the human diet.

Lucerne is also known to produce phytoestrogens, natural oestrogen mimetics that have been shown to cause infertility in grazing stock. Coumestrol (coumesterol) is the major phytoestrogen in lucerne and is known to cause oestrogen-related disorders in animals. Significant genetic variation exists in alfalfa for coumestrol, with levels in lucerne forage ranging from 2.99 - 104.37 ppm.

Food uses of alfalfa are minor and include the use of sprouted seeds in salads, young alfalfa shoots as a vegetable and alfalfa teas.

3. DESCRIPTION OF THE GENETIC MODIFICATION

3.1 Method used in the Genetic Modification

Transformation events J101 and J163 were generated by *Agrobacterium*-mediated transformation using a two-step procedure adapted from earlier methods (Walker and Sato, 1981; Austin *et al*., 1995). The recipient for transformation was a lucerne clone R2336 that was selected from an elite, high-yielding, autumn-dormant lucerne breeding population using a tissue culture screen for callus formation and somatic embryo induction.

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.

Plasmid PV-MSHT4 was used to generate transformation events J101 and J163. The transformation vector PV-MSHT4 contains the *cp4 epsps* coding region under the control of a constitutive promoter. PV-MSHT4 also contains both the left and right transfer-DNA (T-DNA) border sequences to facilitate transformation. *Agrobacterium tumefaciens* strain ABI contains a disarmed Ti plasmid that is incapable of inducing tumour formation because of the deletion of the phytohormone genes originally present in the *Agrobacterium* Ti plasmid.

Each callus resulting from the *Agrobacterium*-mediated transformation was cultured on media containing glyphosate to select for the glyphosate-tolerant trait. Following induction of somatic embryos, glyphosate was removed and embryos were allowed to develop. The resulting plantlets were transferred to soil. Cuttings were taken from the primary transgenic plants were propagated and selected for vegetative tolerance to glyphosate through a 3.0 lb a.e./acre application of Roundup Ultra herbicide. Subsequent F1 and MBC1 (modified back cross 1) generations were treated with herbicide at the two to three trifoliate stages.

Plants were subsequently screened for glyphosate tolerance, and field performance. Of the many transformation events screened, J101 and J163 were selected. Introgression of transformation events J101 and J163 into new lucerne varieties was performed using FGI's breeding process.

These steps are summarized in Figure 1.

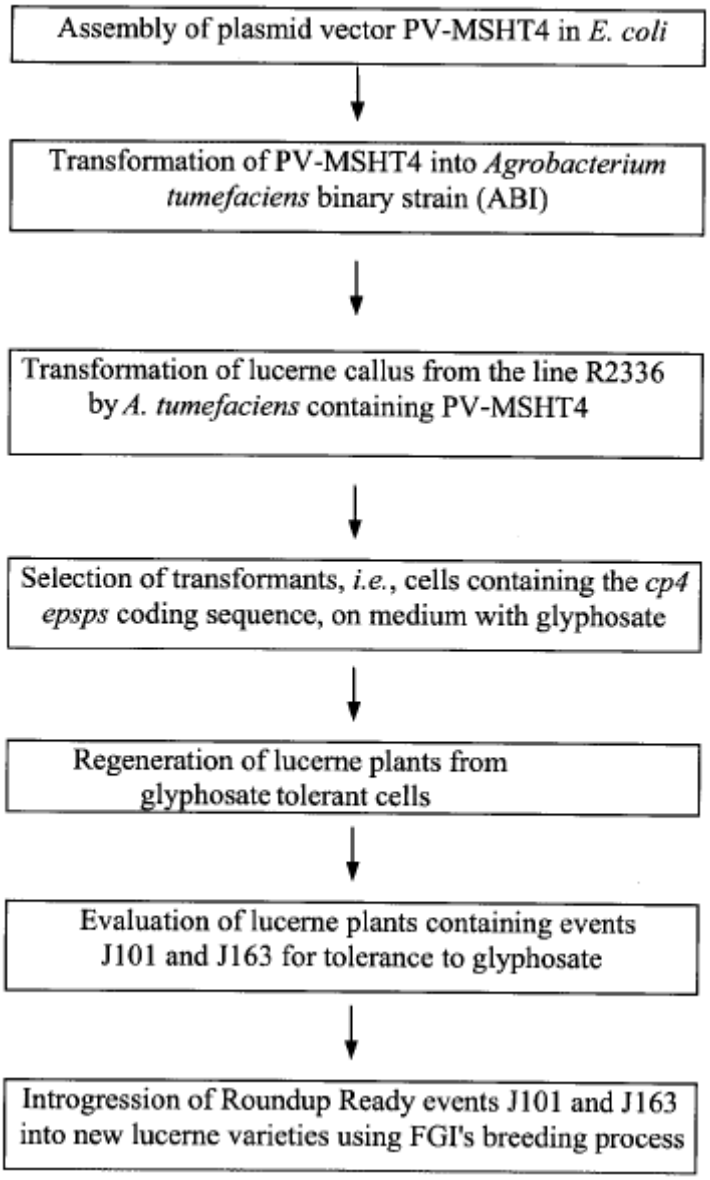

Figure : . Development of glyphosate-tolerant lucerne varieties.
The flow diagram illustrates the steps in the development of the glyphosate-tolerant lucerne varieties.

3.2 Genetic elements in vector

Plasmid PV-MSHT4 is a disarmed, binary *Agrobacterium tumefaciens* transformation vector that contains both the left and right T-DNA border sequences necessary for insertion into the plant genomic DNA. The T-DNA region in PV- MSHT4 contains the *cp4 epsps* gene and regulatory elements necessary for expression in plants. The *cp4 epsps* gene cassette contains an enhanced figwort mosaic virus promoter, HSP70 leader, a chloroplast transit peptide (CTP2) sequence, the *cp4 epsps* coding sequence and the pea rbcS E9 3' polyadenylation sequence, as shown in Table 1.

The *ctp2-cp4 epsps* coding region used to produce both J101 and J163 is the same as that employed in several other glyphosate-tolerant crops, the foods from which have been previously approved by FSANZ.

Table 1: Genetic elements in plasmid PV- MSHT4

Genetic element	Size (bp)	Function
RIGHT BORDER	24	Right border sequence essential for T-DNA transfer (Depicker *et al*., 1982)
P-eFMV	980	The 35S promoter from the figwort mosaic virus (FMV) with duplicated enhancer region (Richins *et al*., 1987)
HSP70 Leader	105	The petunia heat shock protein 70 5' untranslated leader sequence
CTP2	227	Chloroplast transit peptide from Arabidopsis thaliana (Klee *et al*., 1987) present to direct the CP4 EPSPS protein to the chloroplast, the site or aromatic amino acid synthesis
cp4 epsps	1367	Coding sequence for the synthetic CP4 EPSPS enzyme from *Agrobacterium* sp. strain CP4 (Padgette *et al*., 1996)
E9 3'	632	A 3' non-translated region of the pea ribulose-1,5-bisphosphate carboxylase, small subunit (rbc) E9 gene (Coruzzi *et al*., 1984), which terminates transcription and directs polyadenylation of the mRNA.
LEFT BORDER	23	Left border sequence essential for T-DNA transfer (Barker *et al*., 1983)

3.3 Function and regulation of novel genes

The only novel gene introduced into glyphosate-tolerant lucerne J101 and J163 is *cp4 epsps*. Expression of the *cp4 epsps* gene in the lucerne plants confers tolerance to the herbicide glyphosate.

Since the early 1990s it has been known that the *cp4 epsps* gene from *Agrobacterium* sp. strain CP4 has the potential to provide high levels of tolerance to glyphosate when introduced into plants. Glyphosate normally binds to the plant EPSPS enzyme, blocking biosynthesis of essential aromatic amino acids by the shikimate pathway, which is common to plants, bacteria and fungi. The bacterial CP4 EPSPS protein has a lower binding affinity with glyphosate compared to most other EPSPS enzymes and therefore retains its high catalytic efficiency in the presence of the herbicide. The bacterial *cp4 epsps* gene has been modified to create a synthetic gene, which allows for higher expression in plants. These changes to the DNA sequence produce an identical CP4 EPSPS protein (Harrison *et al*., 1996g) and do not affect the functional activity of the expressed protein.

In lines J101 and J163, the use of the figwort mosaic virus (FMV) promoter directs a high level of constitutive expression of the *cp4 epsps* gene in lucerne, conferring tolerance to the herbicide at the whole plant level.

The active site of the EPSPS enzyme in higher plants is the chloroplast (della-Cioppa *et al*., 1986). The CP4 EPSPS protein is produced in the cytoplasm and then targeted to the chloroplasts via an N-terminal fusion with a chloroplast transit peptide sequence (CTP2). The CTP is typically cleaved on uptake of the mature protein into the chloroplast, and is subsequently rapidly degraded.

The *cp4 epsps* gene together with these plant regulatory elements has been used previously to confer glyphosate-tolerance in a range of food crops including canola, cotton, soybean, sugarbeet, and corn.

3.4 Characterisation of the Genes in the Plant

Studies submitted:
Petersen EA, Reiser, SE, Cavato TA and Lirette RP. Molecular Analysis of Roundup Ready® Alfalfa Events J101 and J163. Monsanto Study Report, MSL-17612. completed November 2002.

Integrity of the cp4 epsps *transgene*

Analysis of the DNA introduced into glyphosate-tolerant lucerne J101 and J163 was undertaken using a range of established molecular techniques. Southern blot analyses were performed on genomic DNA extracted from lucerne J101 and J163 and the parent lucerne cultivar R2336 as a control to assess the following:

(i) Number of insertions of the integrated expression cassettes;
(ii) Number of copies of the integrated expression cassettes;
(iii) Integrity of gene expression cassettes;
(iv) Absence of plasmid backbone; and
(v) Stability of the inserted DNA with conventional breeding over several generations.

Genomic DNA from lucerne J101 and J163 was digested with a variety of restriction endonucleases and subjected to Southern blot analyses. The Southern blot hybridisations (based on the method described by (Southern, 1975) involved both short and long gel runs in order to improve the resolution of different size molecular fragments. Individual Southern blots were tested with probes corresponding to the *cp4 epsps* gene of interest, the promoter and polyadenylation sequence, and the transforming plasmid backbone. In all, six separate radiolabelled probes corresponding to segments of DNA spanning the entire length of the plasmid PV-MSHT4 were used in the analyses.

The primary test substance was genomic DNA isolated from leaf tissue of J101 and J163 from the primary transgenic plants generated during transformation. The control substance was the conventional, non-transgenic lucerne cultivar R2336 used to generate the transgenic lines. The plasmid PV-MSHT4 was used as a reference substance serving as a positive hybridisation control.

The combined results from these multiple Southern blot analyses establish that both glyphosate-tolerant lucerne J101 and J163 are characterised by the presence of one copy of the gene cassette, inserted at a single locus in the lucerne genome. No unexpected hybridisation bands were detected. These results indicate that lucerne J101 and J163 do not contain any additional DNA elements other than those expected from the insertion of the *cp4 epsps* expression cassette. Fragments corresponding to partial genes, regulatory elements or backbone sequences derived from the transforming plasmid were not detected in either lucerne J101 or J163. A linear map of the inserted DNA in lucerne J101 and J163 is presented below (Figure 2).

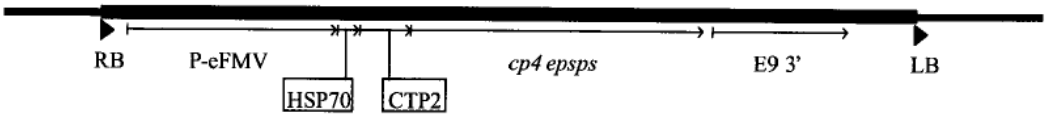

Figure 2: Insert map of the insertion events in glyphosate-tolerant lucerne J101 and J163

A schematic of the insert in glyphosate-tolerant lucerne is shown above. The same insert is present in both J101 and J163. The bold heavy line represents the genetic material inserted into the lucerne genome. The lighter line to the left and right of the insert represents genomic DNA. Individual genetic elements are identified below the insert. The map was developed on the basis of Southern blot characterisation data for both J101 and J163 and confirmed by DNA sequence analysis.

The precise boundaries of the transferred DNA within the insert in J101 and J163 were confirmed using polymerase chain reaction (PCR) and DNA sequencing. Oligonucleotide primers specific to lucerne genomic DNA sequence flanking the 5' and 3' ends of the T-DNA insertion event were paired with insert-specific oligonucleotide primers. The PCR amplifications generated DNA products of the expected sizes. The sequences of the PCR products representing the 5' and 3' insert-to-plant genomic DNA junctions in both J101 and J163 confirmed the 5' and 3' ends of the T-DNA insertion.

According to the Southern hybridisation analyses and the results of DNA sequencing across T-DNA-genomic DNA borders at both ends of the insert, the arrangement of genetic elements in the plant correlates exactly with those present in the transforming plasmid PV-MSHT4.

The DNA sequence of the entire insert in both J101 and J163 was determined and aligned to the corresponding DNA sequence of the transformation vector PV-MSHT4 using the BestFit function in SeqLab. The DNA sequence of the insert in J101 is 100% identical to the corresponding DNA sequence in PV-MSHT4, indicating that the DNA sequence of the J101 insert was not altered during the transformation process. The DNA sequence of the insert in J163 has one base pair change from that in PV-MSHT4. The single A to C base pair change is located in the intervening sequence and does not cause a change in the coding region of the inserted DNA sequence.

Sequence of putative polypeptides encoded at the junction of transgene and host DNA

Studies submitted:
McCoy, R.L. and A. Silvanovich. Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of the Roundup Ready® Alfalfa Event J101 Insert: Assessment of Putative polypeptides. Monsanto Study Report, MSL-18200. completed March 2003.

McCoy, R.L. and A. Silvanovich. Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of the Roundup Ready® Alfalfa Event J163 Insert: Assessment of Putative polypeptides. Monsanto Study Report, MSL-18202. completed March 2003.

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 glyphosate-tolerant lucerne J101 and J163, the transformation event has not resulted in any additions, deletions, rearrangements or partial insertions of the genes of interest, or their regulatory elements, as determined by the Southern blot, PCR analyses and direct DNA sequencing of the entire insert region. Nonetheless, the applicant has provided a bioinformatic evaluation of DNA sequences flanking the junctions of the inserted DNA in J101 and J163 for assessment of putative polypeptides.

PCR was used to determine the genomic DNA sequence flanking each transformation event. The lucerne DNA sequences flanking the transgene junctions of insert J101 and J163 were analysed to determine whether any novel open reading frames had been generated that were capable of encoding new proteins. For both J101 and J163, the lucerne DNA sequence at each end of the transgene insertion was analysed in every reading frame to identify all potential open reading frames. The sequences of these potential polypeptides were evaluated for similarities to known protein toxins and allergens.

Potential toxicity of putative polypeptides encode by the insert-genomic DNA junction sequences was assessed using the FASTA algorithm to search the TOXIN5 database. No alignments with any of the query sequences generated an E score[5] of less than 1×10^{-5}. Further visual inspection of sequence alignments also indicated that none of the putative polypeptides are likely to be structurally similar to known toxin proteins.

Similarly, the bioinformatic analysis of the potential polypeptides for similarities with known allergens using the FASTA sequence alignment tool did not identify any significant sequence similarity. In addition, potential allergenicity of putative polypeptides was assessed using the ALLERGENSEARCH algorithm to screen for smaller immunologically significant epitopes. Using this algorithm to search the AD3_1 database, no alignments of eight of more identical amino acids to known or suspected allergens were obtained. Therefore, these putative polypeptides are unlikely to contain any cross-reactive IgE-binding epitopes.

All potential polypeptides were also analysed for similarity to all known proteins in publicly available genetic databases to identify any relevant similarity to other pharmacologically active proteins. Using the FASTA sequence alignment tool, no E scores less than 10 were recorded. Further visual inspection of all sequence alignments also indicated that the potential chimaeric polypeptides are not likely to be structurally similar to proteins of adverse activity.

The results of these bioinformatic analyses demonstrate that even in the highly unlikely event that any of the junction polypeptides were translated, they would not share a sufficient degree of sequence similarity or identity to indicate that they would be potentially toxic, allergenic or have other health implications.

[5] The E score reflects the degree of similarity between a pair of sequences and can be used to evaluate the significance of an alignment. The calculated E score depends on the overall length of joined (gapped) local sequence alignments, the quality (percent identity/similarity) of the overlap and the size of the database used for the FASTA search (Pearson and Lipman, 1988). For a pair of sequences, very small E score values may indicate a structurally relevant similarity. Conversely, large E score values are typically associated with poor alignments that do not represent a biologically relevant structural similarity.

3.5 Stability of the Genetic Changes

Segregation data

Lucerne is a perennial, autotetraploid plant with four sets of eight individual chromosomes (x=8). In lucerne, there are four copies of each chromosome (2n=4x=32); each set of four chromosomes is assumed to segregate randomly in meiosis, producing gametes with a pair of each chromosome (n=2x=16).

Lucerne is an outcrossing species, showing various forms of genetic self-incompatibility or self-sterility, and displays inbreeding depression (poor vigour and low seed yield). Commercial lucerne breeding programs are built around the need to avoid significant inbreeding. A typical lucerne variety may have ten to 200 parent plants that are allowed to randomly intercross through open pollination to produce breeder seed. A lucerne variety is maintained through multiple generations via open pollination in isolation from other lucerne varieties. While a lucerne variety displays traits within certain defined ranges, individual plants within a variety are genotypically and phenotypically heterogeneous.

Since lucerne is adversely affected by inbreeding, a conventional backcross of a hybrid to either one of its parents is likely to produce plants with a dramatic reduction in forage and seed yield. Lucerne breeders therefore employ a modified backcross (MBC), whereby a hybrid plant is crossed to a plant from either one of its parent's source populations. In this way, vigorous, non-inbred MBC1 seed and progeny are produced.

The segregation of glyphosate-tolerant lucerne J101 and J163 was tested in a hybrid F1 population and through four subsequent modified backcross generations (MBC1 through MBC4). The number of glyphosate-tolerant individuals was recorded in each generation. Expected and observed segregation frequencies of glyphosate tolerance (*cp4 epsps*) were recorded and subjected to chi square analysis. The heritability and stability of the inserted *cp4 epsps* gene was demonstrated through five generations. The results for both J101 and J163 are consistent with a single locus of insertion segregating according to predicted Mendelian patterns of inheritance.

In order to develop lucerne varieties displaying high trait purity, Forage Genetics International (FGI) have developed a proprietary conventional breeding method (patent pending)[6] that combines two separate copies of the *cp4 epsps* transgene at independently segregating loci. The intercrossing of plants carrying at least one copy of each of the two independent transformation events results in populations with greater than 90% trait purity in the commercial seed generation as outlined in Figure 3.

[6] FGI has filed a U.S. patent application (US-2002-0042928-A1) relating to a novel conventional method of breeding lucerne with high transgene trait transmission in the commercial product: "Methods for maximizing Expression of Transgenic Traits in autopolyploid Plants".

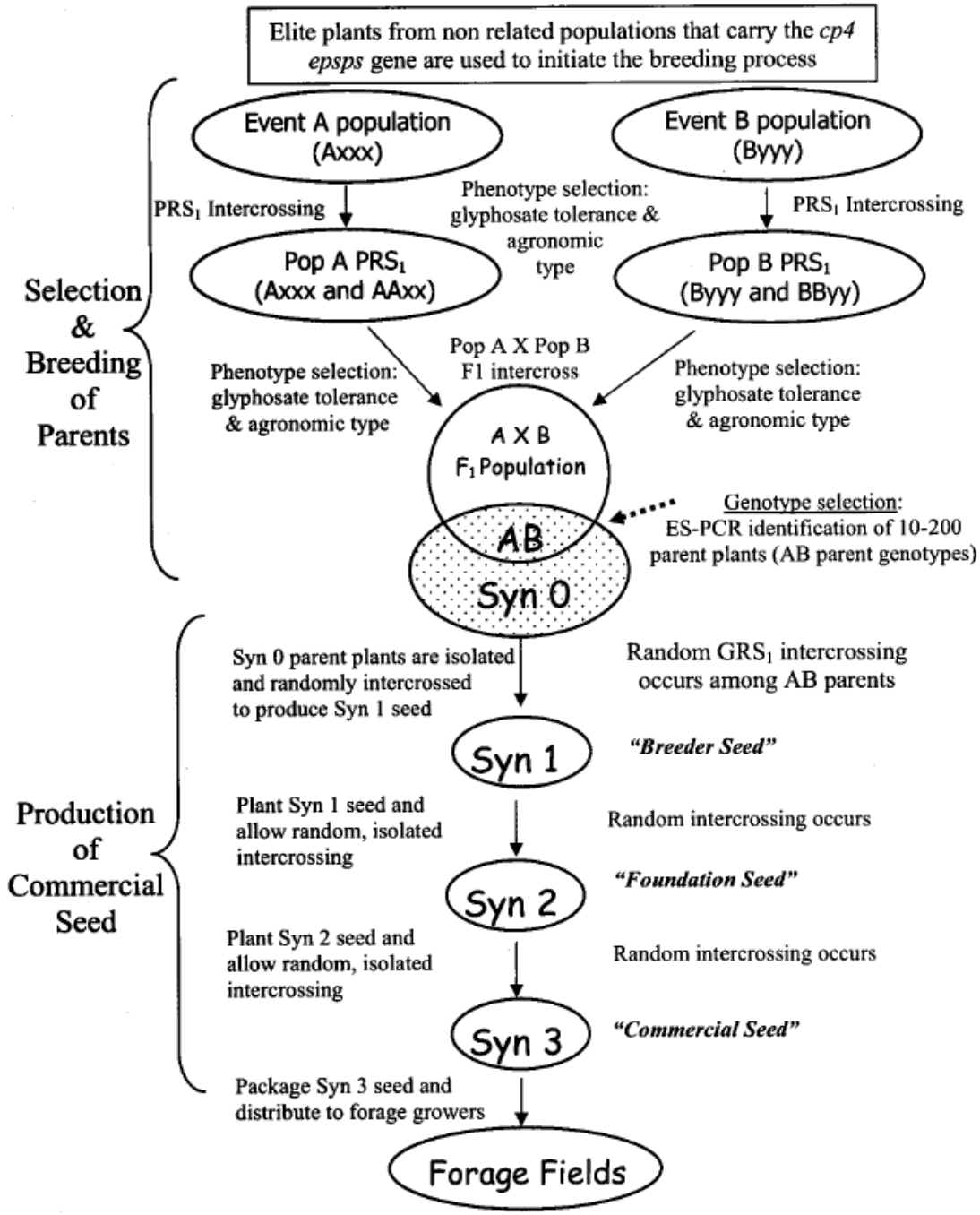

Figure 3: FGI glyphosate-tolerant lucerne synthetic variety breeding schematic.

PRS1 = first cycle of Phenotypic Recurrent Selection - an application of Roundup herbicide is used to eliminate nulliplex plants containing no copies of the *cp4 epsps* transgene.
GRS1 = first cycle of Genotypic Recurrent Selection - event specific PCR (ES-PCR) is used to identify plants carrying at least one copy of both event A (e.g. J101) and event B (e.g. J163).
These dihomogenic plants (A---, B---) within the synthetic population Syn0 are randomly intercrossed to produce breeder seed with greater than 90% trait purity.

Two separate populations are developed; one carrying J101 and the other carrying J163. In each population, glyphosate-tolerant plants are selected and subsequently cross-pollinated. The progeny are again selected for glyphosate tolerance, yielding individual plants carrying either one or two copies of the *cp4 epsps* transgene.

Individual plants from these two separate populations, each carrying one or two copies of either J101 or J163, are randomly crossed. The seed from these F1 plants is bulked and the progeny grown. Event-specific PCR markers were used to genotype individual plants to identify plants carrying one or more copies of the transgene at each of the two loci; these comprise the Syn0 seed. The Syn0 plants were randomly intercrossed to produce Syn1 seed. Syn1 plants (3661) were grown and evaluated phenotypically for herbicide tolerance and genotypically by event-specific PCR.

Statistical significance of the segregation data was determined using Chi square analysis. The expected frequency was calculated using a model for Mendelian inheritance of two independent loci. The data behave as predicted for normal Mendelian inheritance, confirming that the J101 and J163 loci are not genetically linked and are stably inherited over multiple (eight) generations (Table 2).

Table 2: Genotypic Segregation Data for a Dihomogenic (J101 x J163) glyphosate-tolerant lucerne Syn1 Population[a]

Genotype[b]	Actual	Predicted	Chi Square Value (χ^2)	Significance of χ^2
Null	170	170	0.00	NS[c]
J101 only	659	632	1.17	NS
J163 only	641	632	0.13	NS
J101 x J163	2191	2227	0.60	NS
Total	3661			

[a]Table shows actual segregation data for a Syn1 population resulting from the two-event breeding strategy shown in Figure 3. Eight generations of crossing have occurred beyond the T_0 (three generations followed the MBC4 generation.
[b]Null progeny identified in phenotypic assay, herbicide resistant progeny genotype determined by event-specific PCR.
[c]NS = Not significant ($p<0.05$)

The data further show that glyphosate-tolerant lucerne J101 and J163 are genetically stable when combined in dihomogenic populations. Lucerne plants carrying two identical copies of the *cp4 epsps* gene at distinct loci do not display any gross chromosomal rearrangement or genetic instability due to recombination through meiotic pairing of the homologous insert regions on non-homologous chromosomes. Any such events would result in distorted segregation ratios because of embryo abortion and reproductive instability.

The number of *cp4 epsps* gene copies in any individual plant within the glyphosate-tolerant variety can range from zero to eight. However, the same glyphosate tolerance phenotype is observed whether one or more than one copy of the *cp4 epsps* gene is present. The average number of *cp4 epsps* gene copies per plant is calculated to be 2.28 in the Syn1 population.

Stability of the inserted DNA

In order to demonstrate the stability of the genetic change in J101 and J163 over multiple generations, additional Southern blot analyses were performed comparing the original transformants (T_0) and dihomogenic Syn1 generations. Since lucerne can be vegetatively propagated, the original T_0 plants that were regenerated from the R2336 callus tissue have been maintained. Tissue was obtained from the T_0 plants and from plants in the advanced breeding program.

The conventional R2336 cultivar was used as a control. DNA from cultivar R2336 spiked with DNA from plasmid PV-MSHT4 served as a positive hybridisation control. The breeding history of glyphosate-tolerant lucerne is shown in Figure 4.

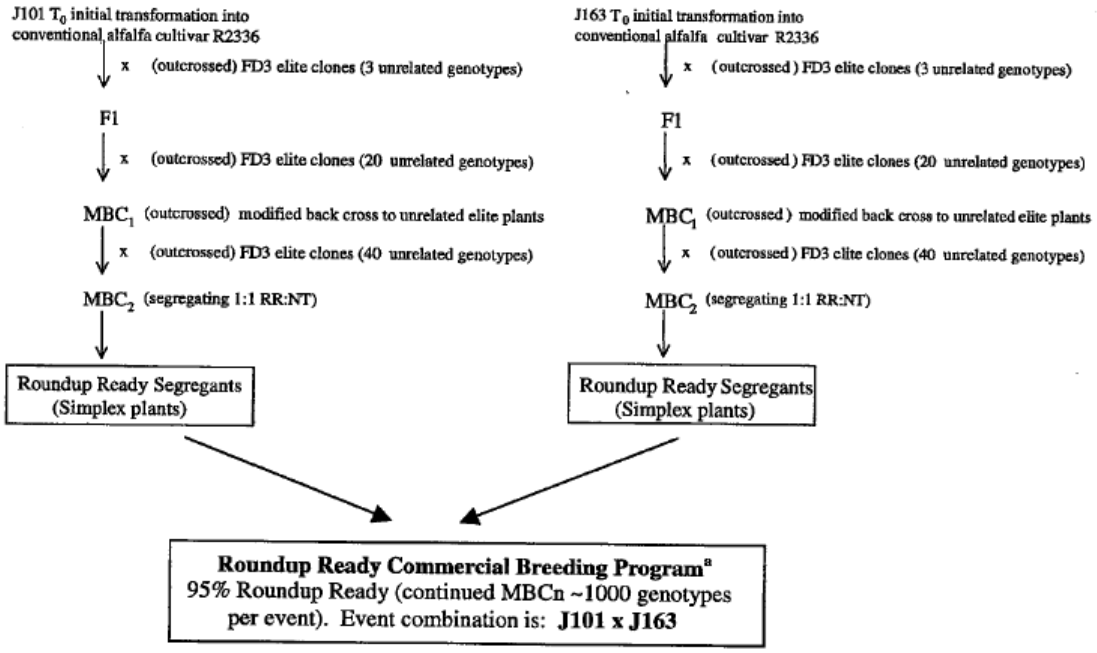

Figure 4: Glyphosate-tolerant lucerne breeding history

aGeneration of the dihomogenic Syn1 line is in bold
FD - fall (autumn) dormancy; x - hybrid cross; MBC - modified backcross (cross to a plant from its parent's source population rather than a parent, to avoid inbreeding depression); RR - Roundup Ready; NT - non-transgenic

Genomic DNA digested with the restriction enzyme SphI was probed with PV-MSHT4 fragments corresponding to the P-eFMV and *cp4 epsps* region. Glyphosate-tolerant lucerne J101 yielded the expected hybridising band sizes of 13.0 kb and 6.5 kb. Glyphosate-tolerant lucerne J163 yielded the expected size bands of 3.6 kb and 1.9 kb. DNA from the dihomogenic J101 x J163 Syn1 generation produced hybridisation bands of 13.0 kb, 6.5 kb, 3.6 kb and 1.9 kb. This demonstrated that both J101 and J163 are stable in the T_0 and dihomogenic Syn1 generations.

3.6 Antibiotic Resistance Genes

No antibiotic marker genes are present in glyphosate-tolerant lucerne J101 or J163. The molecular characterisation shows that the region outside the T-DNA of plasmid PV- MSHT4 was not integrated into the lucerne genome during transformation generating J101 and J163, and therefore genetic elements located on the plasmid backbone were not transferred to the plants. Consequently, the bacterial selectable marker gene, *aad* (which confers resistance to the antibiotics spectinomycin and streptomycin), is not present in glyphosate-tolerant lucerne J101 or J163. The absence of the bacterial marker gene in the plant was confirmed by Southern hybridisation analysis using a probe encompassing the *aad* gene.

4. CHARACTERISATION OF NOVEL PROTEINS

4.1 Function and phenotypic effects

Expression of the CP4 EPSPS protein in lucerne J101 and J163 plants confers tolerance to the herbicide glyphosate. This protein is one of many EPSPS proteins found in nature in a broad range of organisms including plants, bacteria and fungi. The bacterial CP4 EPSPS is naturally highly tolerant to inhibition by glyphosate and continues to have high catalytic efficiency in the presence of the herbicide. Plant cells producing the CP4 EPSPS protein are therefore tolerant to glyphosate because the enzyme continues to function when the plant's own EPSPS has been inactivated by the herbicide.

Several glyphosate-tolerant varieties of corn, canola and soybean expressing CP4 EPSPS have been assessed for safety previously and are permitted on the market for use in food.

The mature 47.6 kDa CP4 EPSPS protein consists of a single polypeptide of 455 amino acids. In lucerne J101 and J163, the pre-protein consists of 531 amino acids including the CTP2 transit peptide of 76 amino acids, which is cleaved on uptake into the plant chloroplasts.

4.2 Protein Expression Analysis

Studies submitted:
Watson, J.A., F.S. Sayegh and R.P. Lirette. CP4 EPSPS Protein Levels in Forage Collected from Roundup Ready ® Alfalfa Lines produced in United States Field Trials in 2001 and 2002. Monsanto Study Report, MSL-17692. completed February 2003.

The levels of the CP4 EPSPS protein in forage of glyphosate-tolerant lucerne J101 and J163 plants were estimated using an enzyme-linked immunosorbent assay (ELISA). For detection of CP4 EPSPS, mouse monoclonal antibodies were raised. A goat polyclonal CP4 EPSPS antibody was used for detection, with quantitation of protein levels accomplished by interpolation from a CP4 EPSPS protein seven point standard curve. The analytical accuracy of the ELISA was validated by spiking extracts from forage of non-transgenic control lucerne plants with known quantities of the CP4 EPSPS protein at three concentrations. The limit of detection of the ELISA was estimated to be 0.18 μg/g fresh weight.

To produce the material for analysis, lucerne populations containing either J101 or J163, as well as J101 x J163 populations and a non-transgenic control line, were planted at six field sites in the spring of 2001. Sites represented geographies where lucerne is typically grown in the United States - California, Illinois, Iowa, New York, Washington and Wisconsin. Because lucerne is a perennial plant that can be harvested multiple times over the growing season, the CP4 EPSPS protein level was determined at two different times during the growing season and from two different years of forage growth (2001 and 2002). Forage was harvested at all sites when plants were at the early to late bud stage, corresponding to the growth stage where lucerne is typically harvested for maximum quality (Marten *et al*., 1988).

The mean level of the CP4 EPSPS protein across two seasons and from multiple cuttings was 257 and 270 μg per gram of tissue fresh weight (tfw) for lucerne plants containing J101 and J163 respectively (Table 3). The mean level of the CP4 EPSPS protein in the lucerne J101 x J163 population across two seasons and from multiple cuttings, was 252 μg/gram tfw.

Combining the two inserts in the J101 x J163 population did not cause an additive effect on the level of the CP4 EPSPS protein, although there was greater variation in the levels of the CP4 EPSPS protein in forage from plants containing J101 x J163.

Table 3: CP4 EPSPS levels in glyphosate-tolerant lucerne J101, J163 and J101 x J163

	Levels of CP4 EPSPS Protein in Forage (μg/g TFW)								
	Event and Year of Forage Sampling								
	Combined years			**2001**			**2002**		
	J101	**J163**	**J101 x J163**	**J101**	**J163**	**J101 x J163**	**J101**	**J163**	**J101 x J163**
Mean across sites	**257**	**270**	**252**	276	317	312	238	223	192
Range low	**160**	**140**	**120**	220	270	260	160	140	120
Range high	**340**	**340**	**390**	340	380	390	340	340	310

The field trials also confirmed that tolerance to glyphosate was excellent and consistent from J101, J163 and J101 x J163.

As one of the major food uses of lucerne is as sprouted seeds, the level of CP4 EPSPS protein in alfalfa sprouts was also evaluated. Surface sterilised alfalfa seeds were grown for four days and the level of CP4 EPSPS in a protein extract was estimated by Western blot analysis using a goat anti-CP4 EPSPS antibody. CP4 EPSPS represents approximately 7-8 ng/500 ng total protein in alfalfa sprouts derived from populations containing J101 and J163. For comparison, a leaf extract from line J163 expressed CP4 EPSPS at 6 ng/500 ng total protein. These results indicate that the level of CP4 EPSPS protein in sprouted tissue is slightly higher than that in leaf tissue.

4.3 Characterisation of the novel protein in J101 and J163

Studies submitted:
Karunanandaa, K., J.L. Lee, M. Tran, J.J. Thorp, R.S. Thoma, T.C. Lee, J.N. Leach, C. George, A. Silvanovich, J.D. Astwood. Assessment of the Physicochemical and Functional Equivalence of the CP4 EPSPS Protein Purified from the Forage of Roundup Ready® Alfalfa Event J101 Grown in Year 2002 and the *E. coli*-produced CP4 EPSPS. Monsanto Report MSL Number: 18295, completed February 2003.

Karunanandaa, K., J.L. Lee, M. Tran, J.J. Thorp, R.S. Thoma, T.C. Lee, J.N. Leach, C. George, A. Silvanovich, J.D. Astwood. Assessment of the Physicochemical and Functional Equivalence of the CP4 EPSPS Protein Purified from the Forage of Roundup Ready® Alfalfa Event J163 Grown in Year 2002 and the *E.coli*-produced CP4 EPSPS. Monsanto Report MSL Number: 18296, completed March 2003.

Quantities of the CP4 EPSPS protein were produced as reference material by expression in *E. coli*. This microbially-produced reference material is used in toxicity and allergenicity studies and to establish that the CP4 EPSPS protein isolated from the forage of glyphosate-tolerant lucerne plants corresponds biochemically to the reference material produced in the laboratory. A panel of analytical tests was used to identify, characterise and compare the plant- and microbially-produced CP4 EPSPS proteins.

The tests included:

(1) N-terminal sequence analysis;
(2) matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (MS);
(3) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis;
(4) CP4 EPSPS activity assay and densitometry; and
(5) glycosylation analysis.

The results are summarised as follows:

(1) N-terminal sequence – The N-terminal sequence for the CP4 EPSPS protein produced in both J101 and J163 confirmed the expected amino acid N-terminal sequence. Two sequences were observed; one starting at residue six, and the other at residue five. Alternate N-terminal sequences for plant produced CP4 EPSPS have been observed previously in soybean, canola and cotton (Harrison *et al*., 1996f) and the loss of the first few N-terminal amino acid residues may be due to protease action when plant cells are homogenised.

(2) MALDI-TOF – The average masses of the CP4 EPSPS protein present in J101 and J163 were 47,037 Da, and 47,032 Da respectively, which compares well with the predicted mass of CP4 EPSPS (corrected for the loss of the five N-terminal amino acid residues) of 47,104 Da.

MALDI-TOF MS analysis of the tryptic digest of the plant derived CP4 EPSPS yielded peptide sequences consistent with the peptide sequences of the *E. coli*-produced CP4 EPSPS. Identification of a protein through MALDI-TOF is dependent on matching a sufficient number of observed masses of tryptic digest fragments to theoretical mass fragments. A total of 20 and 21 observed mass fragments for J101- and J163-purified CP4 EPSPS respectively matched the expected tryptic digest mass fragments from the deduced amino acid sequence of the CP4 EPSPS protein. The identified masses provided 53.4% and 54.7% coverage of the J101 and J163 plant-produced CP4 EPSPS protein, which was sufficient to confirm the chemical equivalence of the plant-produced and *E. coli*-produced CP4 EPSPS protein.

(3) Western blot analysis – Using a goat anti-CP4 EPSPS antibody, the electrophoretic mobility and immunoreactivity of the plant-produced CP4 EPSPS protein were shown to be similar to the *E. coli*-produced CP4 EPSPS reference standard. This serum has previously been shown to be specific for the CP4 EPSPS protein.

(4) Enzyme activity – The functional activities of the plant-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS reference standard were determined using a phosphate release assay. The specific activities of the J101 and *E. coli*-produced CP4 EPSPS proteins were
5.5 U/mg total protein and 3.9 U/mg total protein, respectively. In a separate assay, the specific activities of the J163 and *E. coli*-produced CP4 EPSPS proteins were 7.3 U/mg total protein and 4.7 U/mg total protein, respectively. The enzyme assay demonstrated that the plant-produced CP4 EPSPS protein was as active as *E. coli*-produced CP4 EPSPS protein and thus the plant-produced protein is functionally equivalent to the *E. coli*-produced protein.

(5) Glycosylation – The isolated plant-produced CP4 EPSPS protein was analysed for post-translational modification through covalently bound carbohydrate moieties. The *E. coli*-produced CP4 EPSPS protein was used as a non-glycosylated negative control and the transferrin protein as a positive control.

There is no detectable glycosylation of the plant-derived CP4 EPSPS protein, indicating it is equivalent to the *E. coli*-produced CP4 EPSPS protein with respect to glycosylation.

(6) Molecular weight and purity – The apparent molecular weight of the plant-produced CP4 EPSPS protein, estimated by comparison to molecular weight markers on a stained gel, was 43.6 kDa for J101 and 43.3 for J163 (calculated as the average of 3 loadings). The plant- and *E. coli*-produced proteins co-migrated on the gradient gel. The purity of the plant-produced CP4 EPSPS protein was calculated to be 65.2% for J101 and 72.2% for J163.

A combination of N-terminal sequence analysis, MALDI-TOF and Western blot have confirmed the identity of the plant-produced CP4 EPSPS protein. The characterisation of the *E. coli*-produced CP4 EPSPS protein indicates it is equivalent to the plant-produced CP4 EPSPS protein based on comparable electrophoretic mobility, enzyme activity, immunoreactivity and absence of detectable glycosylation. Based on the similarity of the results from the plant and microbial preparations, the *E. coli*-produced protein is chemically and functionally equivalent to CP4 EPSPS protein expressed in J101 and J163.

4.4 Potential toxicity of novel proteins

Studies submitted:
McCoy, R.L., A. Silvanovich. Bioinformatics Analysis of the CP4 EPSPS Protein utilizing the AD4, TOXIN5 and ALLPEPTIDES Databases. Monsanto Report MSL Number: 18752, completed October 2003.

The mature CP4 EPSPS protein in glyphosate-tolerant lucerne J101 and J163 is substantially similar to the EPSPS proteins naturally present in a variety of food crops (e.g. soybean and corn), which have a history of safe consumption by humans (Padgette *et al*., 1996; Harrison *et al*., 1996e). Also, the mature CP4 EPSPS protein in glyphosate-tolerant lucerne J101 and J163 is identical to, or shares greater than 99% sequence identity to, the amino acid sequence of the CP4 EPSPS protein produced in a number of other glyphosate-tolerant crops that have previously been approved for food use by FSANZ.

The CP4 EPSPS protein has previously undergone assessment by FSANZ when present in other GM (glyphosate-tolerant) crop varieties including soybean, cotton, canola, sugarbeet and corn. The data submitted for an assessment of potential toxicity have therefore been comprehensively appraised (see Final Assessment Reports for FSANZ Applications A338, A355, A362, A363, A378, A416, A525, A548 and A553).

These assessments considered history of previous exposure to the protein through the diet, bioinformatic analysis of the primary and secondary structure of the CP4 EPSPS protein to examine any similarities with known protein toxins, biochemical tests (heat stability), and acute oral toxicity studies in animals. The previous assessments concluded that the CP4 EPSPS protein is not toxic and is therefore safe for human consumption.

Acute toxicity studies

To generate sufficient quantities of the CP4 EPSPS protein required for toxicity, and biochemical studies, it is necessary to produce the protein in bacterial expression systems. Prior to use, the bacterially produced protein is compared to the protein produced in the plant, to demonstrate their equivalence.

The CP4 EPSPS used for further analyses was produced in the laboratory using recombinant *E. coli*. As outlined in the previous section, a range of biochemical methods was used to establish that *E. coli* -produced CP4 EPSPS protein is equivalent to the protein produced by glyphosate-tolerant lucerne J101 and J163.

The acute toxicity of the CP4 EPSPS protein has been previously tested by acute gavage exposure in mice and no deleterious effects were observed (Harrison *et al*., 1996d). The CP4 EPSPS protein was administered at levels 1000 fold of those in anticipated consumption of food products; the no effect level (NOEL) for oral toxicity in mice is 572 mg/kg body weight, and was the highest dose tested. Despite this high dose, there was no mortality or morbidity, and there were no significant differences in terminal body weights of animals in the treated and control groups. Upon necropsy, body cavities were opened and organs examined *in situ* and removed. There were no pathological findings attributable to the treatment with the CP4 EPSPS protein.

4.5 Potential allergenicity of novel proteins

The potential allergenicity of a novel protein is evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity. The assessment focuses on:

(i) the source of the novel protein,
(ii) any significant amino acid sequence similarity of the novel protein with that of known allergens, and
(iii) structural properties of the novel protein, including susceptibility to degradation in simulated digestion models.

Using a decision tree approach, when indicated, additional *in vitro* and *in vivo* immunological testing can be conducted. Applying such criteria systematically provides reasonable evidence on the potential of the novel protein to act as an allergen.

Source of protein

The CP4 EPSPS protein in J101 and J163 is derived from a naturally occurring, glyphosate-degrading bacterium, *Agrobacterium tumefaciens*, identified by the American Type Culture Collection. Species of *Agrobacterium* are not known human or animal pathogens, nor known to be allergenic.

Similarity to known allergens

Potential structural similarities between the CP4 EPSPS enzyme and proteins in the allergen database were evaluated using the FASTA sequence alignment tool. Inspection of the results showed no significant similarities between the CP4 EPSPS protein and known allergens. No immunologically relevant sequences (identity across eight contiguous amino acids) were detected when the CP4 EPSPS sequence was compared to the AD4 sequence database. Previous bioinformatic analyses of the CP4 EPSPS protein have yielded the same negative results.

In vitro digestibility

Typically, food proteins that are allergenic tend to be stable to enzymes such as pepsin and the acidic conditions of the digestive system, exposing them to the intestinal mucosa and leading to an allergic response (Metcalfe *et al*., 1996; Astwood *et al*., 1996; Kimber *et al*., 1999). Novel proteins are therefore investigated for their digestibility in simulated digestion models.

Previous assessment of the CP4 EPSPS protein found that it is rapidly degraded in simulated digestive fluids. The half-life of CP4 EPSPS was less than 15 seconds in the gastric system and less than 10 minutes in the intestinal system, based on Western blot analysis (Harrison *et al*., 1996b; Harrison *et al*., 1996c). Subsequent experiments to assess the *in vitro* digestibility of the CP4 EPSPS protein in simulated gastric fluid (SGF) showed that 95-98% of the CP4 EPSPS protein was digested within 15 seconds. Similarly, the EPSPS activity was reduced to <10% within 15 seconds of incubation in SGF.

4.6 Conclusion

The CP4 EPSPS protein is expressed in J101 and J163 at a mean of 257 and 270 μg/g tfw, respectively. Combining the two lines does not cause an additive effect on the level of the protein; the mean level of CP4 EPSPS protein in the synthetic lucerne population J101 x J163 was 252 μg/g tfw. When grown under normal field conditions, mean concentrations of the CP4 EPSPS protein in lucerne forage range from a high of 390 μg/g tfw, to a low of 120 μg/g tfw. The CP4 EPSPS protein is estimated to represent about 0.49% and 0.52% of the total protein in J101 and J163 respectively. The level of CP4 EPSPS protein in sprouted alfalfa seeds is only slightly higher than that in forage tissue.

The characterisation of the CP4 EPSPS protein in J101 and J163 indicates it is chemically and functionally equivalent to the *E. coli*-produced CP4 EPSPS protein based on comparable electrophoretic mobility, enzyme activity, immunoreactivity and absence of detectable glycosylation. Therefore, previous studies of the acute toxicity carried out using *E. coli*-produced CP4 EPSPS protein are applicable to the protein produced by glyphosate-tolerant lucerne J101 and J163. No deleterious effects of CP4 EPSPS protein were observed in the toxicity study (Harrison *et al*., 1996a).

The CP4 EPSPS protein is structurally and biochemically similar to other EPSPS enzymes from various plant food sources that are currently part of the human diet and have been consumed over a long period of time without health concerns. The mature CP4 EPSPS protein in glyphosate-tolerant lucerne J101 and J163 is identical to, or shares greater than 99% sequence identity to, the amino acid sequence of the CP4 EPSPS protein produced in a number of other glyphosate-tolerant crops that have previously been approved for food use by FSANZ. The potential toxicity and allergenicity of the CP4 EPSPS protein has been assessed by FSANZ on numerous occasions and no adverse findings have been reported. Its use is approved in food derived from specific lines of soybean, sugarbeet, corn, cotton and canola.

5. COMPARATIVE ANALYSES

A comparison of similarities and differences in composition between a GM plant and its conventional counterpart aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy for the safety and nutritional assessment of GM foods (WHO, 2000). Ideally, the comparative approach to establishing substantial equivalence compares the modified variety to the near isogenic parental line grown under identical conditions. In this case, the transgene is the only genetic difference between the two tested varieties. Lucerne is an outcrossing autotetraploid that shows pronounced inbreeding depression. As it is not possible to self-pollinate a single genotype to generate seed with equivalent vigour to the new variety, the non-transgenic progenitor is unsuitable as a comparator. Instead, a closely related population of non-transgenic control plants was developed from null segregants within the glyphosate-tolerant lucerne breeding program for use as a comparator. Since the null segregant population is derived from the same ancestor population as the transgenic lines, it has background genetics representative of the transgenic populations but does not express the CP4 EPSPS protein. A breeding map describing the development of the glyphosate-tolerant lucerne J101, J163 and J101 x J163 populations and the null segregant comparator population is shown in Figure 5. PCR analysis was used to confirm the presence or absence of each test event in test, control and reference substances.

Twelve different commercially available conventional lucerne varieties were also used to establish comparable ranges for compositional constituents.

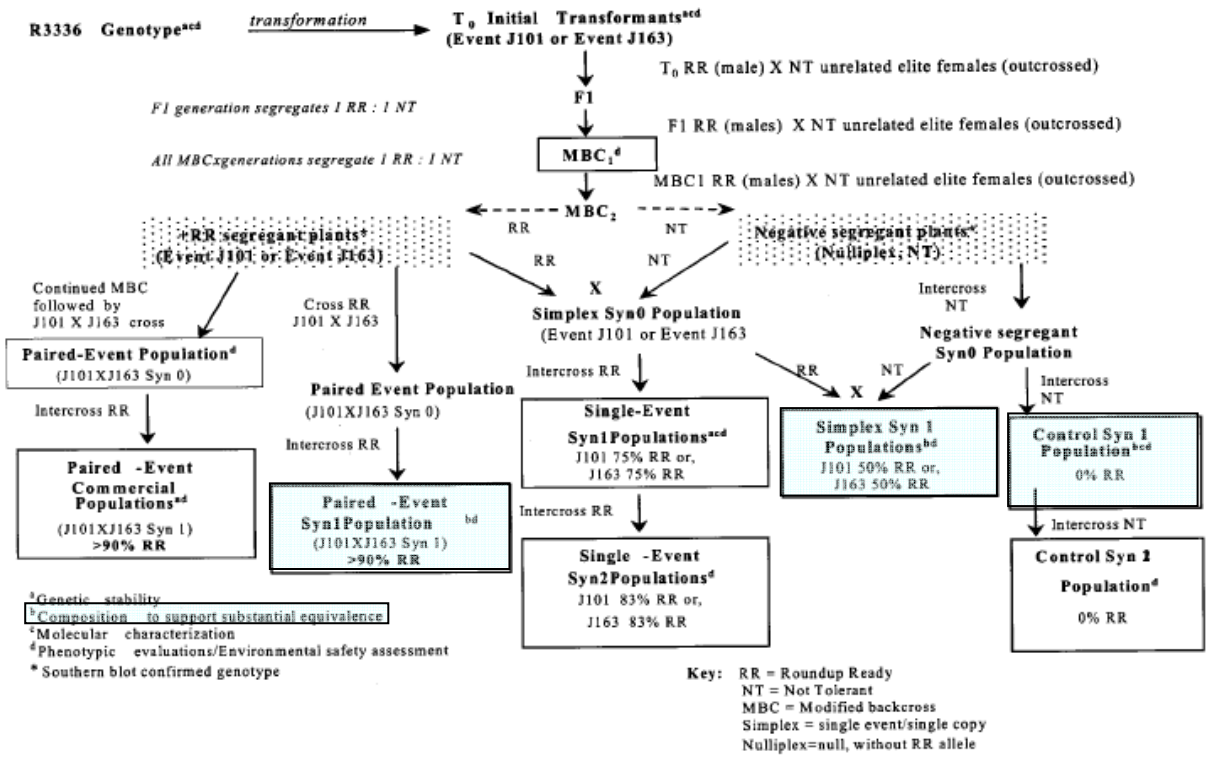

Figure 5: Breeding Map for Glyphosate-tolerant and Control Lucerne Populations

5.1 Levels of key nutrients and other constituents

Studies submitted:
McCann, M., M.A. Nemeth, W.A. Trujillo and R. Sorbet. Compositional Analysis of Forage Collected from Selected Roundup Ready® Alfalfa Events Grown in 2001 U.S. Field Trials. Monsanto Study Report, MSL-18145, completed March 2003.

When determining similarities and differences in composition between a GM plant and its conventional counterpart, the critical components measured are determined by identifying key nutrients, key toxicants and anti-nutrients for the food source in question (FAO, 1996). The key nutrients and toxicants/anti-nutrients are those components in a particular food that have a substantial impact in the overall diet. These can be major constituents (e.g., fats, proteins, carbohydrates) or minor components (e.g., minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose potency and level may be significant to health (e.g., increased levels of solanine in potatoes). As a minimum, the key components of lucerne appropriate for this comparative study include the proximates (crude protein, fat and ash), digestibility factors (neutral detergent fibre, acid detergent fibre and lignin), minerals and amino acids (OECD, 2005).

The primary food uses of lucerne are sprouted alfalfa seeds in salads, young alfalfa shoots as a vegetable and alfalfa teas. The major compositional analysis of glyphosate-tolerant lucerne with its non-transgenic control was performed on forage, which is the material commonly harvested for hay. The OECD guidelines on compositional considerations for new varieties of alfalfa (OECD, 2005) suggest that "a decision regarding the importance of assessing the nutrient composition of forage legumes used as sprouted seeds in human diets should be guided by the frequency and quantity of such sprouts in a given country and their contribution to nutrient intake." Further, that "a comparison of the nutrient composition of one cup of alfalfa sprouts to recommended intakes of these nutrients suggests that the contribution is minor." It is also expected that sprouts or other forms of alfalfa would be consumed in minor quantities on an occasional basis. The OECD consensus document on forage legumes (OECD, 2005) suggests that a comparison of lucerne for food use include analysis of fresh forage or sprouted alfalfa seed for the factors outlined above for forage with the addition of vitamin C, beta-carotene, folate and phytoestrogens as a basis for the assessment of potential unintended effects.

Lucerne Forage

Field conditions

The composition of forage from lucerne J101, J163 and the combined J101 x J163 Syn1 population was evaluated and compared to a non-transgenic control population with a similar genetic background. The lucerne varieties were grown at five replicated field sites across the lucerne producing regions of the U.S. (Illinois, New York, Washington, Wisconsin and California) during the 2001 field season. To provide additional reference material representative of the agricultural conditions, four commercially available lucerne varieties were grown at each of these five field sites (twelve commercially available lucerne varieties in total).

At each field site, the three test populations, the control and four reference populations were grown in single plots randomly assigned within each of four replication randomised complete blocks. All plants were grown under normal agronomic field conditions for the respective geographical regions. All test substances received multiple applications of glyphosate herbicide (Roundup ® Ultra) according to proposed label instructions. Second cutting forage samples were collected from all plots at the early to late bloom stage and analysed for nutritional components.

Compositional analysis

Compositional analyses of the forage samples (above ground parts) included proximates (protein, fat, ash and moisture), acid detergent fibre (ADF), neutral detergent fibre (NDF), lignin, amino acids and minerals (calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium and zinc) and carbohydrates by calculation. In all, 35 analytical components of lucerne forage were measured according to established protocols (either USDA or AOAC methods).

Statistical analyses of the forage compositional data were conducted using a mixed model analysis of variance method. The five replicated sites were analysed both separately and combined, giving six sets of comparisons. Statistical evaluation of the composition data compared the forage from the three lucerne test populations to the non-transgenic control population. Statistically significant differences were determined at the 5% level of significance ($p<0.05$). SAS® software was used to generate all summary statistics and perform all analyses.

Data from commercial varieties were not included in the final statistical analysis. The reference population data were used to develop population tolerance intervals. For each compositional component, 99% tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of commercial lines.

In a study of this magnitude, a small percentage (approximately 5%) of statistically significant differences is expected to occur due to chance alone. For those comparisons in which the glyphosate-tolerant lucerne test result was statistically different from the control, the test range was compared to the 99% tolerance interval derived from the commercial varieties. This determines whether the range of values for each test population is within the variance of a population of the commercial lucerne varieties.

Results of the combined sites analyses

The results of the combined site comparisons are presented in Table 4. A summary of the statistically significant differences between lucerne J101, J163 and J101 x J163 and the control line is presented in Table 5.

Results from the analyses conducted on forage samples derived from lucerne plants containing J101 indicated that there were three statistically significant differences observed between the test and non transgenic control: cystine, glutamic acid and tyrosine. Results from the analyses conducted on forage samples derived from lucerne plants containing J163 indicated that there were seven statistically significant differences observed between the test and non transgenic control: cystine, histidine, lysine, tyrosine, acid detergent fibre, lignin and neutral detergent fibre.

Results from the analyses conducted on samples derived from the J101 x J163 population indicated that there were eleven statistically significant differences observed between the test and non transgenic control: cystine, isoleucine, phenylalanine, praline, tyrosine, neutral detergent fibre, calcium, iron, ash, carbohydrates and moisture.

For the means of the analytes that were statistically significantly different ($p<0.05$) from the control, the values were within the 99% tolerance interval developed from the conventional lucerne varieties grown at the same locations (Table 5). Hence, these differences are unlikely to be biologically meaningful. These data are consistent with the conclusion that forage produced by lucerne plants containing J101, J163 or J101 x J163 is comparable to forage produced by control or conventional lucerne varieties and is compositionally equivalent to forage derived from conventional lucerne varieties currently on the market.

Table 4: Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Alanine (% total AA)	Control	6.19 ± 0.097 (6.01 - 6.56)				(5.93 - 6.93) [5.55, 6.80]
	J101	6.19 ±0.097 (5.99 - 6.69)	0.0015 ± 0.063 (-0.22 - 0.36)	-0.13, 0.13	0.981	
	J163	6.27 ± 0.097 (5.96 - 6.93)	0.084 ± 0.063 (-0.19 - 0.75)	-0.044, 0.21	0.190	
	J101 x J163	6.20 ± 0.097 (6.00 - 6.79)	0.011 ± 0.063 (-0.20 - 0.61)	-0.12, 0.14	0.866	
Arginine (% total AA)	Control	5.64 ± 0.063 (5.40 - 6.23)				(5.40 5.90) [4.98, 6.21]
	J101	5.60 ± 0.063 (5.34 - 5.84)	-0.049 ± 0.057 (-0.64 - 0.25)	-0.17, 0.068	0.399	
	J163	5.58 ± 0.063 (5.32 - 5.82)	-0.060 ± 0.057 (-0.51 - 0.27)	-0.18, 0.056	0.299	
	J101 x J163	5.56 ± 0.063 (5.10 - 5.99)	-0.088 ± 0.058 (-0.75 - 0.44)	-0.21, 0.029	0.137	
Aspartic Acid (% total AA)	Control	12.86 ± 0.37 (10.95 - 16.22)				(11.83 - 15.40) [9.75, 16.61]
	J101	13.28 ± 0.37 (12.02 - 17.22)	0.42 ± 0.25 (-1.49 - 3.13)	-0.090, 0.93	0.103	
	J163	13.34 ± 0.37 (11.63 - 15.62)	0.48 ± 0.25 (-1.67 - 2.27)	-0.023, 0.99	0.060	
	J101 x J163	13.16 ± 0.37 (12.05 - 14.34)	0.31 ± 0.25 (-1.22 - 2.40)	-0.21, 0.82	0.234	

A.A. = Amino acid; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Cystine (% total AA)	Control	1.41 ± 0.057 (1.17 - 1.59)				(1.23 - 1.76) [1.01, 1.96]
	J101	1.56 ± 0.057 (1.36 - 1.86)	0.15 ± 0.042 (-0.16 - 0.64)	0.065, 0.23	<0.001	
	J163	1.56 ± 0.057 (1.35 - 1.90)	0.15 ± 0.042 (-0.15 - 0.69)	0.062, 0.23	<0.001	
	J101 x J163	1.57 ± 0.057 (1.41 - 1.84)	0.16 ± 0.042 (-0.091 - 0.63)	0.070, 0.24	<0.001	
Glutamic Acid (% total AA)	Control	11.10 ± 0.077 (10.85 - 11.79)				(10.75 - 11.62) [10.28, 11.77]
	J101	10.95 ± 0.077 (10.64 - 11.34)	-0.15 ± 0.069 (-0.77 - 0.30)	-0.29, 0.015	0.031	
	J163	11.02 ± 0.077 (10.64 - 11.42)	-0.075 ± 0.069 (-0.53 - 0.35)	-0.21, 0.065	0.285	
	J101 x J163	11.03 ± 0.077 (10.70 - 11.33)	-0.069 ± 0.069 (-0.89 - 0.38)	-0.21, 0.072	0.327	
Glycine (% total AA)	Control	5.56 ± 0.044 (5.39 - 5.97)				(5.35 - 5.64) [5.11, 5.84]
	J101	5.52 ± 0.044 (5.37 - 5.77)	-0.034 ± 0.039 (-0.43 - 0.14)	-0.11, 0.044	0.381	
	J163	5.54 ± 0.044 (5.35 - 5.79)	-0.023 ± 0.039 (-0.30 - 0.20)	-0.10, 0.056	0.562	
	J101 x J163	5.61 ± 0.044 (5.46 - 6.23	0.051 ± 0.039 (-0.36 - 0.62)	-0.028, 0.13	0.195	

A.A. = Amino acid; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Histidine (% total AA)	Control	2.76 ± 0.044 (2.57 - 3.01)				(2.43 - 2.96) [2.25, 3.22]
	J101	2.74 ± 0.044 (2.43 - 2.91)	-0.028 ± 0.032 (-0.42 - 0.14)	-0.094, 0.038	0.391	
	J163	2.67 ± 0.044 (2.44 - 2.85)	-0.098 ± 0.032 (-0.56 - 0.088)	-0.16, -0.032	0.004	
	J101 x J163	2.70 ± 0.045 (2.44 - 2.88)	-0.064 ± 0.033 (-0.39 - 0.15)	-0.13, 0.0017	0.055	
Isoleucine (% total AA)	Control	4.94 ± 0.052 (4.65 - 5.31)				(4.60 - 5.20) [4.25, 5.58]
	J101	4.93 ± 0.052 (4.48 - 5.17)	-0.010 ± 0.037 (-0.52 - 0.34)	-0.083, 0.062	0.784	
	J163	4.91 ± 0.052 (4.69 - 5.29)	-0.029 ± 0.037 (-0.56 - 0.47)	-0.10, 0.044	0.434	
	J101 x J163	4.86 ± 0.052 (4.64 - 5.14)	-0.083 ± 0.037 (-0.60 - 0.20)	-0.16, -0.0093	0.027	
Leucine (% total AA)	Control	8.66 ± 0.059 (8.32 - 9.12)				(8.36 - 8.90) [8.08, 9.07]
	J101	8.60 ± 0.059 (8.08 - 8.87)	-0.056 ± 0.057 (-0.48 - 0.26)	-0.17, 0.059	0.327	
	J163	8.59 ± 0.059 (8.25 - 8.97)	-0.072 ± 0.057 (-0.61 - 0.25)	-0.19, 0.044	0.214	
	J101 x J163	8.55 ± 0.060 (8.24 - 8.88)	-0.11 ± 0.057 (-0.59 - 0.27)	-0.23, 0.0020	0.053	

A.A. = Amino acid; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Lysine (% total AA)	Control	7.05 ± 0.098 (6.62 - 7.34)				(6.27 - 7.48) [6.26, 7.85]
	J101	7.07 ± 0.098 (6.43 - 7.53)	0.026 ± 0.060 (-0.77 - 0.45)	-0.093, 0.14	0.669	
	J163	6.89 ± 0.098 (6.50 - 7.37)	-0.16 ± 0.060 (-0.76 - 0.28)	-0.28, -0.039	0.009	
	J101 x J163	6.94 ± 0.098 (6.55 - 7.39)	-0.11 ± 0.061 (-0.70 - 0.31)	-0.23, 0.013	0.079	
Methionine (% total AA)	Control	1.89 ± 0.031 (1.57 - 2.16)				(1.67 - 2.10) [1.56, 2.30]
	J101	1.88 ± 0.031 (1.64 - 2.17)	-0.015 ± 0.040 (-0.37 - 0.27)	-0.096, 0.065	0.701	
	J163	1.91 ± 0.031 (1.64 - 2.16)	0.017 ± 0.040 (-0.29 - 0.36)	-0.064, 0.098	0.672	
	J101 x J163	1.90 ± 0.031 (1.71 - 2.21)	0.011 ± 0.040 (-0.32 - 0.31)	-0.070, 0.093	0.778	
Phenylalanine (% total AA)	Control	5.67 ± 0.065 (5.32 - 6.47)				(5.40 - 6.16) [4.64, 6.61]
	J101	5.61 ± 0.065 (5.20 - 6.23)	-0.062 ± 0.049 (-0.73 - 0.48)	-0.16, 0.039	0.220	
	J163	5.57 ± 0.065 (5.33 - 5.99)	-0.096 ± 0.049 (-0.88 - 0.24)	-0.20, 0.0044	0.060	
	J101 x J163	5.54 ± 0.066 (5.39 - 6.06)	-0.12 ± 0.050 (-0.92 - 0.31)	-0.23, -0.023	0.017	

A.A. = Amino acid; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Proline (% total AA)	Control	5.28 ± 0.11 (4.32 - 5.97)				(4.86 - 5.73) [4.57, 6.06]
	J101	5.29 ± 0.11 (4.93 - 5.82)	0.011 ± 0.079 (-0.46 - 1.24)	-0.15, 0.17	0.889	
	J163	5.37 ± 0.11 (4.75 - 5.91)	0.090 ± 0.079 (-0.22 - 1.27)	-0.071, -0.25	0.264	
	J101 x J163	5.49 ± 0.11 (5.06 - 6.16)	0.21 ± 0.080 (-0.51 - 0.97)	0.048, 0.37	0.012	
Serine (% total AA)	Control	5.36 ± 0.11 (4.87 - 5.73)				(4.92 - 5.91) [4.31, 6.57]
	J101	5.41 ± 0.11 (4.93 - 5.97)	0.051 ± 0.073 (-0.63 - 0.70)	-0.096, 0.20	0.485	
	J163	5.32 ± 0.11 (4.78 - 5.80)	-0.041 ± 0.073 (-0.79 - 0.54)	-0.19, 0.11	0.578	
	J101 x J163	5.45 ± 0.11 (5.05 - 5.92)	0.086 ± 0.073 (-0.37 - 0.72)	-0.063, 0.23	0.248	
Threonine (% total AA)	Control	4.57 ± 0.067 (4.07 - 4.79)				(4.10 - 4.85) [3.63, 5.48]
	J101	4.54 ± 0.067 (4.23 - 4.84)	-0.029 ± 0.051 (-0.37 - 0.27)	-0.13, 0.074	0.575	
	J163	4.60 ± 0.067 (4.36 - 4.81)	0.035 ± 0.051 (-0.38 - 0.31)	-0.068, 0.14	0.497	
	J101 x J163	4.59 ± 0.067 (4.13 - 4.88)	0.023 ± 0.051 (-0.30 - 0.30)	-0.081, 0.13	0.661	

A.A. = Amino acid; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Tryptophan (% total AA)	Control	1.22 ± 0.056 (0.81 - 1.48)				(0.86 - 1.38) [0.62, 1.84]
	J101	1.15 ± 0.056 (0.73 - 1.42)	-0.073 ± 0.044 (-0.49 - 0.36)	-0.16, 0.016	0.104	
	J163	1.15 ± 0.056 (0.78 - 1.48)	-0.075 ± 0.044 (-0.38 - 0.38)	-0.16, -0.013	0.093	
	J101 x J163	1.19 ± 0.057 (0.86 - 1.45)	-0.036 ± 0.044 (-0.36 - 0.40)	-0.12, 0.054	0.424	
Tyrosine (% total AA)	Control	3.83 ± 0.045 (3.46 - 4.51)				(3.30 - 3.94) [3.33, 4.07]
	J101	3.68 ± 0.045 (3.23 - 3.94)	-0.15 ± 0.052 (-0.79 - 0.41)	-0.25, -0.044	0.005	
	J163	3.69 ± 0.045 (3.19 - 3.86)	-0.14 ± 0.052 (-0.80 - 0.15)	-0.24, -0.036	0.008	
	J101 x J163	3.69 ± 0.046 (3.18 - 3.89)	-0.14 ± 0.053 (-1.14 - 0.36)	-0.25, -0.037	0.007	
Valine (% total AA)	Control	6.01 ± 0.051 (5.58 - 6.41)				(5.69 - 6.26) [5.36, 6.63]
	J101	6.01 ± 0.051 (5.60 - 6.24)	-0.00012 ± 0.052 (-0.44 - 0.56)	-0.10, 0.10	0.998	
	J163	6.01 ± 0.051 (5.74 - 6.35)	0.0071 ± 0.052 (-0.37 - 0.70)	-0.096, 0.11	0.892	
	J101 x J163	6.00 ± 0.052 (5.82 - 6.27)	-0.010 ± 0.053 (-0.59 - 0.44)	-0.11, 0.094	0.842	

A.A. = Amino acid; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Acid Detergent Fiber (% DW)	Control	25.79 ± 1.61 (18.81 - 33.47)				(23.12 - 33.39) [15.76, 40.19]
	J101	26.83 ± 1.61 (21.65 - 32.38)	1.04 ± 0.92 (-5.04 - 5.77)	-0.78, 2.86	0.259	
	J163	28.31 ± 1.61 (20.00 - 39.67)	2.52 ± 0.92 (-5.54 - 12.86)	0.70, 4.35	0.006	
	J101 x J163	27.01 ± 1.62 (22.09 - 33.91)	1.22 ± 0.94 (-5.13 - 5.75)	-0.62, 3.07	0.192	
Lignin (% DW)	Control	5.07 ± 0.56 (1.64 - 8.10)				(3.86 - 9.65) [0, 12.92]
	J101	5.78 ± 0.56 (3.86 - 9.11)	0.71 ± 0.39 (-1.70 - 4.12)	-0.063, 1.48	0.071	
	J163	6.01 ± 0.56 (3.94 - 8.13)	0.94 ± 0.39 (-1.43 - 5.51)	0.17, 1.71	0.017	
	J101 x J163	5.31 ± 0.56 (3.48 - 8.16)	0.24 ± 0.40 (-2.00 - 2.06)	-0.54, 1.03	0.543	
Neutral Detergent Fiber (% DW)	Control	28.09 ± 1.37 (22.25 - 32.07)				(26.53 - 35.72) [20.01, 41.80]
	J101	29.49 ± 1.37 (25.22 - 34.05)	1.40 ± 1.02 (-3.68 - 5.79)	-0.68, 3.47	0.181	
	J163	30.94 ± 1.37 (24.49 - 43.57)	2.85 ± 1.02 (-4.07 - 14.78)	0.77, 4.92	0.008	
	J101 x J163	30.64 ± 1.38 (21.87 - 39.73)	2.54 ± 1.03 (-8.13 - 11.55)	0.45, 4.64	0.018	

DW = dry weight; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Calcium (% DW)	Control	1.12 ± 0.070 (0.88 - 1.44)				(0.90 - 1.53) [0.48, 1.89]
	J101	1.14 ± 0.070 (0.94 - 1.51)	0.022 ± 0.044 (-0.29 - 0.28)	-0.067, 0.11	0.623	
	J163	1.12 ± 0.070 (0.91 - 1.58)	0.0049 ± 0.044 (-0.20 - 0.40)	-0.084, 0.094	0.911	
	J101 x J163	1.01 ± 0.070 (0.81 - 1.38)	-0.10 ± 0.044 (-0.40 - 0.22)	-0.19, -0.015	0.023	
Copper (mg/kg DW)	Control	9.41 ± 0.68 (6.76 - 17.10)				(5.29 - 10.18) [3.12, 12.64]
	J101	8.95 ± 0.69 (6.32 - 11.72)	-0.46 ± 0.60 (-9.20 - 4.65)	-1.68, 0.76	0.451	
	J163	9.15 ± 0.68 (6.66 - 19.49)	-0.25 ± 0.59 (-7.39 - 10.49)	-1.45, 0.95	0.672	
	J101 x J163	8.24 ± 0.68 (6.42 - 12.28)	-1.17 ± 0.59 (-9.22 - 3.85)	-2.37, -0.039	0.057	
Iron (mg/kg DW)	Control	410.19 ± 230.60 (184.32 - 764.23)				(235.53 - 1538.46) [0, 892.57]
	J101	563.39 ± 230.60 (240.21 - 1553.40)	153.20 ± 115.24 (-123.45 - 876.41)	-80.90, 387.30	0.192	
	J163	614.37 ± 230.60 (218.23 - 1882.35)	204.18 ± 115.24 (-259.76 - 1230.18)	-29.91, 438.28	0.085	
	J101 x J163	730.93 ± 230.85 (199.10 - 2196.43)	320.74 ± 115.74 (-176.38 - 1530.12)	85.75, 555.73	0.008	

DW = dry weight; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Magnesium (% DW)	Control	0.26 ± 0.051 (0.11 - 0.54)				(0.11 - 0.45) [0, 0.68]
	J101	0.27 ± 0.051 (0.12 - 0.60)	0.012 ± 0.015 (-0.073 - 0.17)	-0.019, 0.042	0.447	
	J163	0.27 ± 0.051 (0.12 - 0.52)	0.011 ± 0.015 (-0.045 - 0.15)	-0.020, 0.042	0.471	
	J101 x J163	0.24 ± 0.051 (0.10 - 0.38)	-0.019 ± 0.015 (-0.16 - 0.062)	-0.050, 0.012	0.230	
Manganese (mg/kg DW)	Control	54.04 ± 8.57 (32.97 - 81.01)				(34.60 - 109.50) [0, 120.37]
	J101	56.72 ± 8.57 (35.20 - 95.45)	2.68 ± 4.29 (-19.59 - 47.89)	-6.03, 11.39	0.535	
	J163	62.36 ± 8.57 (30.29 - 117.23)	8.32 ± 4.29 (-18.90 - 53.03)	-0.38, 17.03	0.060	
	J101 x J163	61.83 ± 8.60 (35.90 - 112.95)	7.80 ± 4.34 (-8.69 - 32.46)	-1.01, 16.60	0.080	
Phosphorus (% DW)	Control	0.33 ± 0.027 (0.25 - 0.45)				(0.22 - 0.45) [0.095, 0.54]
	J101	0.34 ± 0.027 (0.22 - 0.48)	0.0057 ± 0.0075 (-0.082 - 0.14)	-0.0096, 0.021	0.456	
	J163	0.33 ± 0.027 (0.24 - 0.49)	0.0016 ± 0.0075 (-0.090 - 0.077)	-0.014, 0.017	0.832	
	J101 x J163	0.32 ± 0.027 (0.22 - 0.42)	-0.012 ± 0.0076 (-0.088 - 0.12)	-0.027, 0.0035	0.124	

DW = dry weight; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Potassium (% DW)	Control	3.08 ± 0.41 (1.57 - 4.30)				(1.39 - 4.31) [0.38, 5.75]
	J101	3.07 ± 0.41 (1.48 - 4.61)	-0.011 ± 0.10 (-0.74 - 1.14)	-0.22, 0.19	0.914	
	J163	3.01 ± 0.41 (1.18 - 4.41)	-0.074 ± 0.10 (-0.50 - 0.53)	-0.28, 0.13	0.468	
	J101 x J163	2.96 ± 0.41 (0.85 - 4.32)	-0.12 ± 0.10 (-1.37 - 1.08)	-0.33, 0.083	0.233	
Sodium (% DW)	Control	0.079 ± 0.041 (0.018 - 0.23)				(0.017 - 0.21) [0, 0.31]
	J101	0.087 ± 0.041 (0.018 - 0.25)	0.0085 ± 0.015 (-0.053 - 0.11)	-0.022, 0.039	0.573	
	J163	0.092 ± 0.041 (0.017 - 0.24)	0.013 ± 0.015 (-0.019 - 0.071)	-0.017, 0.043	0.388	
	J101 x J163	0.10 ± 0.041 (0.017 - 0.38)	0.025 ± 0.015 (-0.025 - 0.15)	-0.0060, 0.055	0.112	
Zinc (mg/kg DW)	Control	29.58 ± 2.93 (16.70 - 46.15)				(18.09 - 35.98) [5.05, 50.21]
	J101	30.86 ± 2.93 (18.28 - 44.76)	1.28 ± 1.12 (-10.27 - 11.32)	-0.99, 3.56	0.259	
	J163	29.25 ± 2.93 (16.45 - 40.36)	-0.33 ± 1.12 (-17.06 - 9.51)	-2.60, 1.95	0.771	
	J101 x J163	28.61 ± 2.94 (17.01 - 37.28)	-0.98 ± 1.14 (-11.19 - 10.66)	-3.28, 1.33	0.395	

DW = dry weight; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Ash (% DW)	Control	11.31 ± 2.46 (8.44 - 15.04)				(8.58 - 15.25) [5.59, 16.40]
	J101	13.48 ± 2.46 (8.55 - 28.59)	2.18 ± 1.21 (-1.53 - 13.55)	-0.29, 4.64	0.081	
	J163	13.23 ± 2.46 (8.87 - 26.13)	1.92 ± 1.21 (-1.29 - 11.09)	-0.55, 4.38	0.123	
	J101 x J163	14.41 ± 2.46 (8.26 - 32.50)	3.10 ± 1.22 (-1.09 - 18.12)	0.63, 5.58	0.015	
Carbohydrates (% DW)	Control	65.08 ± 3.01 (55.44 - 73.53)				(58.03 - 74.38) [46.29, 85.59]
	J101	63.32 ± 3.01 (50.30 - 73.64)	-1.76 ± 0.93 (-9.89 - 9.32)	-3.64, 0.12	0.065	
	J163	63.29 ± 3.01 (51.37 - 73.39)	-1.78 ± 0.93 (-8.82 - 4.77)	-3.67, 0.097	0.062	
	J101 x J163	63.10 ± 3.01 (48.03 - 74.71)	-1.98 ± 0.93 (-11.57 - 7.00)	-3.88, -0.085	0.041	
Moisture (% FW)	Control	76.77 ± 1.64 (70.70 - 84.20)				(70.90 - 82.10) [62.91, 88.67]
	J101	77.11 ± 1.64 (71.10 - 82.40)	0.34 ± 0.48 (-4.60 - 5.70)	-0.65, 1.32	0.492	
	J163	77.01 ± 1.64 (71.00 - 83.30)	0.24 ± 0.48 (-3.30 - 4.50)	-0.75, 1.22	0.629	
	J101 x J163	75.78 ± 1.64 (70.70 - 83.10)	-0.99 ± 0.49 (-7.80 - 4.70)	-1.98, -0.0023	0.049	

DW = dry weight; FW = fresh weight; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (continued): Composition of Forage Derived from Lucerne population containing J101, J163, and J101 x J163 vs. Control and Reference Varieties (combined field trials)

Component (Units)	Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Protein (% DW)	Control	21.35 ± 1.24 (16.02 - 28.20)				(15.29 - 25.81) [7.98, 33.81]
	J101	21.01 ± 1.24 (15.44 - 24.89)	-0.35 ± 0.52 (-5.99 - 5.85)	-1.40, 0.70	0.505	
	J163	21.21 ± 1.24 (15.80 - 26.32)	-0.15 ± 0.52 (-3.46 - 5.57)	-1.20, 0.91	0.779	
	J101 x J163	20.49 ± 1.24 (15.53 - 27.11)	-0.87 ± 0.52 (-5.93 - 8.85)	-1.93, 0.19	0.105	
Total Fat (% DW)	Control	2.26 ± 0.17 (1.45 - 3.58)				(1.33 - 3.15) [0, 4.61]
	J101	2.19 ± 0.17 (1.27 - 4.01)	-0.065 ± 0.16 (-1.80 - 0.88)	-0.39, 0.26	0.685	
	J163	2.27 ± 0.17 (1.21 - 3.68)	0.014 ± 0.16 (-1.67 - 0.78)	-0.31, 0.34	0.932	
	J101 x J163	2.12 ± 0.17 (1.50 - 3.13)	-0.14 ± 0.16 (-1.24 - 1.37)	-0.47, 0.18	0.387	

DW = dry weight; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
With 95% confidence, tolerance interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 5: Summary of statistically significant differences comparing J101, J163 and J101 x J163 to the Control

Site/Component (Units)[a]	Mean Test Event	Mean Control	Mean Diff. (% of Control Value)	Significance (p-value)	Test Event (Range)	99% Tolerance Interval[b]
Line J101						
Cystine (% total AA)	1.56	1.41	10.61	<0.001	1.36 - 1.86	1.01, 1.96
Glutamic acid (% total AA)	10.95	11.10	-1.39	0.031	10.64 - 11.34	10.28, 11.77
Tyrosine (% total AA)	3.68	3.83	-3.82	0.005	3.23 - 3.94	3.33, 4.07
Line J163						
Cystine (% total AA)	1.56	1.41	10.40	<0.001	1.35 - 1.90	1.01, 1.96
Histidine (% total AA)	2.67	2.76	-3.55	0.004	2.44 - 2.85	2.25, 3.22
Lysine (% total AA)	6.89	7.05	-2.24	0.009	6.50 - 7.37	6.26, 7.85
Tyrosine (% total AA)	3.69	3.83	-3.62	0.008	3.19 - 3.86	3.33, 4.07
Acid detergent fiber (% dw)	28.31	25.79	9.79	0.006	20.00 - 39.67	15.76, 40.19
Lignin (% dw)	6.01	5.07	18.54	0.017	3.94 - 8.13	0, 12.92
Neutral detergent fiber (% dw)	30.94	28.09	10.13	0.008	24.49 - 43.57	20.01, 41.80
Line J101 x J163						
Cystine (% total AA)	1.57	1.41	11.01	<0.001	1.41 - 1.84	1.01, 1.96
Isoleucine (% total AA)	4.86	4.94	-1.67	0.027	4.64 - 5.14	4.25, 5.58
Phenylalanine (% total AA)	5.54	5.67	-2.19	0.017	5.39 - 6.06	4.64, 6.61
Proline (% total AA)	5.49	5.28	3.97	0.012	5.06 - 6.16	4.57, 6.06
Tyrosine (% total AA)	3.69	3.83	-3.70	0.007	3.18 - 3.89	3.33, 4.07
Neutral detergent fiber (% dw)	30.64	28.09	9.05	0.018	21.87 - 39.73	20.01, 41.80
Calcium (% dw)	1.01	1.12	-9.35	0.023	0.81 - 1.38	0.48, 1.89
Iron (mg/kg dw)	730.93	410.19	78.19	0.008	199.10 - 2196.43	0, 892.57
Ash (% dw)	14.41	11.31	27.46	0.015	8.26 - 32.50	5.59, 16.40
Carbohydrates (% dw)	63.10	65.08	-3.04	0.041	48.03 - 74.71	46.29, 85.59
Moisture (% fw)	75.78	76.77	-1.29	0.049	70.70 - 83.10	62.91, 88.67

[a]dw=dry weight; fw=fresh weight; AA=amino acids;
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Results from individual trial sites

The results from individual trial sites were evaluated. Values obtained from the test lines were compared to the control at each of the five replicated trial sites. While data is not presented in this report, the results are summarised below.

For test line J101, 187 of 210 comparisons indicated no statistically significant difference from the non-transgenic control. For the 23 comparisons observed to be statistically different ($p<0.05$) from the control, 20 of the comparisons were within the 99% tolerance interval derived from the non-transgenic commercial reference varieties. The remaining three values that were outside this tolerance interval, for ash, iron and tyrosine, were only noted for one of the six comparisons. Therefore, it is unlikely that these differences are biologically meaningful and the forage from J101 is considered to be compositionally equivalent to commercial lucerne varieties.

Results from the analyses of J163 indicated that 175 of the 210 comparisons were not statistically different from the non-transgenic control values. For the 35 comparisons that indicated a statistically significant difference ($p<0.05$) between line J163 and the control, only six were outside the 99% tolerance interval derived from commercial reference varieties. Of these values, only one of six comparisons for alanine, ash, iron and tyrosine was outside the tolerance interval, so these differences are unlikely to be biologically meaningful. Two comparisons of NDF were outside the 99% tolerance interval, but values were still within the literature ranges. Again, the differences observed for two of six comparisons of NDF content of J163 to the control are unlikely to be biologically meaningful. Thus, the forage from line J163 is considered to be compositionally equivalent to commercial lucerne varieties.

The comparison of forage from the J101 x J163 test population to the non-transgenic control showed no statistically significant differences for 164 of the 210 comparisons made. Of the 46 comparisons observed to be statistically different, all values for J101 x J163 were observed to fall within the 99% tolerance interval generated from the 12 commercial reference varieties, with the following exceptions: glycine, sodium and tyrosine (one comparison each); ash, iron and proline (two comparisons each). Since the ash, glycine, iron, proline, sodium and tyrosine differences were only noted for one or two of the six comparisons and the remaining test values that had statistically significant differences were within the tolerance interval, it is unlikely these differences are biologically meaningful. The forage from lucerne J101 x J163 is considered to be compositionally equivalent to commercial lucerne varieties.

In summary, a total of 630 comparisons were made between glyphosate-tolerant lucerne and the non-GM control population. For 526 of 630 comparisons, there were no statistically significant differences observed. For the 104 of 630 comparisons that were associated with statistical differences, the range of values of the test lines was found to be within the 99% tolerance interval derived from the 12 commercial lucerne varieties, with the following exceptions: alanine, glycine, NDF, proline and sodium (one or two comparisons out of six comparisons, in one test line only); ash, iron and tyrosine (one or two comparisons out of six comparisons, in all three test lines). While individual values were different from the control, the mean was within the 99% tolerance interval developed using conventional reference varieties. Hence, these differences are unlikely to be biologically meaningful.

In a study of this magnitude, a small percentage (approximately 5%) of statistically significant differences is expected to occur due to chance alone. Differences occurring in one of the field sites only which are not repeated at other sites, are not indicative of a pattern of change that could be attributed to the genetic changes and are more likely to be random occurrences. In this comparative study, changes in the levels of some analytes are in this category. Consequently, these differences, although statistically significant for the individual site, are not considered to be biologically meaningful.

Alfalfa Sprouts

In accordance with the OECD guidelines for compositional analysis of legume sprouts for food use (OECD, 2005), compositional data on the levels of beta-carotene, folic acid and vitamin C in alfalfa sprouts was also evaluated.

Growth conditions

Sprouts from glyphosate-tolerant lucerne J101, J163 and J101 x J163 test lines, as well as the null-segregant control population were grown in a laboratory using a randomized complete block design with three replicates per treatment. The conventional lucerne varieties were grown as a single replicate within one block to provide reference substance data. One additional reference variety was purchased as whole fresh sprouts from a grocery store local to the applicant's Creve Coeur facility.

Seeds were surface sterilized in 10% bleach (supplemented with 0.5% surfactant to assist wetting), rinsed thoroughly, soaked overnight then grown in sprouting trays that were rinsed twice a day. Early on day five, batch 1 sprouts were moved to a cold room to slow growth prior to harvest on day seven. Batch 2 sprouts were harvested on day seven. Sprouts were frozen on dry ice and sent to Covance Laboratories (Madison, WI) for analysis.

Compositional analysis

The levels of moisture, beta-carotene, folic acid and vitamin C were determined. Values for vitamin content were converted to dry weight (dw) for comparison. Means and standard errors were calculated for test and control samples, while a range of values was derived from the reference varieties. The values for folic acid and vitamin C are provided in Table 6.

As more than 50% of the beta-carotene values were below the limit of quantitation, these values are not reported.

Results

The levels of folic acid in sprouts derived from test alfalfa seed were comparable to that of the non-GM control and within the reference and literature ranges for folic acid in alfalfa sprouts.

The level of vitamin C in dihomogenic J101 x J163 test sprouts was comparable to the non-GM control and within the reference and literature ranges for vitamin C in alfalfa sprouts. However, the values for vitamin C in J101 and J163 samples were higher than the control value and reference range. Because of the apparent higher level of vitamin C in the J101 and J163 samples for Batch 1, vitamin C analysis was repeated in Batch 2.

The levels of vitamin C in test lines grown in Batch 2 fall within the reference and literature ranges, and are comparable to the control value.

During the growth of Batch 1 sprouts, it was noted that the sprouts were variable in colour, possibly due to variability in lighting within the laboratory. This variability was corrected such that sprouts in Batch 2 were grown under more uniform lighting conditions. Also, sprouts grown in Batch 2 were directly frozen, rather than being placed in the refrigerator prior to harvest, which may also reduce variability in developmental stage of the sprouts. Vitamin C levels are known to be developmentally regulated, being significantly higher in sprouted seeds compared to seeds and so it is likely that differences in growing and pre-harvest conditions contributed to the variability in vitamin C levels in Batch 1.

These data support the conclusion that the levels of vitamin C and folic acid in sprouts from glyphosate-tolerant lucerne are comparable to those in sprouts from conventional lucerne.

Table 6: Comparison of Vitamin Levels in Glyphosate-tolerant, Control and Conventional Alfalfa Sprouts

Line	Batch 1 Folic Acid (mg/100g dw)	Batch 1 Vitamin C (mg/100g dw)	Batch 2 Vitamin C (mg/100g dw)
J101 (Mean ± S.E)	0.97 ± .0.06	283 ± 71	89 ± 7
J163 (Mean ± S.E)	0.95 ± 0.03	205± 67	96 ± 1
J101 x J163 (Mean ± S.E)	0.76 ± 0.13	165 ± 25	97 ± 3
Control (Mean ± S.E)	0.99 ± 0.13	117 ± 11	83 ± 5
Reference (Range)	0.61 - 1.02	63 - 110	91 - 130
Literature (Range)	0.4[a] - 1.53[b]	82[c] - 205[d]	82[c] - 205[d]

[a] USDA, 2004, Values converted to dry weight using reported moisture of 91.14%
[b] Magaram et al, 1985, Values converted to dry weight using reported moisture of approximately 80%
[c] Plaza et al, 2003
[d] Yamaguchi, 1983

5.2 Level of naturally occurring toxicants and anti-nutrients

Lucerne contains several toxicants and anti-nutrients, including lignin, phytoestrogens, tannins, saponins and soluble forage proteins. The OECD consensus document on forage legumes (OECD, 2005) suggests that additional components that may be considered for particular forage legumes are the phytoestrogens, the anti-bloat factor tannins, and the secondary metabolite saponins and antinutrient cyanogenic glycosides. Condensed tannins can reduce protein digestibility, while high levels of readily digestible protein, along with saponins, are associated with bloat. Tannins, saponins and soluble protein impact on the quality of animal feed when legume forage comprises a large proportion of the feed. They are unlikely to be of concern in food uses of alfalfa, since alfalfa is expected to be consumed in minor quantities.

Lignin levels are commonly used to establish the forage quality of lucerne feed as high lignin levels reduce digestibility. Lignin content increases as the plant matures, so lignin levels are unlikely to be anti-nutritional in alfalfa sprouts, particularly as alfalfa comprises a small proportion of the human diet.

Nevertheless, lignin levels were measured in lucerne forage from the transgenic varieties, the null control population and conventional lucerne varieties as presented in Table 4. Lignin levels were not statistically different between J101 and the control, or between J101 x J163 and the control. The lignin level of J163 was significantly higher than the null population, but the level was within the 99% tolerance interval derived from the conventional varieties. Therefore, the lignin levels in glyphosate-tolerant lucerne are comparable to lignin levels in conventional lucerne.

Phytoestrogens are plant compounds similar in structure to mammalian oestrogen which can stimulate oestrogen receptors in animals and humans. Coumestrol is the most estrogenically active compound of the naturally occurring plant phytoestrogens (Reinli and Block, 1996; Dodge, 1998). The level of the anti-nutrient coumestrol in glyphosate-tolerant lucerne was measured.

Field Conditions and Compositional Analysis

Levels of coumestrol were determined in lucerne plants grown in field trials at four locations in the United States. Plants at each site were grown using a randomised complete block design with four replicates. Bulk forage was harvested at the late bud to early bloom stage from the first field cutting of 2003. The plants were in their third season of growth (established spring 2001) and all plants had been treated with glyphosate. Data were analysed for statistical significance using a mixed model analysis of variance. Coumestrol levels in glyphosate-tolerant lucerne were compared to levels in the control population within and across all field sites. Values obtained from the forage from conventional reference varieties were used to generate a 99% tolerance interval. The data are summarised in Table 7.

Results

The majority of observations were not significantly different from the control. Where differences were observed, they were within the 99% tolerance interval derived from conventional varieties grown in the same field trial. In addition, the data were not consistent across all locations, and are thus considered to be due to inherent biological variation. Combined data showed that there were no statistically significant differences between coumestrol levels in glyphosate-tolerant lucerne and control lucerne. The coumestrol levels in glyphosate-tolerant lucerne are equivalent to those in non-transgenic control and conventional lucerne forage.

Table 7: Results from the Statistical Evaluation of the Content of Coumestrol in Forage from glyphosate-tolerant lucerne J101, J163 and J101 x J163 compared to the Null Segregant Control

Line	Mean ± S.E. (Range)	Difference (Test Minus Control)			Commercial (Range) [99% T.I.][a]
		Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
J101	48.71 ± .15.05 (3.21 - 104.31)	11.05 ± 6.02 (-20.18 - 53.46)	-1.08, 23.19	0.073	
J163	45.98 ± 15.05 (3.49 - 94.56)	8.32 ± 6.02 (-79.87 - 45.52)	-3.81, 20.46	0.173	
J101 x J163	47.42 ± 15.10 (3.07 - 108.00)	9.76 ± 6.14 (-16.50 - 53.20)	-2.60, 22.12	0.118	
Control	37.66 ± 15.05 (3.66 - 124.50)				(2.99 - 104.37) [0, 145.77]

[a]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Conclusion

The comparative analyses do not indicate any compositional differences of biological significance in the forage derived from glyphosate-tolerant lucerne J101 x J163, compared to the non-genetically modified control when grown in a range of geographical regions. Although a difference in the levels of cystine and tyrosine was observed for J101, J163 and the combined J101 x J163 populations compared to the control population, the absolute levels were well within the range expected for these amino acids for conventionally produced commercial lucerne varieties. The levels of other components of lucerne J101 x J163 that are statistically significantly different from the null control population are also within the 99% tolerance interval derived from conventional commercial varieties. The differences therefore are not considered to raise any nutritional concerns. Overall, forage derived from glyphosate-tolerant lucerne J101 x J163 can be considered equivalent in composition to forage from conventionally produced lucerne varieties and the levels of vitamin C and folic acid in sprouts from glyphosate-tolerant lucerne are comparable to those in sprouts from conventional lucerne.

6. NUTRITIONAL IMPACT

Establishing that a GM food is safe for human consumption is generally achieved through an understanding of the genetic modification and its direct consequences in the plant, together with an extensive comparative analysis of the food components derived from the GM plant and the non-GM counterpart.

To date, all approved GM plants with modified agronomic production traits (e.g. herbicide tolerance) have been shown to be compositionally equivalent to their conventional counterparts. Feeding studies in animals using feeds derived from the approved GM plants have shown equivalent nutritional performance to that observed with the non-GM feed. Thus the evidence to date is that where GM varieties have been shown to be compositionally equivalent to conventional varieties, feeding studies using target livestock species contribute minimally to a safety assessment.

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

In the case of glyphosate-tolerant lucerne J101 and J163, the extent of the compositional and other available data is considered sufficient to establish the nutritional adequacy of the food.

REFERENCES

Astwood, J.D., Leach, J.N. and Fuchs, R.L. (1996) Stability of food allergens to digestion *in vitro*. *Nat.Biotechnol.* 14(10):1269-1273.

Austin, S., Bingham.E.T., Mathews, D.E., Shahan, M.N., Will, J. and Burgess, R.R. (1995) Production and field performance of transgenic alfalfa (*Medicago sativa* L.) expressing alpha-amylase and manganese-dependent lignin peroxidase. *Euphytica* 85:381-393.

Barker, R.F., Idler, K.B., Thompson, D.V. and Kemp, J.D. (1983) Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. *Plant molecular biology* 2:335-350.

Coruzzi, G., Broglie, R., Edwards, C. and Chua, N.H. (1984) Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. *EMBO J.* 3(8):1671-1679.

della-Cioppa, G., Bauer, S.C., Klein, B.K., Shah, D.M., Fraley, R.T. and Kishore, G.M. (1986) Translocation of the precursor of 5-enolpyruvylshikimate-3-phosphate synthase into chloroplasts of higher plants in vitro. *Proc.Natl.Acad.Sci.U.S.A* 83(18):6873-6877.

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and Goodman, H.M. (1982) Nopaline synthase: transcript mapping and DNA sequence. *J Mol Appl Genet* 1(6):561-573.

Dodge, J.A. (1998) Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment. Structure/activity relationships. *Pure and Applied Chemistry* 70(9):1725-1733.

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.*

Harrison, L.A., Bailey, M.R., Naylor, M.W., Ream, J.E., Hammond, B.G., Nida, D.L., Burnette, B.L., Nickson, T.E., Mitsky, T.A., Taylor, M.L., Fuchs, R.L. and Padgette, S.R. (1996d) The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. *J.Nutr.* 126(3):728-740.

Harrison, L.A., Bailey, M.R., Naylor, M.W., Ream, J.E., Hammond, B.G., Nida, D.L., Burnette, B.L., Nickson, T.E., Mitsky, T.A., Taylor, M.L., Fuchs, R.L. and Padgette, S.R. (1996e) The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-

phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. *J.Nutr.* 126(3):728-740.

Harrison, L.A., Bailey, M.R., Naylor, M.W., Ream, J.E., Hammond, B.G., Nida, D.L., Burnette, B.L., Nickson, T.E., Mitsky, T.A., Taylor, M.L., Fuchs, R.L. and Padgette, S.R. (1996f) The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. *J.Nutr.* 126(3):728-740.

Harrison, L.A., Bailey, M.R., Naylor, M.W., Ream, J.E., Hammond, B.G., Nida, D.L., Burnette, B.L., Nickson, T.E., Mitsky, T.A., Taylor, M.L., Fuchs, R.L. and Padgette, S.R. (1996g) The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. *J.Nutr.* 126(3):728-740.

Harrison, L.A., Bailey, M.R., Naylor, M.W., Ream, J.E., Hammond, B.G., Nida, D.L., Burnette, B.L., Nickson, T.E., Mitsky, T.A., Taylor, M.L., Fuchs, R.L. and Padgette, S.R. (1996a) The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. *J.Nutr.* 126(3):728-740.

Harrison, L.A., Bailey, M.R., Naylor, M.W., Ream, J.E., Hammond, B.G., Nida, D.L., Burnette, B.L., Nickson, T.E., Mitsky, T.A., Taylor, M.L., Fuchs, R.L. and Padgette, S.R. (1996b) The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. *J.Nutr.* 126(3):728-740.

Harrison, L.A., Bailey, M.R., Naylor, M.W., Ream, J.E., Hammond, B.G., Nida, D.L., Burnette, B.L., Nickson, T.E., Mitsky, T.A., Taylor, M.L., Fuchs, R.L. and Padgette, S.R. (1996c) The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. *J.Nutr.* 126(3):728-740.

Kimber, I., Kerkvliet, N.I., Taylor, S.L., Astwood, J.D., Sarlo, K. and Dearman, R.J. (1999) Toxicology of protein allergenicity: prediction and characterization. *Toxicol.Sci* 48(2):157-162.

Klee, H.J., Muskopf, Y.M. and Gasser, C.S. (1987) Cloning of an Arabidopsis thaliana gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. *Mol.Gen.Genet.* 210(3):437-442.

Marten, G.C., Buxton, D.R. and Barnes, R.F. (1988) Feeding value (forage quality). In: Hanson, A.A., Barnes, D.K., and Hill, R.R. eds. *Alfalfa and Alfalfa Improvement*. American Society of Agronomy, Madison, Wisconsin, pp465-484. Agronomy.

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.

OECD (2005) *Consensus document on Compositional Considerations for New Varieties of Alfalfa and other Temperate Forage Legumes: Key Feed Nutrients, Anti-Nutrients and*

Secondary Plant Metabolites. Series on the Safety of Novel Foods and Feeds. 13, Organisation for Economic Co-operation and Development, Paris.

Padgette, S.R., Re, D.B., Barry, D.B., Eichholtz, D.A., Delannay, X., Fuchs, R.L., Kishore, G.M. and Fraley, R.T. (1996) New Weed Control Opportunities: Development of Soybeans with a Roundup Ready(TM) Gene. In: Duke, S.O. eds. *Herbicide Resistant Crops*. CRC Press Inc, pp53-84.

Reinli, K. and Block, G. (1996) Phytoestrogen content of foods--a compendium of literature values. *Nutr.Cancer* 26(2):123-148.

Richins, R.D., Scholthof, H.B. and Shepherd, R.J. (1987) Sequence of figwort mosaic virus DNA (caulimovirus group). *Nucleic Acids Res.* 15(20):8451-8466.

Rumbaugh, M.D., Caddell, J.L. and Rowe, D.E. (1988) Breeding and Quantitative Genetics. In: Hanson, A.A., Barnes, D.K., and Hill, R.R. eds. *Alfalfa and Alfalfa Improvement*. American Society of Agronomy, Madison, Wisconsin, pp777-808. Agronomy.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* 98(3):503-517.

Viands, D.R., Sun, P. and Barnes, D.K. (1988) Pollination control: mechanical and sterility. In: Hanson, A.A., Barnes, D.K., and Hill, R.R. eds. *Alfalfa and Alfalfa Improvement*. American Society of Agronomy, Madison, Wisconsin, pp931-960. Agronomy.

Walker, K.A. and Sato, S.J. (1981) Morphogenesis in callus tissue of *Medicago sativa*: the role of ammonium ion in somatic embryogenesis. *Plant cell, tissue and organ culture* 1(2):109-121.

WHO (2000) *Safety aspects of genetically modified foods of plant origin. Report of a Joint FAO/WHO Expert Consultation, World Health Organization, Geneva.*

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.

ATTACHMENT 3

SUMMARY OF FIRST ROUND PUBLIC SUBMISSIONS

A total of 9 submissions were received – 3 from New Zealand and 6 from Australia.

Submissions from New Zealand

1. Kathryn Liddell

- Opposed to the application because the repercussions of GM are not known and this will be the thin edge of the wedge. Not enough is known about the effect of GE food on animals or on humans.
- Concerned that USDA APHIS assessments do not inspire confidence and NZ should not accept product simply because USDA has.

2. GE Free New Zealand (Claire Bleakley)

- Opposed to the use of GE crops for human food and concerned about contamination of human food by GM animal feed and deleterious effects on animals.
- Cites example of CSIRO GM field peas indicating potential for unintended changes in protein structure. Concerned that FSANZ only has data from protein from original soil organism not the GM food containing the transgene.
- FSANZ must seek independent assessment.
- Data provided does not warrant a variation of a food regulatory measure.
- Concerned by report that *cp4 epsps* transgene survives passage through the small intestine (Netherwood *et al* (2004) Nature Biotechnology, **22**:204-209).
- No assurance that occurrence of non-specific digestive illness could not be attributed to ingestion of GE.
- Concerned gene promoters similar to Cauliflower mosaic virus 35S are unstable and associated with integration into some human genomes with possible link to Hepatitis B. Fragmentation of the CaMV virus could cause DNA rearrangements leading to new toxins and proteins in the food (Independent Science Panel (2003) The case for a GM-Free Sustainable World, Institute of Science in Society, London UK).
- Concerned about the lack of long term data about introduction of GE foods.
- Raises several issues regarding high lysine corn (A549).

3. New Zealand Food Safety Authority

- Agrees with the issues identified by FSANZ in the Initial Assessment Report.
- Requests that DAR makes clear that approval for import of glyphosate-tolerant Lucerne lines J101 and J163 into New Zealand only requires approval by the Environmental Risk Management Authority (ERMA) if the imported lucerne is viable; importation of non-viable lucerne does not require ERMA approval.
- Suggests clarification in DAR of FSANZ Memorandum of Understanding (MOU) with OGTR, such that no split approvals will be made.

Submissions from Australia

4. Ivan Jeray

- Does not support approval as GM ingredients have not been proven safe. Mentions example of CSIRO GM field pea causing illness in mice.
- Concerned GM derived food may not be required to be labelled as such.

5. Food Technology Association of Victoria

- Supports approval of glyphosate-tolerant Lucerne lines J101 and J163.

6. New South Wales Food Authority

- No particular concerns and does not object to further consideration of the application.

7. Victorian Department of Human Services

- No concerns expressed in relation to the assessment of glyphosate-tolerant Lucerne lines J101 and J163.

8. Queensland Health

- Awaits the Draft Assessment Report before stating support or opposition.
- Expresses concerns with the cost of testing if food derived from glyphosate-tolerant Lucerne lines J101 and J163 is approved, and the applicant should be obliged to provide methodology and reference material to assist with enforcement capabilities.

9. Australian Food and Grocery Council (AFGC)

- Supports approval of glyphosate-tolerant Lucerne lines J101 and J163, contingent upon satisfactory safety assessment by FSANZ.