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

2 September 2009
[14-09]

APPLICATION A1006
FOOD DERIVED FROM HERBICIDE-TOLERANT SOYBEAN LINE DP-356043-5
SECOND ASSESSMENT REPORT

Executive Summary

Purpose

Food Standards Australia New Zealand (FSANZ) received an Application from Pioneer Hi-Bred International, Inc. (Pioneer), a DuPont Company, on 18 March 2008. The Applicant requested an amendment to Standard 1.5.2 – Food produced using Gene Technology, in the *Australia New Zealand Food Standards Code* (the Code), to permit the sale and use of food derived from a new genetically modified (GM) variety of soybean, dual herbicide-tolerant soybean line DP-356043-5.

Soybean line DP-356043-5 is tolerant to the broad-spectrum herbicide glyphosate and to acetolactate synthase (ALS)-inhibiting herbicides. Tolerance is conferred by expression in the plant of two novel proteins: GAT4601 and GM-HRA. The GAT4601 protein confers tolerance to glyphosate-containing herbicides by the acetylation of glyphosate, thereby rendering it non-phytotoxic. The GM-HRA protein is a modified soybean ALS enzyme that is able to function in the presence of the ALS-inhibiting class of herbicides, thereby conferring tolerance to those herbicides.

This Application is being assessed as a Major Procedure, which includes two rounds of public consultation. FSANZ has considered all submissions received in the first consultation period and has addressed issues, particularly those relevant to the safety of food derived from soybean DP-356043-5. Where necessary, additional information has been incorporated into this Second Assessment Report.

Safety Assessment

FSANZ completed a comprehensive safety assessment of food derived from soybean line DP-356043-5, which was released in the First Assessment Report. This assessment included consideration of (i) the genetic modification to the plant; (ii) the potential toxicity and allergenicity of the novel proteins; (iii) the composition of soybean DP-356043-5 compared with that of conventional soybean varieties; and (iv) the nutritional adequacy of soybean DP-356043-5 when incorporated into the diet.

No public health and safety concerns were identified in the pre-market safety assessment. On the basis of the available evidence, including detailed studies provided by the Applicant,

food derived from dual-herbicide tolerant soybean line DP-356043-5 is considered as safe and wholesome as food derived from other commercial soybean varieties.

Novel herbicide residues

Two novel herbicide residues are generated from the use of glyphosate on soybean DP-356043-5. Following a comprehensive toxicological assessment, no public health and safety concerns were identified with regard to *N*-acetyl glyphosate (NAG) and *N*-acetyl aminomethylphosphonic acid (*N*-acetyl AMPA), which are less toxic than glyphosate itself.

As stated in the First Assessment Report, the US EPA recently included NAG in tolerance limits, to reflect use of glyphosate on soybean line DP-356043-5. While noting this decision, FSANZ considers that a change to the residue definition for glyphosate as applies in Australia and New Zealand is not necessary. There would be no food safety benefit in amending the existing residue definition, and such an amendment would result in costs that are not justified given the proportion of soybean line DP-356043-5 that is likely to be present in foods imported into either country. Therefore, the existing residue definition and Maximum Residue Limit (MRL) for glyphosate on soybean in the Code should continue to apply, and these are appropriate for soybean line DP-356043-5. In adopting this approach, FSANZ consulted the Australian Pesticides and Veterinary Medicines Authority, the New Zealand Food Safety Authority, and the Applicant.

Labelling

If approved, food derived from soybean line DP-356043-5 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 novel proteins are present in the raw seed.

Soybean DP-356043-5 has elevated levels of two minor fatty acids, heptadecanoic acid (C17:0) and heptadecenoic acid (C17:1), and of the acetylated amino acids *N*-acetyl glutamate (NAGlu) and *N*-acetyl aspartate (NAAsp). Standard 1.5.2 of the Code states that there could be additional labelling requirements for GM food where *the genetic modification has resulted in one or more significant composition or nutritional parameters having altered levels.* However, FSANZ has examined this issue and is not recommending any additional labelling requirements for foods derived from soybean DP-356043-5, as the elevated components are not considered *significant composition or nutritional parameters* based on their demonstrated safety, low abundance, lack of nutritional impact, and presence in other commonly consumed foods.

Labelling addresses the objective set out in paragraph 18(1)(b) of the *Food Standards Australia New Zealand Act 1991* (FSANZ Act); that is, the provision of adequate information relating to food to enable consumers to make informed choices. The general labelling requirements will provide consumers with relevant information about the GM status of foods.

Impact of regulatory options

Following satisfactory completion of the safety assessment, two regulatory options were considered: (1) no approval; or (2) approval of food derived from soybean DP-356043-5.

Following analysis of the potential costs and benefits of each option on affected parties (consumers, the food industry and government), option 2, approval of this Application, is the preferred option. Under option 2, the potential benefits to all sectors outweigh any costs associated with the approval.

Assessing the Application

In assessing the Application, FSANZ has had regard to the following matters as prescribed in section 29 of the *Food Standards Australia New Zealand Act 1991* (FSANZ Act):

- The costs that would arise from an amendment to the Code approving food derived from dual herbicide-tolerant soybean line DP-356043-5 do not outweigh the direct and indirect benefits to the community, Government and industry that would arise from the development or variation of the food regulatory measure

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

- Any relevant New Zealand standards including for residue limits

- Any other relevant matters

Preferred Approach

Proceed to formally amend Standard 1.5.2 – Food produced using Gene Technology, to include food derived from dual herbicide-tolerant soybean line DP-356043-5 in the Table to clause 2.

Reasons for Preferred Approach

The development of an amendment to the Code to give approval to the sale and use of food derived from dual herbicide-tolerant soybean line DP-356043-5 in Australia and New Zealand is proposed on the basis of the available scientific evidence, for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce dual herbicide-tolerant soybean line DP-356043-5;

- the novel herbicide residues generated on soybean DP-356043-5 plants following glyphosate application are less toxic than glyphosate and pose no food safety concerns;

- labelling of certain foods derived from dual herbicide-tolerant soybean line DP-356043-5 will be required if novel DNA and/or protein is present in the final food;

- a regulation impact assessment process has been undertaken that fulfils the requirement in Australia and New Zealand for an assessment of compliance costs. The assessment concluded that the preferred option is Option 2, an amendment to the Code; and

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

Consultation

Consultation on the First Assessment was conducted over a period of six weeks; nine submissions were received. Summaries of these are in **Attachment 4** of this report.

FSANZ has taken all submitters' comments into consideration in completing the Second Assessment Report. Specific issues relating to the safety of food derived from dual herbicide tolerant soybean line DP-356043-5 have been addressed. Public comment is now invited on this Report, which includes a draft variation to Standard 1.5.2. Comments received in the second consultation period will be used to assist in preparing the Approval Report, to complete the Application.

Invitation for Submissions

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

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

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

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

DEADLINE FOR PUBLIC SUBMISSIONS: 6pm (Canberra time) 30 September 2009

SUBMISSIONS RECEIVED AFTER THIS DEADLINE WILL NOT BE CONSIDERED

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

Questions relating to making submissions or the application process can be directed to the Standards Management Officer at standards.management@foodstandards.gov.au. If you are unable to submit your submission electronically, hard copy submissions may be sent to one of the following addresses:

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

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

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INTRODUCTION

On 18 March 2008, Pioneer Hi-Bred International, Inc. (Pioneer), a DuPont Company, submitted an Application seeking approval for food derived from dual herbicide-tolerant soybean line DP-356043-5 (also referred to as soybean 356043) under Standard 1.5.2 – Food produced using Gene Technology, in the *Australia New Zealand Food Standards Code* (the Code).

Soybean 356043 has been genetically modified for tolerance to the broad-spectrum herbicide glyphosate and to acetolactate synthase (ALS)-inhibiting herbicides. Protection is conferred by expression in the plant of two novel proteins: GAT4601 (glyphosate acetyltransferase) and GM-HRA (modified version of a soybean ALS). The GAT4601 protein, encoded by the *gat4601* gene, confers tolerance to glyphosate-containing herbicides by acetylating glyphosate and thereby rendering it non-phytotoxic. The GM-HRA protein, encoded by the *gm-hra* gene, is able to function in the presence of the ALS-inhibiting class of herbicides, thereby conferring tolerance to those herbicides.

The dual herbicide tolerance traits of soybean DP-356043-5 are intended to enable growers to choose an optimal combination of the herbicides to manage weed populations. An existing glyphosate-tolerant soybean, 40-3-2, currently accounts for 60% of the global soybean area and is the most cultivated GM plant product to date. Extending tolerance to ALS-inhibiting herbicides is intended to provide growers with an additional management tool for weeds that are difficult to control with glyphosate alone.

The First Assessment Report included a full scientific evaluation of food derived from soybean DP-356043-5 according to FSANZ guidelines[1] to assess its safety for human consumption. Following a six week period of public consultation, the issues raised in submissions have been considered and addressed in this Second Assessment. Additional information has been included in the safety assessment at **Attachment 3.** Public comment is now sought on this Second Assessment Report, which includes the draft variation to Standard 1.5.2 of the Code, prior to preparation of the Approval Report and completion of the Application. All submissions relating to the First Assessment have been summarised in **Attachment 4** of this Report.

1. The Issue / Problem

The Applicant has developed GM soybean line DP-356043-5 that is tolerant to the broad-spectrum herbicide glyphosate and to ALS-inhibiting herbicides. Pre-market approval is necessary before this product may enter the Australian and New Zealand food supply. An amendment to the Code granting approval to food derived from soybean 356043 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.

Soybean line DP-356043-5 is intended to be grown in North America. Before release onto commercial agricultural markets, the Applicant is seeking regulatory approval for soybean DP-356043-5 in key trading markets for soybean, including Australia and New Zealand. This is necessary because once it is cultivated on a commercial-scale, soybean products imported into Australia and New Zealand could contain ingredients derived from soybean 356043 as a result of comingling practices at harvest or later processing stages.

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

The Applicant has therefore sought the necessary amendments to Standard 1.5.2 to include food derived from soybean line DP-356043-5 prior to any decision to commercialise this line. The Application is being assessed as a Major Procedure.

2. Current Standard

2.1 Background

Approval of genetically modified foods under Standard 1.5.2 is contingent upon completion of a comprehensive pre-market safety assessment. Foods that have been assessed under the Standard, if approved, are listed in the Table to clause 2 of the Standard.

2.2 Overseas approvals

Soybean line DP-356043-5 is intended for commercialisation in the United States and Canada. Soybean 356043 has been approved for food and feed use and environmental release in the United States (US Food and Drug Administration and the USDA-Animal and Plant Health Inspection Service) and Japan (Ministry of Agriculture, Forestry & Fisheries, Ministry of the Environment, and Ministry for Health, Labour & Welfare). Approval for food/feed use has been obtained in Mexico (Secretary of Health) and Taiwan (Department of Health). Submissions have been made to the appropriate agencies for food, feed and environmental approvals in Canada (Health Canada and the Canadian Food Inspection Agency). Regulatory submissions for food import approvals have also been made in the European Union. The Applicant has advised that further submissions for import approvals in key international markets will also be made.

The US Environmental Protection Agency (US EPA) has only recently amended the tolerance (i.e. maximum residue limits) for herbicide residues on soybean 356043 treated with glyphosate to also include the novel metabolite *N*-acetyl glyphosate.

3. Objectives

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

- the protection of public health and safety; and
- 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.

RISK ASSESSMENT

Food derived from dual-herbicide tolerant soybean line DP-356043-5 has been evaluated according to the safety assessment guidelines prepared by FSANZ[2]. The summary and conclusions from the safety assessment (at **Attachment 2**) and hazard assessment of glyphosate residues (at **Attachment 3**) 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 in the assessment.

4. Risk Assessment Summary

4.1 Safety Assessment Process

In conducting a safety assessment of food derived from soybean line DP-356043-5, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the soybean genome; the changes at the level of DNA, protein and in the whole food; detailed compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic in humans. The safety evaluation of soybean 356043 also included a separate assessment of two novel herbicide residues, namely *N*-acetyl glyphosate (NAG) and *N*-acetyl aminomethylphosphonic acid (*N*-acetyl AMPA), generated on the plants following glyphosate application.

The safety assessment applied to food from soybean line DP-356043-5 addresses only food safety and nutritional issues. It does not address any risks related to the release into the environment of GM plants used in food production, the safety of animal feed or animals fed with feed derived from GM plants, or the safety of food derived from the conventional (non-GM) plant.

4.2 Outcomes of the Safety Assessment

Soybean 356043 contains two novel genes, *gat4601* and *gm-hra*. Detailed molecular analyses indicate that one copy of each novel gene has been inserted at a single site in the plant genome and the genes are stably inherited from one generation to the next. No antibiotic resistance marker genes are present in soybean 356043.

Soybean 356043 expresses two novel proteins: GAT4601 and GM-HRA. The GAT4601 sequence is based on the GAT enzyme sequences from three strains of *B. licheniformis* that were optimised for enhanced glyphosate acetylation activity. The GAT4601 protein is 84% homologous to each of the three native GAT enzymes from which it was derived, compared with 94% amino acid homology between each of the native enzymes. GAT4601 is 146 amino acids in length and has an approximate molecular weight of 17 kDa. The GAT4601 protein is expressed at low levels in soybean 356043 seed, with a mean concentration of 0.24 µg/g of tissue (dry weight).

The GM-HRA protein is a modified version of the native ALS (acetolactate synthase) from soybean. The GM-HRA protein is characterised by two specific amino acid changes in the mature ALS protein that are known to confer tolerance to sulfonylurea herbicides.

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

The GM-HRA protein is 656 amino acids in length with a predicted molecular weight of 71 kDa. Following transport into the chloroplast and cleavage of the transit peptide, the mature protein is 604 amino acids with a predicted molecular weight of 65 kDa. The GM-HRA protein is expressed at low levels in soybean 356043 seed, with a mean concentration of 0.91 µg/g of tissue (dry weight).

Both proteins conform in size and amino acid sequence to that expected, do not exhibit any post-translational modification including glycosylation, and also, for GM-HRA, demonstrate the predicted enzymatic activity.

Bioinformatic studies with the GAT4601 and GM-HRA proteins confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens. Digestibility studies demonstrated that both proteins would be rapidly degraded following ingestion, similar to other dietary proteins. Acute oral toxicity studies in mice with both proteins also confirmed the absence of toxicity. Taken together, the evidence indicates that neither protein is toxic nor likely to be allergenic in humans.

Compositional analyses were done to establish the nutritional adequacy of soybean 356043, and to compare it to a non-transgenic conventional soybean under typical cultivation conditions. For the majority of components, there are no compositional differences of biological significance in forage or seed from transgenic soybean 356043, compared to the non-GM control.

Increased levels of two fatty acids, heptadecanoic acid (C17:0) and heptadecenoic acid (C17:1) were observed. C17:0 and C17:1 in soybean 356043 together constitute around 0.5% of the total fatty acid content, compared to 0.2% in the conventional counterpart. C17:0 and C17:1 are present in other vegetable oils and other commonly consumed foods. As these fatty acids are typical constituents of the human diet and readily metabolised, the increased levels raise no safety or nutritional concerns.

The enzyme GAT4601 also acetylates the amino acids glutamate and aspartate, increasing the levels of N-acetylglutamate (NAGlu) and N-acetylaspartate (NAAsp) in soybean 356043 compared with conventional soybean. NAAsp and NAGlu account for 0.1% of the total amino acid content in soybean 356043 seed. Both NAGlu and NAAsp were found to be present in a number of common foods, indicating that they are normal components of human diets. Both compounds are readily metabolised in humans and raise no safety or nutritional concerns. In addition, exposure to NAGlu and NAAsp through the diet would not be expected to change significantly as neither compound is detectable in soybean oil, which accounts for 94% of all soybean food consumption.

Soybean is one of the major allergenic foods. The potential allergenicity of soybean 356043 was compared to that of the parental soybean variety by assessing IgE binding responses using sera from known soybean allergic individuals. These studies indicated that soybean 356043 does not have any greater potential to be allergenic than conventional soybean varieties.

Based on the scientific information, the introduction of herbicide-tolerant soybean 356043 into the food supply would not be expected to have any nutritional impact. This was supported by the results of a feeding study, where no differences in health and growth performance were found in broiler chickens fed diets containing soybean 356043 meal compared with those fed conventional soybean diets. Similarly, a 90 day sub-chronic toxicity study concluded that there were no diet related adverse effects in rats fed a diet containing soybean 356043.

An assessment was undertaken to establish the safety of the two novel compounds generated on soybean 356043 plants following glyphosate application, namely NAG and *N*-acetyl AMPA. While NAG is the predominant residue detected on soybean 356043 plants treated with glyphosate, parent glyphosate, aminomethylphosphonic acid (AMPA) and *N*-acetyl AMPA are also detectable. Using a weight-of-evidence approach, NAG and *N*-acetyl AMPA were concluded to be less toxic than glyphosate, which itself has low toxicity potential. On this basis, the establishment of a new acceptable daily intake (ADI) for glyphosate and its residues, or a separate ADI for NAG and *N*-acetyl AMPA would be unnecessary.

4.3 Conclusions

No potential public health and safety concerns were identified in the assessment of dual-herbicide tolerant soybean line DP-356043-5. On the basis of the data provided in the Application, and other available information, food derived from soybean line DP-356043-5 is considered as safe and wholesome as food derived from conventional soybean varieties.

The metabolite residues generated by glyphosate-treated soybean 356043 plants are considered less toxic than glyphosate, which itself is considered of very low potential toxicity in animals. Hence, there is no increase in overall toxicity arising from the presence of novel glyphosate residues on soybean 356043, and the current ADI for glyphosate is considered to be protective of public health and safety.

RISK MANAGEMENT

5. Issues raised

5.1 Impact on other Standards

As part of its pre-market safety assessment of food derived from herbicide-tolerant GM crops, FSANZ has regard to the generation of new residues or increased concentrations of known residues on the crop, following application of the herbicide. The potential toxicity of any new residues that have not previously been assessed is relevant to food safety and could also have implications for the existing MRLs[3]. The purpose of these MRLs is to ensure the legitimate and safe use of agricultural chemicals on commodities grown in, or imported into, Australia or New Zealand.

In Australia, the MRLs for agricultural and veterinary chemical residues present in food are listed in Standard 1.4.2, an Australia only Standard. The current MRL in the Code for soybean (dry) for glyphosate is 10 mg/kg; the current residue definition is the *sum of glyphosate and aminomethylphosphonic acid (AMPA) metabolite, expressed as glyphosate*. These are the same as listed in the Australian Pesticides and Veterinary Medicines Authority (APVMA) MRL Standard and are the requirements used for monitoring compliance with the use of glyphosate containing formulations in Australia. The Applicant states that the residues in soybean 356043 will not exceed the current MRL for glyphosate and therefore an amendment to the current MRL in the Code for glyphosate on soybean is not necessary.

In New Zealand, MRLs are established by the Agricultural Compounds and Veterinary Medicines Group (ACVMG) within the NZ Food Safety Authority (NZFSA).

[3] The MRL is the maximum concentration of a residue, resulting from the registered use of an agricultural or veterinary chemical legally permitted or recognized as acceptable in or on a food, agricultural commodity, or animal feed.

There is no MRL for glyphosate on soybean currently listed in the NZ MRL Standard[4], however, there is a provision for residues of up to 0.1 mg/kg for agricultural compound/food combinations not specifically listed. In addition, the NZ MRL Standard recognises Codex standards for imported food. The Codex MRL for glyphosate in soybean seed is 20 mg/kg (the Codex and New Zealand residue definition includes only parent glyphosate).

In this case, the Applicant provided information to enable a separate hazard assessment of residues of glyphosate and metabolites in soybean 356043 seed. This assessment concluded that glyphosate is the only toxicologically-significant compound of the four residues considered in the assessment, and is detectable on commodities derived from herbicide-treated soybean 356043 plants. On this basis, the current residue definition for glyphosate in Standard 1.4.2, the sum of glyphosate and AMPA expressed as glyphosate, remains appropriate from a safety perspective.

In the First Assessment report, FSANZ acknowledged that there is a need to consider the existing MRL and residue definition for glyphosate, given that the US EPA recently amended the existing tolerances[5] for glyphosate residues on soybean to include the combined residues of the herbicide glyphosate and its metabolite N-acetyl-glyphosate (NAG) on soybean 356043[6].

While noting the US EPA decision, FSANZ considers that the costs of amending the existing residue definition for glyphosate solely in relation to soybean 356043, do not outweigh the benefits of pursuing such an amendment to Standard 1.4.2. There is no approval, nor any application under consideration, to grow soybean line DP-356043-5 plants in Australia or New Zealand. Therefore, food commodities derived from soybean 356043 will only be present in foods in Australia or New Zealand if they are imported as food or food ingredients, most likely from the US. In addition, the presence of NAG and *N*-acetyl AMPA raises no safety concerns. On this basis and consistent with the view expressed by the Applicant, FSANZ proposes that the existing residue definition and MRL for soybean in the Code should continue to apply and should apply to soybean line DP-356043-5.

FSANZ considers this approach to be appropriate because:

- there is no food safety basis for modifying the existing MRL or residue definition for soybean in the Code to incorporate a separate residue definition or MRL for soybean line DP-356043-5;
- incorporating a new residue definition with novel residues may have compliance implications in relation to the availability of analytical standards or analytical capability for the novel residues– thereby imposing costs on compliance agencies for an issue that does not have a food safety imperative;
- this approach would maintain consistency between the Code and the APVMA MRL Standard in relation to MRLs and residue definitions, and thereby continue the existing requirements for industry and government agencies with no additional costs in relation to glyphosate analysis of soybean or soybean products;
- unnecessarily complicating Standard 1.4.2 with a separate residue definition for soybean line DP-356043-5 is not justified when it is considered that this line of soybean is unlikely to constitute a major component of the soybean or soybean products that may be consumed in Australia or New Zealand.

[4] http://www.nzfsa.govt.nz/policy-law/legislation/food-standards/nz-mrl-fs-2008-consolidation.pdf
[5] The term 'tolerances' is used in the United States and is equivalent to the term Maximum Residue Limit in Australia.
[6] http://edocket.access.gpo.gov/2008/E8-28571.htm

5.5.1 Tolerance to other herbicides

Soybean line DP-356043-5 also carries a second genetic modification conferring tolerance to ALS-inhibiting herbicides. FSANZ has not previously assessed any GM lines that are tolerant to ALS-inhibiting herbicides. If approved, soybean line DP-356043-5 would need to comply with the existing MRLs in the Code.

5.2 Risk Management Strategy

If approved, food derived from dual-herbicide tolerant soybean line DP-356043-5 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 novel proteins are present in the seed. Highly refined products, such as soybean oil, are exempt from this general labelling requirement if they do not contain novel protein or DNA.

Standard 1.5.2 also contains provision for additional labelling requirements in cases where 'the genetic modification has resulted in one or more significant composition or nutritional parameters having values outside the normal range of values for existing counterpart food not produced using gene technology.' In developing the GM food labelling standard, it was recognised that there may be instances where additional labelling would be appropriate, for example where a property or characteristic of the food means that it is no longer equivalent to an existing counterpart food (Proposal P97).

Soybean 356043 has elevated levels of two minor fatty acids (C17:0 and C17:1) and two acetylated amino acids (NAGlu and NAAsp). FSANZ therefore considered whether additional labelling requirements would be appropriate in this case. Following a detailed evaluation of the issues, FSANZ has concluded that additional labelling requirements for soybean 356043 are not warranted, based on the following considerations.

The levels of C17:0 and C17:1 in soybean 356043 together constitute around 0.5% of the total fatty acid content, compared to 0.2% in the conventional counterpart. NAAsp levels are increased over 200-fold, and NAGlu around 7-fold, although NAAsp and NAGlu together account for only 0.14% of the total amino acid content in soybean 356043 seed. Although elevated compared to the conventional counterpart, these constituents remain minor components of soybean 356043. In addition, after specific consideration of any possible impact on food safety, no nutritional issues could be identified as a result of the increased levels in soybean 356043 as C17:0, C17:1, NAGlu and NAAsp are natural constituents of commonly eaten foods in the human diet and are readily metabolised.

In this case, these components are not considered to be *significant composition or nutritional parameters* for the purposes of labelling GM foods.

Labelling is intended to address the objective set out in subsection 18(1)(b) of the FSANZ Act; the provision of adequate information relating to food to enable consumers to make informed choices. Labelling for changes in the levels of C17:0, C17:1, NAGlu and NAAsp would be unlikely to provide consumers with useful information, particularly as the changes are of no safety or nutritional consequence and do not change the nature of the food. In this context, additional labelling is likely to be confusing and potentially misleading to consumers. The general labelling provisions of the Standard would provide consumer information on the GM status of the food.

The costs to the agricultural and food industry sectors of applying additional labelling requirements in the absence of a clear consumer benefit were also considered. Soybean 356043 has been approved for cultivation and as food in the US.

The US FDA has not imposed a requirement for labelling of soybean 356043 and soybean 356043 will be treated as other GM soybean varieties. In order to comply with any additional labelling requirements in Australia and New Zealand, soybean 356043 would need to be segregated from other soybean, including other GM soybean, varieties. This would involve considerable additional costs associated with food production, which could be passed on to consumers. It is also important to note from an enforcement perspective that where comingling of soybean varieties occurs, either at harvest or at a later processing stage, the altered levels of C17:0 and C17:1 would not be detectable in soybean oil products.

Studies conducted by the Applicant clearly show that NAGlu and NAAsp are below the limit of quantitation in soybean oil, the major food fraction of soybean. Therefore, any need for additional labelling for increased levels of acetylated amino acids would not apply to refined soybean oil. Other soybean fractions that are likely to contain NAGlu and NAAsp would already be captured under existing general labelling requirements for novel DNA and/or novel protein. This means that products such as soy flour and soy milks would require labelling in any case, notwithstanding the increase in levels of acetylated amino acids.

6. Options

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

6.1 Option 1 – Maintain the *status quo*

Reject the Application, thus maintaining the *status quo*.

6.2 Option 2 – Proceed to the development of a food regulatory measure

Proceed to development of a food regulatory measure to amend Standard 1.5.2 to permit the sale and use of food derived from dual herbicide-tolerant soybean line DP-356043-5, with or without specified conditions in the Table to clause 2 of the Standard.

7. Impact Analysis

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

7.1 Affected Parties

The affected parties may include the following:

- Consumers of soybean-containing food products, particularly those concerned about the use of biotechnology to generate new crop varieties.

- Industry sectors:

    - food importers and distributors of wholesale ingredients
    - processors and manufacturers of soybean-containing food products
    - food retailers

- Government:

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

Soybean line DP-356043-5 has been developed primarily for agricultural production overseas and, at this stage, the Applicant has no plans for cultivation of this variety in either Australia or New Zealand. The cultivation of soybean 356043 in Australia or New Zealand could have an impact on the environment, which would need to be independently assessed by the Office of the Gene Technology Regulator (OGTR) in Australia, and by various New Zealand government agencies including the Environmental Risk Management Authority (ERMA) and the Ministry of Agriculture and Forestry (MAF) before commercial release in either country could be permitted.

7.2 Benefit Cost Analysis

7.2.1 Option 1 – reject the Application, thus maintaining the status quo

Consumers: Possible restriction in the availability of imported soybean products to those products that do not contain soybean line DP-356043-5.

No impact on consumers wishing to avoid GM foods, as food from soybean line DP-356043-5 is not currently permitted in the food supply.

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

Industry: Possible restriction on imports of soybean food products once soybean line DP-356043-5 is commercialised overseas.

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

7.2.2 Option 2 – approve food from soybean line DP-356043-5

Consumers: Broader availability of imported soybean products as there would be no restriction on imported foods containing soybean line DP-356043-5.

Potentially, no increase in the prices of imported foods manufactured using comingled soybean products.

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

Government: Benefit that if soybean line DP-356043-5 was detected in soybean imports, approval would ensure compliance of those products with the Code. This would ensure no potential for trade disruption on regulatory grounds.

Approval of soybean line DP-356043-5 would ensure no conflict with WTO responsibilities.

This option could impact on monitoring resources, as certain foods derived from soybean line DP-356043-5 will be required to be labelled as genetically

modified. There may also be an impact on compliance resources associated with detection of soybean line DP-356043-5.

Industry: Importers of processed foods containing soybean derivatives would benefit as foods derived from soybean line DP-356043-5 would be compliant with the Code, allowing broader market access and increased choice in raw materials.

Retailers may be able to offer a broader range of soy products or imported foods manufactured using soybean derivatives.

Possible cost to food industry as some food ingredients derived from soybean line DP-356043-5 would be required to be labelled as genetically modified.

7.3 Comparison of Options

As food from dual herbicide-tolerant soybean line DP-356043-5 has been found to be as safe as food from conventional varieties of soybean, Option 1 is likely to be inconsistent with Australia's and New Zealand's WTO obligations. Option 1 would also offer little benefit to consumers, as approval of soybean line DP-356043-5 by other countries could limit the availability of imported soy products in the Australian and New Zealand markets. In addition, Option 1 would result in the requirement for segregation of any products containing soybean 356043 from those containing approved soybean varieties, which would be likely to increase the costs of imported soy foods.

As the novel herbicide residues generated on soybean 356043 plants following glyphosate application are less toxic than glyphosate itself, glyphosate is considered the only toxicologically-significant residue associated with seed derived from soybean 356043 plants. Detection and measurement of glyphosate residues on material derived from soybean 356043 plants is adequate from a safety perspective. Consultation between the APVMA, NZFSA and the Applicant has concluded that consequential amendments to Standard 1.4.2 are not necessary.

Based on the conclusions of the safety assessments, the potential benefits of Option 2 outweigh the potential costs. A variation to Standard 1.5.2 giving approval to dual herbicide-tolerant soybean line DP-356043-5 is therefore the preferred option.

COMMUNICATION AND CONSULTATION STRATEGY

8. Communication

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

As normally applies to all GM food assessments, this Second Assessment Report will be available to the public on the FSANZ website and distributed to major stakeholders. Public comments on this Second Assessment will be used in preparing an Approval Report that will be considered by the FSANZ Board.

The Applicant and individuals and organisations that make submissions on this Application will be notified at each stage of the assessment. After the FSANZ Board has considered the Approval Report, if the draft variation to the Code is approved, the decision will be notified to the Ministerial Council. If the approval of food derived from dual herbicide-tolerant soybean

line DP-356043-5 is not subject to review, the Applicant and stakeholders, including the public, will be notified of the gazettal of changes to the Code in the national press and on the website.

9. Consultation

9.1 Public consultation

Public submissions were invited on the First Assessment Report between 20 March and 1 May 2009. Comments were specifically sought on the scientific aspects of this Application, in particular, information relevant to the safety assessment of food derived from dual herbicide-tolerant soybean line DP-356043-5, and the novel herbicide residues (NAG and *N*-acetyl AMPA). Comments on the proposed labelling requirements for food derived from soybean line DP-356043-5 were also invited. Nine submissions were received. A summary of these is provided in **Attachment 4** to this Report. Responses to the main issues raised regarding any risks to human safety if soybean 356043 were to be approved for food use, are provided below. Where necessary, FSANZ has addressed the issue through a change to the safety assessment report for soybean 356043.

As this Application is being assessed as a major procedure, there are two rounds of public comment. Submissions from the public are invited on this Second Assessment Report, including the proposed draft variation to the Code.

9.1.1 General Issues

A number of issues were raised concerning GM foods and their assessment in a general context. The majority of issues falling within FSANZ responsibilities have been addressed in previous assessments and specific information is available from the FSANZ website[7] (refer to Table 1).

FSANZ has been asked to not approve any GM foods by some stakeholders. It must be acknowledged however that FSANZ has a statutory obligation to consider all applications seeking to amend the Code on their individual merits, subject to the application meeting detailed criteria concerning format and inclusion of information. An open and transparent process of assessment is then used to develop or amend food standards as may be appropriate in Australia and New Zealand. In particular, public consultation periods are considered integral to this process, and comments received from submitters contribute to the overall effectiveness of the risk assessment.

In relation to GM foods, novel foods or substances added to foods requiring a comprehensive pre-market assessment, a scientific, evidence-based assessment is used to establish that the food or substance is safe for human consumption. For GM foods, this requires evidence to show that the proposed food is as safe as the existing counterpart food, on a case-by-case basis. FSANZ will not approve a GM food if any public health and safety concerns have been identified in the assessment.

9.1.2 Specific Issues

A number of issues specific to the assessment of soybean line DP-356043-5 were raised in submissions and have been addressed in the following responses.

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

Table 1: Sources of Information, available on the FSANZ website, regarding GM Food

Issue	General area of FSANZ website where information can be found	Specific web link
Safety of GM food	Safety Assessment of Genetically Modified Foods	http://www.foodstandards.gov.au/_srcfiles/GM%20Foods_text_pp_final.pdf
	Frequently Asked Questions on GM foods	http://www.foodstandards.gov.au/foodmatters/gmfoods/frequentlyaskedquest3862.cfm
	Response to article by Dr Judy Carman	http://www.foodstandards.gov.au/foodmatters/gmfoods/chiefscientistrespon3993.cfm
Lack of independent data used to inform the Safety Assessment	Food Matters • GM Foods	http://www.foodstandards.gov.au/foodmatters/gmfoods/
	Frequently Asked Questions on GM foods • Part II. Safety Assessment	http://www.foodstandards.gov.au/foodmatters/gmfoods/frequentlyaskedquest3862.cfm
Labelling of GM food	Appendix 3: Safety Assessment of Genetically Modified Foods	http://www.foodstandards.gov.au/_srcfiles/GM%20Foods_text_pp_final.pdf
	Frequently Asked Questions on GM foods Part III. Labelling of GM Foods	http://www.foodstandards.gov.au/foodmatters/gmfoods/frequentlyaskedquest3862.cfm
	GM Labelling Review Report	http://www.foodstandards.gov.au/newsroom/publications/gmlabellingreviewrep2460.cfm
Safety of food products derived from livestock fed GM feeds	Section 7.7: Safety Assessment of Genetically Modified Foods	http://www.foodstandards.gov.au/_srcfiles/GM%20Foods_text_pp_final.pdf
	Frequently Asked Questions on GM foods Part IV. Other Questions	http://www.foodstandards.gov.au/foodmatters/gmfoods/frequentlyaskedquest3862.cfm
	Safety Assessment of Genetically Modified Foods Guidance Document	http://www.foodstandards.gov.au/_srcfiles/GM%20FINAL%20Sept%2007L%20_2_.pdf
The need for animal feeding studies	Food Matters • GM Foods	http://www.foodstandards.gov.au/foodmatters/gmfoods/
	Frequently Asked Questions on GM foods • Part II. Safety Assessment	http://www.foodstandards.gov.au/foodmatters/gmfoods/frequentlyaskedquest3862.cfm
	Role of Animal Feeding Studies in the Safety Assessment of GM Foods	http://www.foodstandards.gov.au/foodmatters/gmfoods/roleofanimalfeedings3717.cfm
	Safety Assessment of Genetically Modified Foods Guidance Document	http://www.foodstandards.gov.au/_srcfiles/GM%20FINAL%20Sept%2007L%20_2_.pdf
Horizontal Gene Transfer/Antibiotic Resistance Genes	Safety Assessment of Genetically Modified Foods Guidance Document	http://www.foodstandards.gov.au/_srcfiles/GM%20FINAL%20Sept%2007L%20_2_.pdf
	Section 7.4: Safety Assessment of Genetically Modified Foods	http://www.foodstandards.gov.au/_srcfiles/GM%20Foods_text_pp_final.pdf
Published studies in Austria that have suggested there are adverse effects on laboratory animals fed GM food	Impact of Austrian reproduction study on the safety of GM corn lines MON810 and NK603	http://www.foodstandards.gov.au/newsroom/factsheets/factsheets2009/updateimpactofaustri4157.cfm
Safety of GM feed for livestock.	Section 7.7: Safety Assessment of Genetically Modified Foods	http://www.foodstandards.gov.au/_srcfiles/GM%20Foods_text_pp_final.pdf
	Frequently Asked Questions on GM foods Part IV. Other Questions	http://www.foodstandards.gov.au/foodmatters/gmfoods/frequentlyaskedquest3862.cfm
Herbicide residues in GM foods	Section 7.5: Safety Assessment of Genetically Modified Foods	http://www.foodstandards.gov.au/_srcfiles/GM%20Foods_text_pp_final.pdf

(1) Compositional changes arising from the acetylation of glutamate and aspartate

Several submissions, including from the New South Wales Food Authority and the New Zealand Food Safety Authority, express concerns about the increased levels of NAAsp and NAGlu in soybean line 356043, particularly with regard to dietary exposure.

Response

To complement the data already given in Section 5.6.1 of the Safety Assessment Report, FSANZ has included additional information on the distribution and metabolism of these acetylated amino acids in humans, and the potential toxicity of NAAsp. As well, further information on the natural abundance of NAAsp and NAGlu in commonly consumed foods has been recently published, and, together with soybean processing data, enabled FSANZ to undertake a simple dietary intake assessment for Australian and New Zealand population groups, to estimate any impact on the whole diet if soybean 356043 was permitted in the food supply. The Applicant also provided new information on the levels of NAAsp and NAGlu in the diet of rats in the sub-chronic feeding study, which enabled a review of the safety of soybean 356043 when incorporated into the diet. Based on the additional information and reviews, FSANZ is confident that the increased levels of NAAsp and NAGlu in soybean 356043 are nutritionally insignificant and raise no food safety concerns.

(2) Consideration of the glyphosate MRL in the Code

The New South Wales Food Authority and the New Zealand Food Safety Authority both agree on the need for FSANZ to consider the recent US EPA decision to include N-acetyl glyphosate in the tolerance limits for glyphosate, to determine whether any consequential amendments to Standard 1.4.2 are warranted.

Response

FSANZ has given due consideration to the residue definition currently relating to glyphosate on soybeans in Standard 1.4.2 and concludes that the existing MRL and residue definition should continue to apply. As a result, FSANZ is not proposing to amend the current residue definition for glyphosate on soybeans. This decision followed discussion of the issue with the APVMA, the NZFSA and the Applicant. Further discussion of this issue, together with the reasons for this decision, are presented in Section 5.1 of this Report.

(3) The safety of glyphosate residues on soybean 356043

In one submission, a recent study (Benachour *et al.* 2009)[8] was cited as evidence that soybean DP-356043-5 sprayed with glyphosate could pose a food safety concern for consumers of products derived from that line.

Response

The potential toxicity of herbicide residues is a general issue and not specifically related to GM foods, as herbicides are used on conventional (non-GM) crops as well as GM crops. The new study does not raise any food safety concerns. The key point is that it deals with an *in vitro* system that permits direct exposure of a detergent (POEA) on mammalian cells. The detergent known as POEA is used as a surfactant in some glyphosate formulations, however is not present in formulations used near aquatic environments because of possible adverse effects on aquatic organisms.

[8] Benachour, N. and Seralini, G.E. (2009). Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. *Chem. Res in Toxicol*. **22**, 97-105.

It is well known that detergents effectively disrupt cell membranes, however this type of exposure has no impact on the safety of food products derived from herbicide treated plants.

Concerns expressed about the potential toxicity of the glyphosate metabolite AMPA are also unfounded. I*n vivo* data in rats show that when AMPA is orally ingested, no effects are observed. Both the APVMA and FSANZ have reviewed the relevant toxicological data which shows that even for the whole glyphosate formulation, the potential toxicity in mammals is very low.

This Application included a detailed assessment of the potential toxicity of the novel herbicide residues (NAG and *N*-acetyl AMPA), and no additional food safety concerns were identified. Soybean 356043 plants sprayed with glyphosate would have mostly NAG residue, some N-acetyl AMPA residue and very little glyphosate residue. The NAG and N-acetyl AMPA residues generated on soybean DP-356043-5 plants sprayed with glyphosate, are less toxic than glyphosate, which itself, is regarded as having low potential toxicity in mammals (see Attachment 3). The Applicant does not consider that an amendment to the MRL for glyphosate is necessary in this Application because analysis has shown that soybean 356043 will meet the current MRL that applies to all commercially cultivated soybeans.

(4) Use of ALS-inhibiting herbicides

Queensland Health notes that the only ALS-inhibiting herbicides currently with an MRL for soybean in the Code are tribenuron, imazethapyr and flumetsulam, and seeks assurance that products derived from soybean DP-356043-5 would comply with the existing MRLs.

Response

If approved, soybean line DP-356043-5 would need to comply with the existing MRLs in the Code. Enforcement is the responsibility of the jurisdictions, including the New Zealand government although, for imported foods, operational responsibility resides in Australia with the Australian Quarantine and Inspection Service. FSANZ has previously brought this issue to the Applicant's attention prior to receipt of the Application. To date, Pioneer Hi-Bred International has not applied to FSANZ to extend the range of permitted MRLs for ALS-inhibiting herbicides for soybean which indicates that any products (produced using GAP), in theory would be able to meet the existing limits for the current list of chemicals.

(5) Regulatory approvals

Queensland Health seeks clarification about whether soybean 356043 will be grown in Australia and also requests advice on submissions made by the Applicant to regulatory agencies in other countries.

Response

The Gene Technology Regulator is the authority responsible for the issuing of a licence for the cultivation of GM crops in Australia, following a thorough consideration of any risks to human health and safety, and the environment. To date, Pioneer Hi-Bred International has not applied to the Gene Technology Regulator for a licence to cultivate soybean 356043 in Australia.

Since the preparation of the First Assessment Report, soybean 356043 has been approved for food/feed use in Japan, Mexico and Taiwan, and cultivation in Japan. This information has been added to the Second Assessment Report (Section 2.2).

(6) Compositional analysis

One submitter expressed the view that soybean 356043 cannot be regarded as nutritionally equivalent to non-GM soybean, since the compositional analysis showed a number of analytes in soybean 356043 were significantly different from conventional soybean.

Response

The Safety Assessment acknowledges differences in the levels of several analytes between soybean 356043 and non-GM varieties and does not claim that soybean 356043 is 'nutritionally equivalent'. However, FSANZ does not consider that the changes in the levels of some analytes raise any food safety or nutritional concerns. Despite some differences, food derived from soybean 356043 is as wholesome as food derived from other commercial soybean varieties.

(7) Feeding studies with other GM soybean

One submitter cited studies on a different glyphosate tolerant soybean, as evidence that animals fed GM soybean products had developed abnormalities. The studies include Malatesta *et al.* 2002, Tudisco *et al.* 2006, and unpublished studies by Dr Ermakova (released in 2005[9]), which have all been previously investigated by FSANZ.

Response

These three studies all deal with a different line of GM soybean, known as Roundup Ready soybean line 30-3-2. The novel gene in Roundup Ready soybean is the *cp4 epsps* gene from *Agrobacterium tumefaciens*. The genetic modification conferring tolerance to glyphosate in soybean 356043 involves the use of the GAT gene which is based on three glyphosate acetyltransferase genes from *Bacillus licheniformis*. Notwithstanding these major molecular differences in the plants, the study results have been thoroughly investigated and claims of toxic effects in the animals caused by a diet containing GM soy cannot be substantiated.

(8) Molecular characterisation of soybean 356043

One submitter cites a number of concerns written in February 2008 by the African Centre for Biosafety (ACB), regarding the molecular characterisation and other elements of the safety assessment of soybean DP-356043-5. The concerns are summarised and addressed as follows:

(i) The molecular characterisation should not rely on Southern blots with insufficient sensitivity and a limited number of frequently-cutting restriction enzymes. The positive control did not produce the expected pattern with *Xba1*, probably because it was methylated, meaning that integrity of the cassette could not be accurately determined and fragment sizes could not be accurately compared to the original construct.

Response

While not debating the usefulness and sensitivity of other techniques, it is generally held that properly designed Southern analysis should be the primary method of detection of all complete and/or partial novel gene inserts.

[9] Malatesta, M; Caporaloni, C.;Gavaudan, S.;Rocchi, M.B.L.;Serafini, S.;Tiberi, C.;Gazzanelli, G. (2002). Ultrastructural morphometrical and immunocytochemical analyses of hepatocyte nuclei from mice fed on genetically modified soybean. *Cell Structure and Function* 27: 173 – 180.
Ermakova, I. (2005). Influence of genetically modified soya on the birth-weight and survival of rat pups. Presentation at "Epigenetics, Transgenic Plants & Risk Assessment' available online at http://www.somloquesembrem.org/img_editor/file/Ermakovasoja.pdf
Tudisco, R.;Lombardi, P.;Bovera, F.;d'Angelo, D.;Cutrignelli, M.I.;Mastellone, V.;Terzi, V.;Avallone, L.;Infascelli, F. (2006). Genetically modified soya bean in rabbit feeding: detection of DNA fragments and evaluation of metabolic effects by enzymatic analysis. *Animal Science* 82:193 – 199.

The Applicant acknowledged that *XbaI* could not cut at one site on the PHP20163 fragment (prepared from a Dam$^+$ *E. coli* strain) because this site overlaps a Dam methylation recognition sequence, and that the presence of this site affected size prediction. In order to address this, the Applicant prepared plasmid PHP20163 from a strain of *E. coli* lacking Dam methylase (Dam$^-$). Digestion of this Dam$^-$ plasmid with *XbaI* produced the predicted size bands and confirmed that soybean 356043 contains an intact insertion of the PHP20163 fragment.

(ii) Insert copy number was determined by visual reference to known copies of plasmid DNA and no quantification was made.

Response

To date, the most reliable method for transgene copy number determination is Southern blot hybridisation. While other techniques such as real-time PCR may be useful, there have been concerns about detection limitations. In the study submitted by the Applicant, a standard Southern-based procedure was followed to determine copy number. Restriction enzyme (*BglII*) with only one restriction site in the transgene fragment was chosen to digest genomic DNA from soybean 356043, and the digested DNA was used for Southern blot hybridisation with probes specific to the two transgenes. With such a restriction enzyme and probe sequences, the number of bands obtained equals the number of transgene copies per haploid genome (Bhat & Srinivasan 2002)[10]. Data from another digest (using *HindIII*) supported the conclusion that there is a single copy of the PHP20163A fragment in the genome of soybean 356043.

(iii) Data are insufficient to support the conclusion that the PHP20163 fragment is genetically and phenotypically stable – only a few plants were studied using a few (generally 3) restriction enzymes (which cut infrequently) over only two generations.

Response

The insert stability studies supplied by the Applicant used 14 or 15 plants from each of the T4 and T5 generations as well as 77 plants from the F3 generation. This provided data from multiple generations, and is considered sufficient for testing of genetic stability (eg EFSA 2008; OECD 2009). Additionally, chi square analysis of trait inheritance from 5 generations (T1, F2, F3, BC1F2 and C2F2) not only demonstrated the Mendelian inheritance of the introduced traits but also their stability. Phenotypic stability of soybean 356043 was confirmed by the extensive compositional studies that involved 18 replicates across 6 locations under both herbicide-sprayed and unsprayed conditions.

(iv) No data are available to address the question of whether there have been any unintended rearrangements at the site of integration.

Response

The Applicant provided DNA sequence of the entire insert together with flanking border sequence. This information fully characterises the integrity of the insert and allows detailed open reading frame analysis of the genomic sequence flanking the insertion site present in soybean 356043. These studies satisfactorily confirmed the absence of unintended rearrangements at the insertion site.

(v) The Rsyn7-SynII element of the SCP1 promoter is new to nature and has not been tested for biosafety. The SCP1 promoter also contains a CaMV-35S element; CaMV-35S is associated with increased rearrangements/deletions affecting cassette integrity and genome stability.

Response

The fundamental consideration by FSANZ is whether the scientific evidence supports the safety of a GM food using established assessment methods.

[10] Bhat, S.R.; Srinivasan, S. (2002). Molecular and genetic analyses of transgenic plants: Considerations and approaches. *Plant Science* 163: 673 – 681.

The results from various studies completed by the Applicant (see answers to points raised above) have indicated there are no concerns with unintended rearrangements or genetic/phenotypic instability in soybean 356043.

(vi) A technique such as repPCR, RAPD or comparative genome hybridization could have been used to establish the genome similarity between soybean 356043 and the non-GM parent line.

Response

The information supplied by the Applicant in various studies provided evidence that, apart from the insertion of a single, intact copy of the PHP20163 (flanked by unaltered soybean genomic DNA), the soybean 356043 genome does not differ from that of the non-GM parent. Techniques such as repPCR, RAPD or comparative genome hybridisation are not as useful for a comparative study because the results of such analyses cannot be meaningfully interpreted. Rather, it is important for the risk assessment to rely on studies that focus the attention on the intended differences between the GM and non-GM line, and then further focus the assessment on any unintended effects observed through the data analysis. This more strategic approach is accepted by most international bodies to be the most informative for risk assessment purposes.

Furthermore, it should be noted that it is not clear from the web document cited as reference, whether the ACB is addressing a full package of data as provided to FSANZ. It is possible that the data provided to FSANZ (received 18 March 2008) contained more updated and detailed data than was used by ACB. Additionally, the ACB would not have had access to the A1006 First Assessment prepared by FSANZ since this did not become publicly available until 20 March 2009.

(vii) The ACB also claims to have concerns about whether the supporting data for allergenicity and toxicity studies are sufficient. It claims that a feeding study should have been used to observe the immune response of both novel proteins since bioinformatics and simulated gastric/intestinal fluid studies cannot show this. It is also asserted that the bacterially expressed HRA protein used for the acute oral toxicity study is not sufficiently similar to the plant expressed HRA protein.

Response

As stated in the First Assessment for A1006, the potential allergenicity of novel proteins is evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity. Only in some cases, such as where the novel protein has sequence similarity to a known allergen, may additional *in vitro* and *in vivo* immunological testing be warranted. In the case of soybean 356043 such testing was not deemed to be warranted.

Immune responses are the result of exposure to an intact epitope (sequence or structure) on a protein. The simulated digestibility studies are particularly informative because they indicate whether a protein is likely to survive normal digestion, or be broken down into its constituent amino acids. The more readily a protein degrades under conditions that mimic normal human digestion, the more likely it is that potential epitopes will denature and therefore not present to the immune system.

The equivalence of the GM-HRA protein from plant and microbial sources was established by comparing results from a range of analyses – SDS-PAGE; Western Blot; MALDI-MS; N-terminal amino acid sequencing; protein glycosylation. In addition, electrospray mass spectroscopy established that the molecular mass of the microbially-derived protein was consistent with the expected mass (65 kDa), and the microbially-derived protein exhibited the expected ALS biochemical activity. Taken together, these results confirm that the bacterial-derived GM-HRA protein is suitable to use as a substitute for the plant-derived GM-HRA protein in toxicity testing.

(9) Safety of novel protein source

The NSW Food Authority questioned the general statement that *B. licheniformis* is not associated with safety concerns.

Response

The Safety Assessment has been reworded to reflect the fact that certain isolates of the *B. licheniformis* bacterium produce toxins that have been associated with food poisoning incidents. However, these changes have no direct impact on the conclusions of the safety assessment.

10.2 World Trade Organization (WTO)

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

The inclusion of food derived from soybean 356043 in the Code would have a liberalising effect on trade as it would permit any foods containing this variety of soybean to be imported into Australia and New Zealand and sold, where currently they would be prohibited. For this reason, there was no need to notify this Application under the Sanitary or Phytosanitary Measures (SPS) Agreement.

CONCLUSION

11. Conclusion and Preferred Approach

Preferred Approach

Proceed to develop a food regulatory measure, to amend Standard 1.5.2 – Food produced using Gene Technology, to include food derived from dual herbicide-tolerant soybean line DP-356043-5 in the Table to clause 2.

11.1 Reasons for Preferred Approach

An amendment to the Code giving approval to the sale and use of food derived from soybean line DP-356043-5 in Australia and New Zealand is proposed on the basis of the available scientific evidence, for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce dual herbicide-tolerant soybean line DP-356043-5;
- food derived from soybean line DP-356043-5 is equivalent to food from the conventional counterpart and other commercially available soybean varieties in terms of its safety for human consumption and nutritional adequacy;
- the novel herbicide residues generated on soybean line DP-356043-5 plants following glyphosate application are less toxic than glyphosate;

- labelling of certain foods derived from dual herbicide-tolerant soybean line DP-356043-5 will be required where novel DNA and/or protein is present in the final food;

- a regulation impact assessment process has been undertaken that fulfils the requirement in Australia and New Zealand for an assessment of compliance costs. The assessment concluded that the preferred option is Option 2, the development of a food regulatory measure; and

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

12. Implementation and Review

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

ATTACHMENTS

1. Draft variations to the *Australia New Zealand Food Standards Code*
2. Safety Assessment of dual herbicide-tolerant soybean line DP-356043-5
3. Hazard Assessment of glyphosate residues
4. Summary of submissions

Attachment 1

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

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

To commence: on gazettal

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

Food derived from herbicide-tolerant soybean line DP-356043-5	

Attachment 2

SAFETY ASSESSMENT

SUMMARY AND CONCLUSIONS

Dual herbicide-tolerant soybean DP-356043-5 has been genetically modified for tolerance to the broad-spectrum herbicide glyphosate and to acetolactate synthase (ALS)-inhibiting herbicides. Tolerance is conferred by expression in the plant of two novel proteins: GAT4601 and GM-HRA. The GAT4601 enzyme is an optimised acetyltransferase that results in the inactivation of the glyphosate-containing herbicides, rendering them non-phytotoxic. The GM-HRA enzyme is a modified version of a soybean ALS that can function in the presence of the ALS-inhibiting class of herbicides.

In conducting a safety assessment of food derived from dual herbicide-tolerant soybean 356043, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the soybean genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic in humans.

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

History of Use

The cultivated soybean, *Glycine max* (L.) Merr., is an annual crop grown commercially in over 35 countries. Soybean is the dominant oilseed traded in international markets (OECD, 2001). There are three major soybean products – beans, meal and oil. The principle processed fraction used by the food industry is soybean oil.

The *gat4601* gene is based on the sequence of three genes from the common soil bacterium *Bacillus licheniformis*. *B. licheniformis* is an approved bacterial source for the production of a number of enzymes used as food processing aids. *B. licheniformis* is widespread in the environment, and is not generally associated with any safety concerns.

The GM-HRA protein encoded by the *gm-hra* gene derived from soybean differs from the native soybean ALS protein at two specific amino acids.

Molecular Characterisation

Soybean 356043 contains two novel genes, *gat4601* and *gm-hra*. Detailed molecular analyses indicate that one copy of each novel gene has been inserted at a single site in the plant genome and the genes are stably inherited from one generation to the next. No antibiotic resistance marker genes are present in soybean 356043.

Characterisation of Novel Protein

Two novel proteins, GAT4601 and GM-HRA, are expressed in soybean 356043. The GAT4601 sequence is based on the GAT enzyme sequences from three strains of *B. licheniformis* that have been optimised for enhanced glyphosate acetylation activity.

GAT4601 is 84% homologous to each of the three native GAT enzymes from which it was derived. The GAT4601 protein is 146 amino acids in length and has an approximate molecular weight of 17 kDa. It is expressed at low levels in soybean 356043 seed, with a mean concentration of 0.24 µg/g of tissue (dry weight).

The GM-HRA protein is a modified version of the native ALS from soybean. Two specific amino acid changes in the mature ALS protein are known to confer tolerance to sulfonylurea herbicides. Following transport into the chloroplast and cleavage of the transit peptide, the mature GM-HRA protein is 604 amino acids with a predicted molecular weight of 65 kDa. It is expressed at low levels in soybean 356043 seed, with a mean concentration of 0.91 µg/g of tissue (dry weight).

A large number of studies have been done to confirm the identity and physicochemical and functional properties of the expressed GAT4601 and GM-HRA proteins in the plant, as well as to determine their potential toxicity and allergenicity. Both proteins conform in size and amino acid sequence to that expected, do not exhibit any post-translational modification including glycosylation, and also, for GM-HRA, demonstrate the predicted enzymatic activity.

Bioinformatic studies confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens, and digestibility studies demonstrated that both proteins would be rapidly degraded following ingestion, similar to other dietary proteins. Separate acute oral toxicity studies in mice with GAT4601 and GM-HRA confirmed that neither protein is toxic in mammals. Taken together, the evidence indicates that the proteins are not toxic or likely to be allergenic in humans.

Compositional Analyses

Compositional analyses were done to establish the nutritional adequacy of soybean 356043, and to compare it to a non-transgenic conventional soybean under typical cultivation conditions. The components analysed were protein, fat, carbohydrate, amino acids, fatty acids, vitamins, minerals, isoflavones, and the anti-nutrients stachyose, raffinose, lectins, phytic acid and trypsin inhibitor.

For the majority of components, there are no compositional differences of biological significance in seed from transgenic soybean 356043, compared to the non-GM control. Several minor differences in key nutrients and other constituents were noted, however, the mean levels observed were generally within the range of values observed for the non-transgenic comparator and within the range of natural variation.

Increased levels of two fatty acids, heptadecanoic acid (C17:0) and heptadecenoic acid (C17:1) were observed. C17:0 and C17:1 in soybean 356043 together constitute around 0.5% of the total fatty acid content, compared to 0.2% in the conventional counterpart. C17:0 and C17:1 are present in vegetable oils and other commonly consumed foods. As these fatty acids are typical constituents of the human diet and readily metabolised, the increased levels raise no safety or nutritional concerns.

The GAT4601 enzyme also acetylates the amino acids glutamate and aspartate, producing elevated levels of N-acetylglutamate (NAGlu) and N-acetylaspartate (NAAsp) in soybean 356043 compared with conventional soybean. Together, NAAsp and NAGlu account for only 0.14% of the total amino acid content in soybean 356043 seed. Both NAGlu and NAAsp are endogenously produced and are readily metabolised in humans. Both compounds are naturally present in low amounts in a number of common foods, indicating that they are normal components of the diet. Although commercialisation of soybean 356043 could potentially increase dietary intakes of NAGlu and NAAsp, processing would significantly reduce the amounts of NAGlu and NAAsp in soybean food products. Neither compound is

detectable in soybean oil, which accounts for 94% of soybean food consumption. The levels of NAAsp and NAGlu in soybean 356043 therefore do not raise food safety concerns.

Soybean is one of the major allergenic foods. The potential allergenicity of soybean 356043 was compared to that of the parental soybean variety by assessing IgE binding responses using sera from known soybean allergic patients. These studies indicated that soybean 356043 does not have any greater potential to be allergenic than conventional soybean varieties.

Nutritional Impact

The introduction of dual herbicide-tolerant soybean 356043 into the food supply would be expected to have negligible nutritional impact. This was supported by the results of two feeding studies, one in broiler chickens and another in rats. The results showed no differences in health and growth performance of broiler chickens, and in the health and well-being of rats, fed diets containing soybean 356043 compared with a diet containing conventional soybean.

Conclusion

No potential public health and safety concerns have been identified in the assessment of dual herbicide-tolerant soybean 356043. On the basis of the data provided in the present Application, and other available information, food derived from soybean 356043 is considered as safe for human consumption as food derived from conventional soybean varieties.

1. INTRODUCTION

Dual herbicide tolerant soybean 356043 has been genetically modified for tolerance to the broad-spectrum herbicide glyphosate and acetolactate synthase (ALS)-inhibiting herbicides. The intended product name for this soybean is Optimum™ GAT™.

Soybean 356043 plants express two novel proteins, GAT4601 (glyphosate acetyltransferase) and GM-HRA (modified version of a soybean acetolactate synthase). The GAT4601 protein, encoded by the *gat4601* gene, confers tolerance to glyphosate-containing herbicides by acetylating glyphosate and thereby rendering it non-phytotoxic. The GM-HRA protein, encoded by the *gm-hra* gene, contains two specific amino acid changes to the soybean GM-ALS enzyme, an essential enzyme in the biosynthesis of branched chain amino acids in plants. Expression of the GM-HRA enzyme confers tolerance to the ALS-inhibiting class of herbicides such as the sulfonylureas.

2. HISTORY OF USE

2.1 Donor organisms

2.1.1 The gat4601 gene

The *gat4601* gene is based on the sequence of three gat genes from the common soil bacterium *Bacillus licheniformis*. *B. licheniformis* is an approved bacterial source for the production of a number of enzymes used as food processing aids, such as α-amylase, pullulanase (a glucanase) and serine protease. The U.S. Environmental Protection Agency has determined that this organism presents a low risk to human health and the environment when used under specific conditions for general commercial use (EPA, 1996). However, while *B. licheniformis* is widespread in the environment and people are regularly exposed to it without any associated adverse effects, nonproteinaceous toxins produced by isolates of *B. licheniformis* have been associated with food involving food poisoning incidents (see Salkinoja-Salonen *et al*., 1999 and references therein).

2.1.2 The gm-hra gene

The *gm-hra* gene is derived from the crop plant soybean, which has a long history of use as food (see following section).

2.2 Host organism

Cultivated soybean (*Glycine max* (L.) Merrill) is a diploidised tetraploid (2n=40) of the Leguminosae family. Soybean is an annual crop that is grown commercially in over 35 countries world-wide. Soybean is the major oilseed crop in terms of world production and trade in international markets. In 2005-2006 global production exceeded 219 million tonnes. The major producers are the US, Argentina, Brazil and China; these countries account for 87% of total production (OECD, 2001). In 2005, a GM soybean known as glyphosate-tolerant soybean line 40-3-2 accounted for 60% of global soybean production (James, 2005).

The majority of soybean is processed for soybean meal used in animal feed, and soybean oil for human food uses. Soybeans are a traditional source of protein and oil for human consumption. Foods that contain soybean protein include bakery products, confections, meat products, textured foods and nutritional supplements. Soybean protein isolate is also the protein source for soy–based infant formula, where the amino acid and fatty acid profile is very important (OECD, 2001). The oil is typically used in margarine, shortening, cooking oil, salad oil and mayonnaise. Lecithin, derived from crude soybean oil, is used as a natural emulsifier, lubricant and stabilising agent.

There are no human food uses for raw unprocessed soybeans as they contain high levels of trypsin inhibitor and lectins, both of which have anti-nutritional properties. A significant proportion of both trypsin inhibitor and lectins is destroyed by heat treatment. Phytic acid present in soybean can reduce bioavailability of some mineral nutrients (OECD, 2001).

Soybean also contains phytoestrogens, naturally occurring isoflavone compounds that have a number of biochemical activities in mammals. The low molecular weight carbohydrates stachyose and raffinose are the cause of intestinal gas production resulting in flatulence and are considered to be anti-nutrients.

Soybeans contain several allergenic proteins that can cause severe adverse reaction when present in the diet of hypersensitive individuals (OECD, 2001).

3. DESCRIPTION OF THE GENETIC MODIFICATION

3.1 Method used in the genetic modification

Soybean 356043 was generated by particle bombardment of embryogenic soybean cultures using linear DNA encoding the *gat4601* and *gm-hra* genes.

Clumps of secondary somatic embryos derived from explants from small, immature soybean seeds were used as the targets for transformation. The soybean cultivar Jack was used for transformation, as it has a high embryogenic capacity.

Somatic embryos can be induced from immature cotyledons, proliferated and maintained in liquid medium prior to transformation. Transformation experiments were conducted on soybean somatic embryogenic cultures two to four months after initiation.

A linear DNA fragment PHP20163A containing the *gat4601* and *gm-hra* gene cassettes was used for particle bombardment. The genetic elements within this fragment are described in detail in the following section. Microscopic gold particles coated with the purified fragment PHP20163A DNA were accelerated into the embryogenic soybean cultures using a Biolistics PDS-1000/He particle gun, essentially as described by (Klein *et al*., 1987).

Following transformation, soybean tissue was transferred to liquid culture maintenance medium for recovery. After seven days, the soybean cells carrying the *gm-hra* transgene were selected by culturing in maintenance medium supplemented with the ALS-inhibiting herbicide chlorsulfuron. After several weeks of chlorsulfuron selection, chlorsulfuron-tolerant green tissue became visible as small islands of healthy green tissue growing out of pieces of dying somatic embryogenic tissue. These green embryogenic clumps were excised and regularly subcultured into fresh liquid selection medium until the start of the regeneration process.

Embryogenic tissue samples were analysed by Southern blot hybridisation to confirm the presence of the *gat4601* and *gm-hra* transgenes. Primary transgenic (T0) plants were regenerated and transferred to the greenhouse for seed production. A schematic diagram of the development process for soybean 356043 is shown in Figure 1. The breeding tree of soybean 356043 is shown in Figure 2.

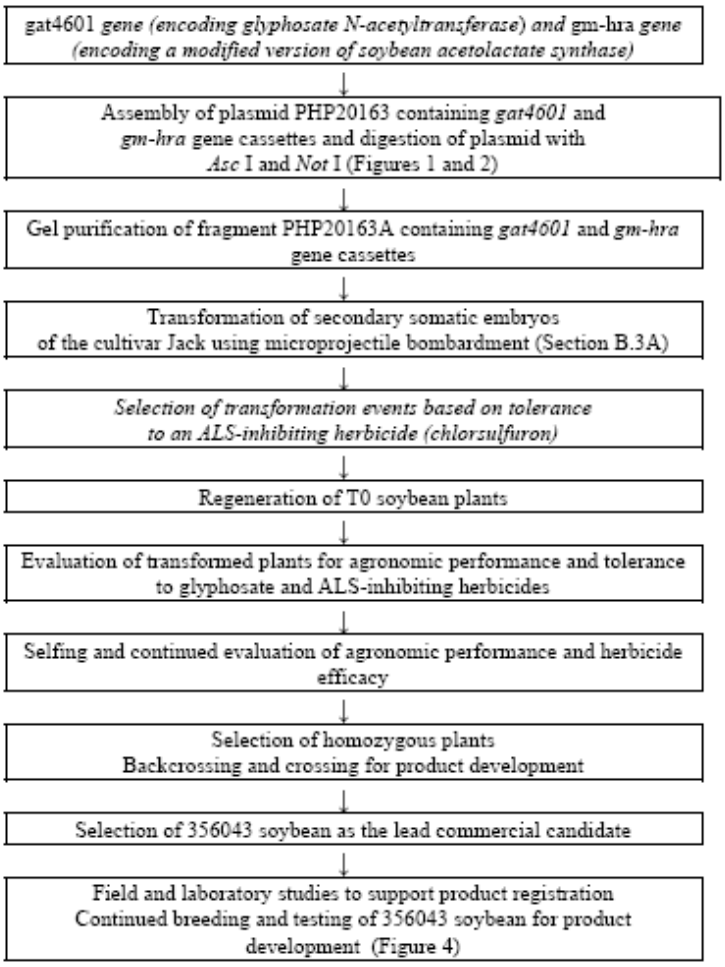

Figure 1: Development of soybean 356043

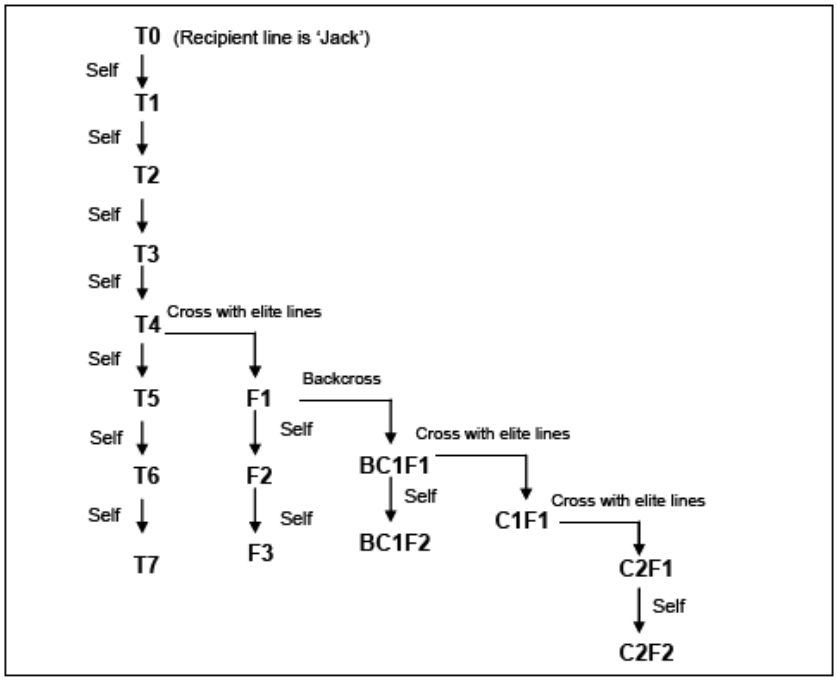

Figure 2: Breeding Diagram for soybean 356043

Molecular characterisation was performed using the T4, T5 and F3 generations. Inheritance analyses were performed on T1, F2, F3, BC1F2 and C2F2 generations. Levels of transgenic

protein expression and compositional assessment were determined in the T5 generation. A feeding study in broiler chickens (see Section 6.1) used material from the T7 generation.

3.2 Genetic elements in DNA fragment

A linear DNA fragment PHP20163A containing two novel genes, *gat4601* and *gm-hra* was used in the transformation. The DNA was isolated from the plasmid PHP20163 by digestion with the restriction enzymes *Asc*I and *Not*I and purified using agarose gel electrophoresis. A schematic map of the PHP20163A fragment and PHP20163 plasmid are shown in Figures 3 and 4. A summary of the genes and regulatory elements and their position on plasmid PHP20163 is provided in Table 1.

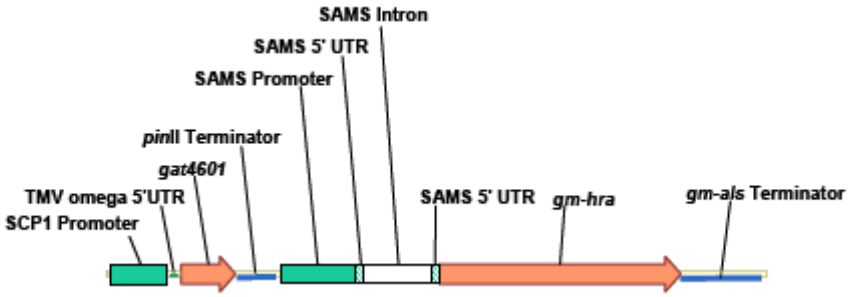

Figure 3: Map of the PHP20163A DNA fragment
Schematic diagram of the fragment used for particle bombardment with the gat4601 gene cassette and gm-hra gene cassette elements indicated. Length of the fragment is 5361 base pairs.

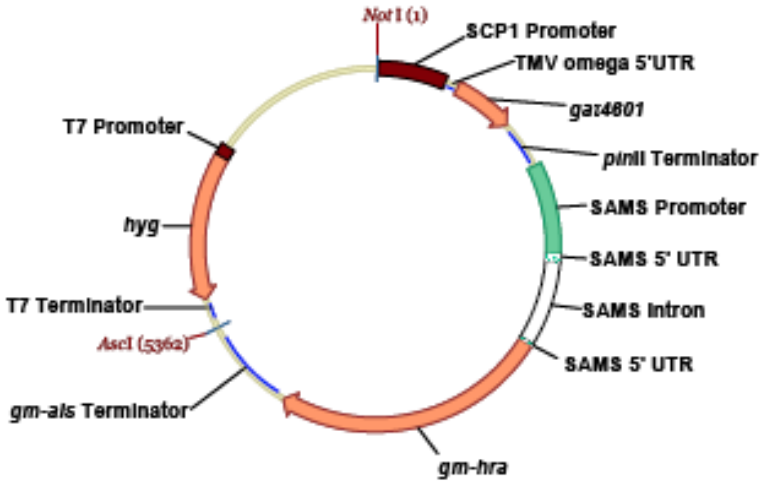

Figure 4: Map of the PHP20163 plasmid
Schematic diagram of plasmid PHP20163 with the location of genes and regulatory elements indicated. Plasmid size is 7953 base pairs. PHP20163A (Figure 3) was isolated from this plasmid by a NotI and AscI double digestion. Enzyme sites for NotI and AscI are indicated at base pair position 1 and 5362, respectively.

The first cassette in the PHP20163A fragment contains *gat4601* a synthetic glyphosate N-acetyltransferase gene encoding the GAT4601 protein. Expression of *gat4601* is controlled by the SCP1 promoter, a synthetic constitutive promoter containing a portion of the CaMV 35S promoter (Odell *et al*., 1985) and the Rsyn7-Syn II Core synthetic consensus promoter (Bowen *et al.*, 2000; Bowen *et al.*, 2003). The omega 5' untranslated leader of the Tobacco Mosaic Virus (TMV omega 5'-UTR) is present to enhance translation (Gallie and Walbot, 1992). The cassette also contains the 3' terminator sequence from the *Solanum tuberosum* proteinase inhibitor II gene (*pin*II terminator) (An *et al*., 1989; Keil *et al*., 1986).

The second cassette in the PHP20163A fragment contains *gm-hra*, a modified version of the

endogenous soybean acetolactate synthase gene (*gm-als*). Expression of the *gm-hra* gene is controlled by the promoter from an S-adenosyl-L-methionine synthetase (SAMS) gene from soybean (An *et al*., 1989; Falco and Li, 2003), consisting of a constitutive promoter and an intron that interrupts the SAMS 5' untranslated region. The native soybean acetolactate synthase terminator (*gm-als* terminator) is used as terminator for the cassette.

3.3 Function and regulation of the novel genes

The *gat4601* gene encodes a synthetic glyphosate N-acetyltransferase that confers tolerance to the herbicide glyphosate by acetylating glyphosate and thereby rendering it non-phytotoxic. The *gm-hra* gene encodes a modified acetolactate synthase that is insensitive to ALS-inhibiting herbicides such as sulfonylureas.

The *gat4601* gene is a synthetic glyphosate N-acetyltransferase gene encoding the GAT4601 protein. The *gat* gene sequences isolated from three strains of *B. licheniformis* were used to produce a novel gene encoding a GAT enzyme with enhanced glyphosate acetylation activity. The relevant gene in these *B. licheniformis* strains was identified using a mass spectrometry method to detect N-acetylglyphosate (Castle *et al*., 2004).

Table 1: Genetic elements in Fragment PHP20163A

Location on Plasmid PHP20163 (base pair position)	Genetic Element	Size (base pairs)	Description
1 to 16	Polylinker region	16	Region required for cloning genetic elements
17 to 502	SCP1 promoter	486	Constitutive synthetic promoter comprising a portion of the cauliflower mosaic virus (CaMV) 35S promoter (Odell *et al*., 1985) and the Rsyn7-Syn II Core consensus promoter (Bowen *et al.,* 2000; Bowen *et al.,* 2003).
503 to 504	Polylinker region	2	Region required for cloning genetic elements
505 to 571	TMV omega 5'-UTR	67	An element derived from the Tobacco Mosaic Virus (TMV) omega 5' untranslated leader that enhances translation (Gallie and Walbot, 1992).
572 to 596	Polylinker region	25	Region required for cloning genetic elements
597 to 1037	*gat4601* gene	441	Synthetic glyphosate N-acetyltransferase (*gat*) gene (Castle *et al*., 2004).
1038 to 1053	Polylinker region	16	Region required for cloning genetic elements
1054 to 1369	*pin*II terminator	316	Terminator region from *Solanum tuberosum* proteinase inhibitor II (*pin*II) gene (An *et al*., 1989; Keil *et al*., 1986).
1370 to 1385	Polylinker region	16	Region required for cloning genetic elements
1386 to 2030	SAMS promoter	645	Promoter of the S-adenosyl-L-methionine synthetase (SAMS) gene from soybean (An *et al*.,

Location on Plasmid PHP20163 (base pair position)	Genetic Element	Size (base pairs)	Description
			1989; Falco and Li, 2003).
2031 to 2089	SAMS 5'-UTR	59	5' untranslated region of the SAMS gene from soybean (Falco and Li, 2003).
2090 to 2680	SAMS intron	591	Intron within the 5'-untranslated region of the SAMS gene from soybean (Falco and Li, 2003).
2681 to 2696	SAMS 5'-UTR	16	5' untranslated region (UTR) of the SAMS gene from soybean (Falco and Li, 2003).
2697 to 4667	*gm-hra* gene	1971	Modified version of the acetolactate synthase gene from soybean with 15 additional nucleotides on the 5' end (2697 to 2711) derived from the *als* 5'UTR and two nucleotide changes within the coding sequence.
4668 to 5318	*gm-als* terminator	651	Native terminator from the soybean acetolactate synthase gene.
5319 to 5361	Polylinker region	43	Region required for cloning genetic elements

The three *gat* genes, representing the GAT enzyme sequence diversity of *B. licheniformis,* were used as parents for fragmentation-based multigene shuffling to create enzymes with higher efficiency and increased specificity for glyphosate. This process recombines genetic diversity from parental genes to create libraries of gene variants that are screened to identify those with improved properties. Further sequence diversity was introduced to enhanced variants using information from natural genetic variability in related hypothetical proteins of the GNAT superfamily of enzymes (see Section 4.1). The GAT4601 protein was identified after seven rounds of shuffling, and the *gat4601* gene was found to generate highly tolerant transgenic soybean.

The herbicide sensitive *gm-als* gene was modified to encode two specific amino acid changes that are known to confer herbicide tolerance to the ALS enzyme, resulting in the herbicide tolerant GM-HRA enzyme.

3.4 Characterisation of the novel genes in soybean 356043

Studies submitted:
Weber N. and Dietrich, N. (2006) Characterization of Soybean Event DP-356043-5: Gene Copy Number and Genetic Stability over Two Generations. Unpublished Pioneer Report PHI-2005-105

Brink, K. and Cogburn, A. (2006) Characterization of Soybean Event DP-356043-5: Detailed Physical Map of Insert Region by Southern Analysis. Unpublished Pioneer Report PHI-2005-106

Weber, N. and Igo, E. (2006) Characterization of Soybean Event DP-356043-5: Genetic Equivalence of the Inserted DNA within a Single Generation. Unpublished Pioneer Report PHI-2005-128

3.4.1 Insert and copy number

Analysis of the DNA introduced into soybean 356043 was undertaken using a range of established molecular techniques. Southern blot analyses were performed on genomic DNA extracted from soybean 356043 and the parent soybean cultivar Jack as a control to assess the following:

(i) number of insertions of the expression cassette;
(ii) number of copies of the expression cassette;
(iii) integrity of the inserted gene expression cassette;
(iv) presence or absence of plasmid backbone; and
(v) stability of the inserted DNA with conventional breeding over several generations.

Genomic DNA from the T4 and T5 generation of soybean 356043 and the parent line Jack was digested with two restriction endonucleases, *Xba*I or *Bgl*II, and subjected to Southern blot analyses. The plasmid PHP20163 was used as a reference substance serving as a positive hybridisation control. The Southern blot hybridisations (Southern, 1975) were performed with probes corresponding to the gat4601 cassette (probes encompassing the SCP1 promoter, *gat4601* coding region and *pin*II terminator), the gm-hra cassette (probes encompassing the SAMS promoter, *gm-hra* coding region and *gm-als* terminator), as indicated schematically in Figure 5.

Two additional probes corresponding to the PHP20163 plasmid backbone (backbone and hygromycin resistance gene) were used to confirm the absence of plasmid sequence from PHP20163 outside of the transformation fragment PHP20163A.

Multiple Southern blot analyses indicate that soybean 356043 is characterised by the presence of one intact copy of the two-gene cassette, inserted at a single locus in the soybean genome. No unexpected hybridisation bands were detected.

These results suggest that soybean 356043 does not contain any additional DNA elements other than those expected from the insertion of the PHP20163A expression cassette. Fragments corresponding to partial genes, regulatory elements or backbone sequences derived from the PHP20163 plasmid were not detected.

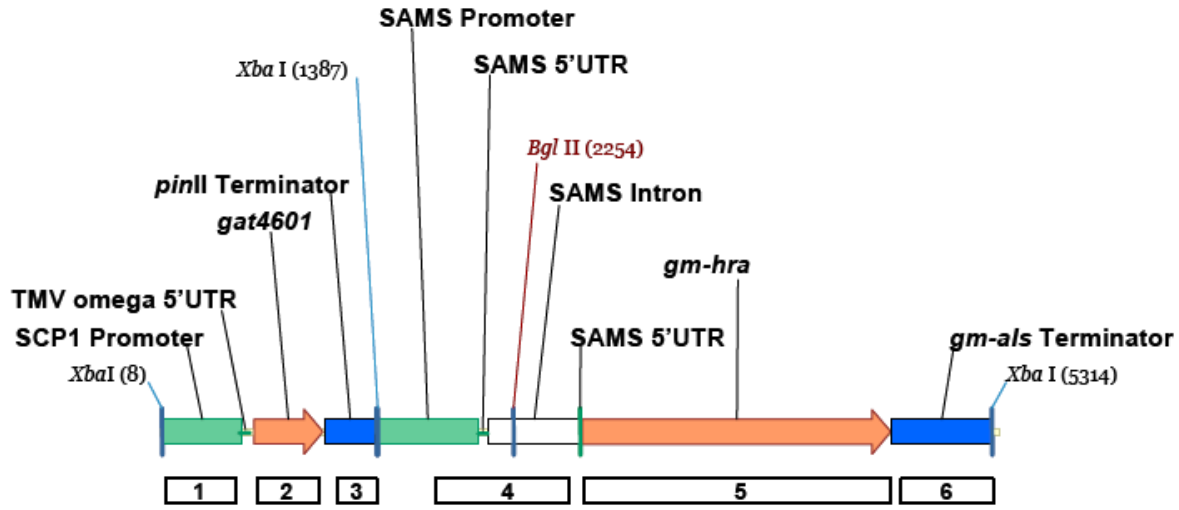

Figure 5: Fragment PHP20163A with genetic probes indicated. Schematic map of fragment PHP20163A indicating location of genetic elements contained in the two gene expression cassettes and base pair positions for BglII *and* XbaI *restriction enzyme sites. Approximate locations of the probes used are shown as numbered boxes below the fragment. The probes are: 1 SCP1 promoter probe; 2 gat4601 probe; 3* pinII *terminator probe; 4 SAMS probe; 5* gm-hra *probe, and; 6* gm-als *terminator probe.*

The Southern blot analyses also indicate that the inserted PHP20163A fragment in soybean 356043 is stably inherited across several generations (T4, T5 and F3 generation, see Figure 2). Based on these results, a map of the inserted DNA is presented below (Figure 6).

3.4.2 PCR and sequence analysis

Studies submitted:
Henderson, N.L. (2006) Insert and Flanking Border Sequence Characterization of Soybean Event DP-356043-5. Unpublished Pioneer Report PHI-2005-115.

The sequence of the DNA insert and flanking genomic border regions was determined to confirm the integrity of the inserted DNA and to characterize the genomic sequence flanking the insertion site present in soybean 356043.

In total, 10849 bp of soybean 356043 genomic sequence was confirmed, comprising 3317 bp of the 5' flanking genomic border sequence, 2170 bp of the 3' flanking genomic border sequence, and 5362 bp of inserted DNA. The insert was found to be intact, and identical to the PHP20163A fragment used for transformation.

The 5' and 3' flanking genomic border regions of soybean 356043 were verified to be soybean genomic DNA by PCR amplification and sequencing of the border regions from both soybean 356043 and control Jack samples. Sequence comparison of the border regions to DNA databases resulted in significant identities to public and proprietary soybean genomic sequences. Such alignment can reveal potential deletion or addition of DNA sequence in comparison to the wild-type genome at the site of the insertion event.

Overall, sequence characterization of the insert and border sequence in soybean 356043 indicated that a single, intact insertion of the PHP20163A fragment is present in the soybean 356043 genome, as shown schematically in Figure 6.

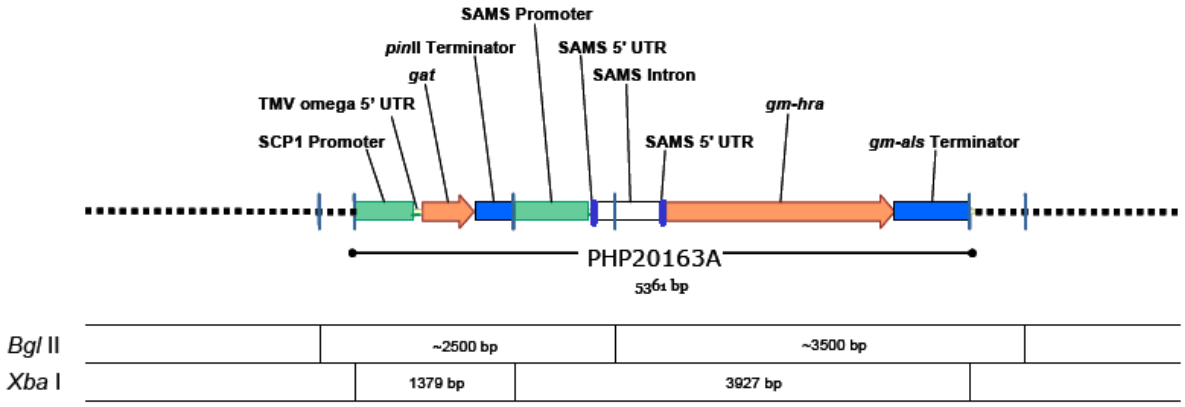

Figure 6: Schematic map of the insertion event in soybean 356043
Schematic map of the transgene insertion in soybean 356043 based on Southern blot analyses. The flanking soybean genome is represented by the horizontal dotted line. Bg*II and* Xba*I restriction enzyme sites are indicated with the sizes of observed fragments on Southern blots shown below the map in base pairs (bp).*

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

Studies submitted:
Cressman, R.F. (2007) Open Reading Frame Analysis of the Insert/Border Junctions in Soybean Event DP-356043-5. Unpublished Pioneer Report PHI-2006-173.

Based on the analysis described in the previous section, it can be concluded that the DNA sequences flanking the soybean 356043 insert are native to the soybean genome. The junction regions between the insert and genomic DNA were further analysed for their potential to be involved in the production of chimeric proteins.

The production of unexpected chimeric proteins as a result of transgene insertion is relevant to food safety. This is because positioning of the insert DNA within the genomic DNA may result in the production of a novel protein with unknown properties from a newly created open reading frame. In cases where there is 100% molecular identity between the transforming DNA and inserted DNA in the plant, and all regulatory elements including termination and polyadenylation signals are intact, production of a chimeric protein is less likely.

In the case of soybean 356043, the transformation event has resulted in the precise insertion of an intact PHP20163A fragment and has not resulted in any additions, deletions, rearrangements or partial insertions of the genes of interest, or regulatory elements, as determined by the Southern blot, PCR analyses and direct DNA sequencing of the entire insert region. Nonetheless, bioinformatics analyses were performed to assess the potential allergenicity and toxicity of any putative polypeptides theoretically encoded by the DNA spanning the junctions between soybean genomic DNA and the 5' and 3' ends of the inserted DNA.

Sequences spanning the 5' and 3' junction regions were translated from stop codon to stop codon in all six reading frames and evaluated for their translation potential, based on the identification of a start codon, necessary for initiating protein translation.

From all twelve reading frames, eight contained stop codon to stop codon translations spanning the transgene junction; only one reading frame spanning a transgene junction with a necessary start codon was identified. This single novel open reading frame (ORF) encodes an 18 residue peptide.

The sequence of this theoretical novel peptide was compared to a publicly available database of 1541 known and putative allergen and celiac protein sequences derived from the FARRP6 data set at the University of Nebraska (www.allergenonline.com). International guidance suggests that the threshold for considering the possibility of IgE cross-reactivity is 35% identity across 80 or more amino acids (Codex, 2004). As the putative novel peptide is significantly less than 80 residues long, no such match was possible. The peptide was also assessed for short polypeptide (eight amino acids) matches using a pair-wise comparison algorithm. Any contiguous identical amino acid matches of 8 amino acids or greater to allergens in the database were identified by generating all possible 8-word peptides from both the query and dataset proteins and evaluating each query 'word' to each dataset 'word' for perfect matches. No eight or greater contiguous identical amino acid matches were observed.

The novel peptide was also assessed for potential toxicity by performing a similarity search using the BLASTP algorithm (Altschul *et al*., 1997) using a cutoff expectation (E) value of 1.0 against the publicly available sequence databases, including the non-redundant dataset from GenBank and RefSeq (GenPept) and the SWISS-PROT and PIR protein sequence databases. The BLASTP program is frequently used for searching protein sequences for sequence similarities. The BLAST algorithm searches for short stretches or domains of sequence similarity by performing local alignments. This detects more similarities than would be found using the entire query sequence length. The E-value reflects the degree of amino acid similarity between a pair of sequences and can be used to evaluate the significance of the alignment. Comparisons between highly homologous proteins yield E-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. A cutoff expectation (E) value of 1.0 was used to identify proteins with even limited similarity. In addition, low complexity filtering was turned off. Even using these relaxed criteria, the BLASTP analysis using the novel peptide sequence as a query against the GenBank non-redundant dataset returned no alignments.

Overall, no biologically relevant structural similarity to allergens or protein toxins was observed. Therefore, even in the unlikely event of low-level transcription occurring across the transgene junction region, and subsequent translation of that transcript, the results of these bioinformatic analyses indicate that any novel peptide produced would be unlikely to be a safety concern.

3.5 Stability of the genetic changes

3.5.1 Segregation data

A number of analyses were performed on plants from five different generations (T1, F2, F3, BC1F2 and C2F2, see Figure 2) to determine the Mendelian heritability and stability of the *gat4601* and *gm-hra* genes in soybean 356043. In each generation tested, plants were expected to segregate 1:2:1 (homozygous positive: hemizygous positive: homozygous negative [null]). The predicted segregation ratio was confirmed using Chi square analysis of inheritance of various traits. In some experiments, positive plants were distinguished from negative plants, and in other experiments, homozygous positive plants were differentiated from hemizygous positive plants.

3:1 Positive (homozygous plus hemizygous): Negative Segregation Ratio

Studies submitted:
Weber, N. and Igo, E. (2006) Characterization of Soybean Event DP-356043-5: Genetic Equivalence of the Inserted DNA within a Single Generation. Unpublished Pioneer Report PHI-2005-128

A number of methods were used to score plants as positive or negative in the T1, F3 and BC1F2 generations. In the T1 generation, PCR analysis identified plants containing the *gat4601* transgene. In the F3 generation, Western analysis identified plants expressing the GAT4601 protein, following by confirmation by Southern analysis that plants carried both the *gat4601* and *gm-hra* genes. In the BC1F2 generation, an ALS seed soak assay (where seeds are soaked in the ALS-inhibiting herbicide chlorsulfuron) identified plants expressing tolerance to ALS-inhibiting herbicides as only those seeds emerge after planting. The segregation ratios observed in these assays are shown in Table 2.

Table 2: Comparison of Observed and Expected 3:1 Segregation Ratios for Soybean 356043

Generation	Method	Observed		Expected		Chi-Square Test
		Positives +/+ or +/-	Negatives -/-	Positives +/+ or +/-	Negatives -/-	P-value
T1	*gat* PCR	59	23	61.5	20.5	0.610
F3	GAT westerns followed by *gat4601* and *gm-hra* Southern analyses	75	15	67.5	22.5	0.088
BC1F2	ALS seed soak					
Elite 7 background		700	222	691.5	230.5	0.543
Elite 8 background		761	273	775.5	258.5	0.315
Elite 9 background		160	54	160.5	53.5	1.000
Elite 10 background		205	79	213	71	0.304

1:2 Homozygous Positive: Hemizygous Positive Segregation Ratio

Two methods were applied to the F2 generation to remove negative plants and to subsequently distinguish positive plants as either homozygous positive or hemizygous positive (Table 3).

Positive F2 plants were selected by spraying emerged plants with the herbicide glyphosate, thus removing homozygous negative (null) plants. The *gm-hra* genotype of these *gat4601* positive F2 plants was determined by scoring the subsequent F3 generation for tolerance to an ALS-inhibiting herbicide. Ten F3 seeds from individual F2 parent plants were rolled into a paper towel wetted with the ALS-inhibiting herbicide chlorsulfuron. An F2 parent plant was scored as homozygous positive if all ten F3 seeds germinated and grew normally.

An F2 parent plant was scored as hemizygous positive if one or more of the germinated F3 seeds produced a seedling with unifoliate leaves (rather than the normal trifoliate) with a wrinkled appearance.

Positive F2 plants were also selected by soaking the F2 seeds in an ALS-inhibiting herbicide prior to planting. Positive F2 plants were subsequently scored as either homozygous positive or hemizygous positive for the *gat4601* gene using a quantitative PCR (qPCR) assay.

Table 3: Comparison of Observed and Expected 1:2 Segregation Ratios for Soybean 356043

Generation	Method	Observed		Expected		Chi-Square Test
		Homozygous +/+	Hemizygous +/-	Homozygous +/+	Hemizygous +/-	P-value
F2						
Elite 1 background	Glyphosate spray to remove nulls, followed by ALS inhibitor rag doll test	16	24	13.3	26.7	0.467
Elite 2 background		32	53	28.3	56.7	0.466
F2						
Elite 3 background	ALS seed soak to remove nulls, followed by qPCR for *gat4601*	110	182	97.3	194.7	0.131
Elite 4 background		124	284	136	272	0.227
Elite 5 background		27	61	29.3	58.7	0.678
Elite 6 background		22	29	17	34	0.181

1:2:1 Homozygous Positive: Hemizygous Positive: Homozygous Negative Segregation Ratios

Segregation analysis of the C2F2 generation using qPCR assays for both the *gat4601* and *gm-hra* genes was used to identify the genotype of all plants as homozygous positive, hemizygous positive or homozygous negative to confirm the expected 1:2:1 segregation ratio (Table 4).

Summary

As the *gat4601* and *gm-hra* gene cassettes are physically linked on the soybean 356043 insert, they are expected to co-segregate; this was confirmed in those experiments where both traits were analysed in the same plants (F3 and C2F2).

The segregation analyses indicate that the observed segregation ratios are not different from the expected values in five separate generations of soybean DP-356043. These results are consistent with a single locus of insertion of the *gat4601* and *gm-hra* genes that segregate according to predicted Mendelian laws. Inheritance of the insert is stable across five generations, including progeny of both self- and cross-pollinations.

Table 4: Comparison of Observed and Expected 1:2:1 Segregation Ratios for Soybean 356043

Generation	Method	Observed			Expected			Chi-Square Test
		Homo-zygous +/+	Hemi-zygous +/-	Homo-zygous -/-	Homo-zygous +/+	Hemi-zygous +/-	Homo-zygous -/-	P-value
C2F2								
Elite 44 background	*gat* and *gm-hra* qPCR	41	76	43	40	80	40	0.799
Elite 45 background		160	294	142	149	298	149	0.550

3.5.2 Stability of the inserted DNA

Studies submitted:
Weber N. and Dietrich, N. (2006) Characterization of Soybean Event DP-356043-5: Gene Copy Number and Genetic Stability over Two Generations. Unpublished Pioneer Report PHI-2005-105

Weber, N. and Igo, E. (2006) Characterization of Soybean Event DP-356043-5: Genetic Equivalence of the Inserted DNA within a Single Generation. Unpublished Pioneer Report PHI-2005-128

The stability of the genetic change in soybean 356043 over multiple generations was demonstrated by Southern blot analyses as described in Section 3.4. Genomic DNA from three generations of 356043 was examined (T4, T5, and F3, see Figure 2). Genomic DNA from the parental line Jack and plasmid PHP20163 were used as negative and positive controls respectively. Probes were used to detect both the *gat4601* and *gm-hra* genes.

The Southern blot analyses confirm that the single, intact PHP20163A fragment inserted into the soybean genome in soybean 356043 is stably inherited across multiple generations.

3.6 Antibiotic resistance genes

No antibiotic resistance marker genes are present in soybean 356043. The molecular characterisation shows that the plasmid sequence from PHP20163 outside of the transformation fragment PHP20163A was not integrated into the soybean genome during transformation. Consequently, the bacterial selectable marker gene, *hyg* (which confers resistance to the antibiotic hygromycin), is not present in soybean 356043. The absence of the bacterial marker gene in the plant was confirmed by Southern blot analysis using a probe specific for the *hyg* gene.

4. CHARACTERISATION OF NOVEL PROTEINS

Soybean 356043 expresses two novel proteins: GAT4601 and GM-HRA.

4.1 Function and phenotypic effects

4.1.1 GAT4601

Expression of the GAT4601 protein in soybean 356043 plants confers tolerance to the broad spectrum herbicide glyphosate. The GAT (glyphosate acetyltransferase) protein detoxifies glyphosate to the non-phytotoxic N-acetylglyphosate, by acetylating the secondary amine of glyphosate using acetyl coenzyme A as an acetyl donor, as shown in Figure 7.

N-acetyl AMPA and AMPA are also formed as minor metabolites during metabolism of glyphosate in soybean 356043 plants.

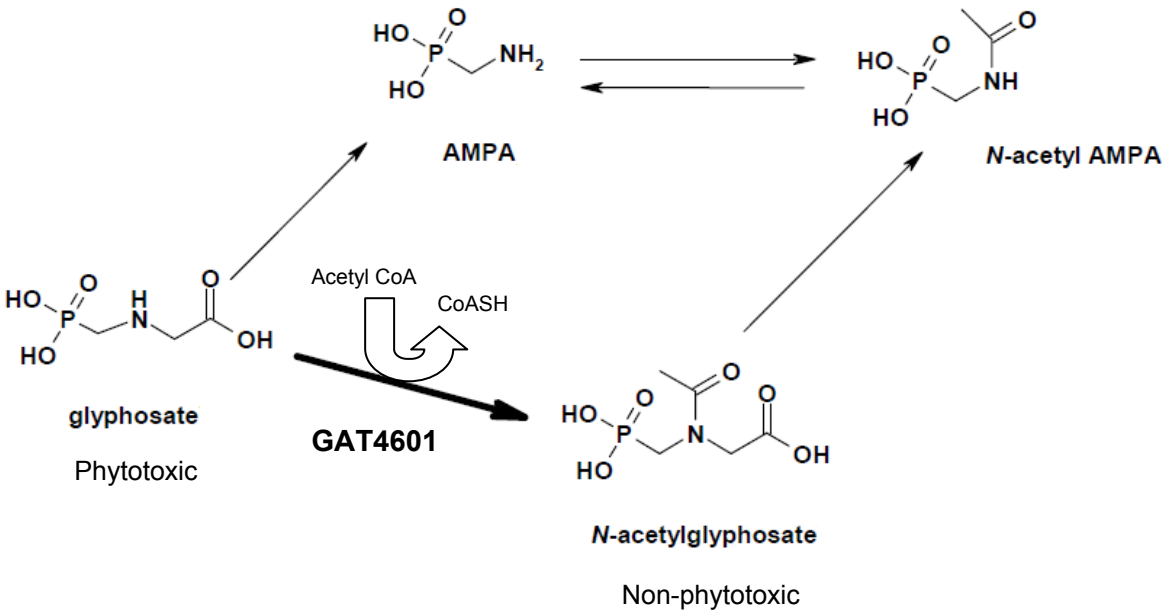

Figure 7: Enzymatic activity of GAT proteins

The GAT protein is a member of the GCN5-related family of N-acetyl transferases, also known as the GNAT superfamily. This large enzyme superfamily contains over 10,000 representatives and is found in plants, animals and microbes. Members of the GNAT superfamily contain a conserved GNAT motif, but are otherwise highly sequence divergent.

GAT4601 is comprised of 146 amino acids and has an approximate molecular weight of 17 kDa.

Derivation of GAT4601

The GAT4601 sequence is the result of a process of selecting optimal GAT enzyme sequences from three strains of *B. licheniformis* that were then further enhanced for glyphosate acetylation activity. This process is described below.

An enzyme with glyphosate acetyltransferase activity was first identified by screening a collection of several hundred bacterial isolates using a mass spectrometry method to detect N-acetylglyphosate. Several strains of *B. licheniformis* exhibited GAT activity reproducibly (Castle *et al*., 2004). Genomic DNA fragments from two of these strains were screened in recombinant *E. coli* to identify the gene encoding GAT activity. Another gene variant was isolated from a third *B. licheniformis* strain.

To generate a GAT enzyme with improved glyphosate acetylation activity, the three *gat* genes were used as the templates for gene shuffling. DNA shuffling recombines genetic diversity, through fragmentation and recombination, to create new gene variants that can be screened for improved properties.

To select for improved GAT activity, libraries of shuffled gene variants were generated, expressed in *E. coli* and screened for glyphosate acetylation. Variants that showed increased accumulation of N-acetylglyphosate were selected for further rounds of shuffling.

In each round, approximately 5,000 gene variants were screened, and typically three to twelve improved variants were used as the parents for the subsequent round of shuffling.

Further sequence diversity was introduced to enhanced variants in later rounds of shuffling using information from natural genetic variability in related hypothetical proteins of the GNAT superfamily of enzymes (Castle *et al*., 2004). .

After seven rounds of gene shuffling, the GAT activity was approximately 2400-fold improved over the native enzymes (Siehl *et al*., 2005).

The GAT4601 protein was identified from this round, and the *gat4601* gene was shown to confer robust glyphosate tolerance when expressed in plants. The GAT4601 protein is 84% identical to each of the three native GAT enzymes from which it was derived (Figure 8).

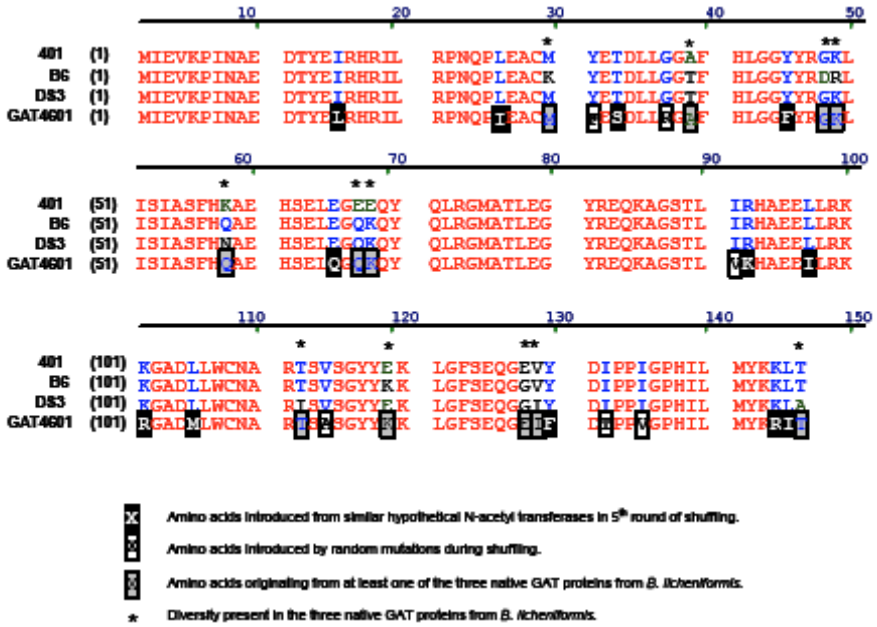

Figure 8: Comparison of Amino Acid Sequence Between GAT4601 and Parental GAT Proteins. GAT4601 pedigree showing diversity introduced from the native gat genes or by PCR using sequence information from B. subtilis *and* B. cereus

4.1.2 GM-HRA

Expression of the GM-HRA protein in soybean 356043 plants confers tolerance to acetolactate synthase (ALS)-inhibiting herbicides such as the sulfonylureas and imidazolinones. The GM-HRA protein is a modified version of the native ALS from soybean normally sensitive to these herbicides. The *gm-hra* gene conferring herbicide tolerance was engineered to encode two specific amino acid changes in the mature ALS protein; P183A and W560L. These two amino acid substitutions in ALS had previously been identified both in a tobacco line selected for tolerance to sulfonylurea herbicides, and as commonly found natural tolerance mutations. The herbicide tolerant GM-HRA also has five additional amino acids at the N-terminus derived from the translation of the normally 5' untranslated region of the soybean *gm-als* gene

ALS is a key enzyme that catalyses the first common step in the biosynthesis of the essential branched-chain amino acids isoleucine, leucine and valine.

ALS enzymes catalyse the conversion of two molecules of pyruvate to acetolactate, leading to the synthesis of leucine and valine, and also catalyse the condensation of pyruvate with 2-ketobutyrate to form 2-acetohydroxybutyrate in the pathway to isoleucine. The involvement

of ALS in these two reactions is shown in Figure 9. The ALS enzyme is also known as acetohydroxyacid synthase (AHAS) or acetolactate pyruvate-lyase.

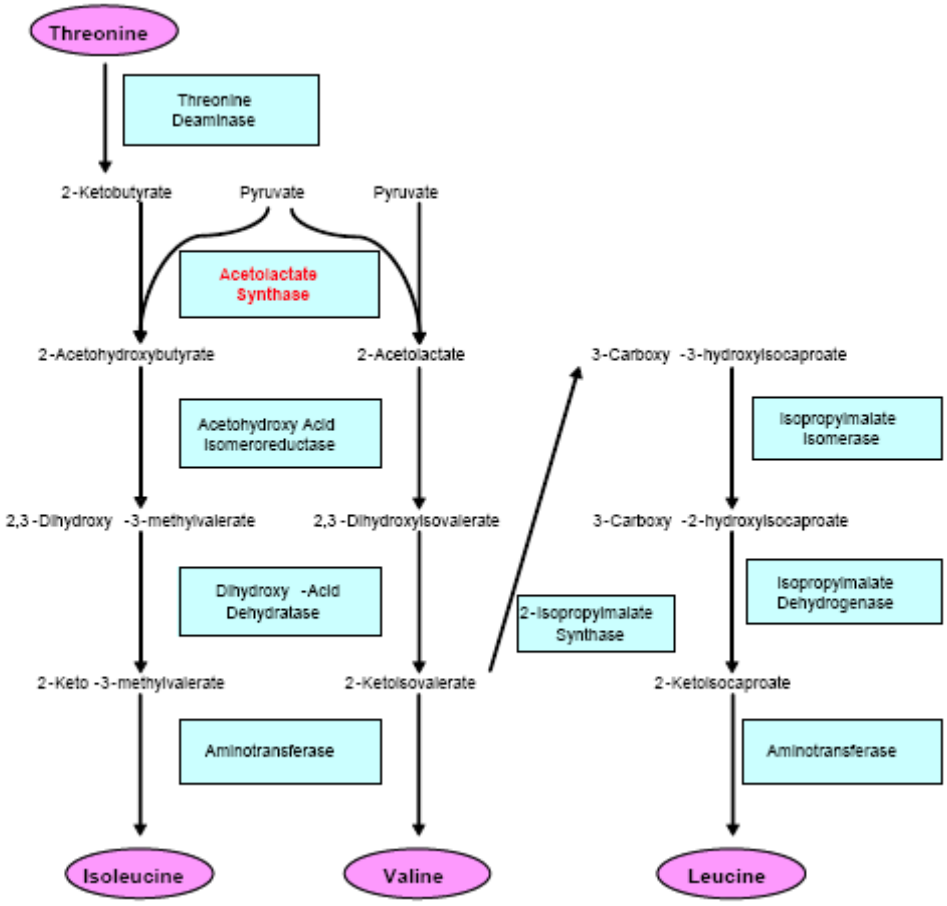

Figure 9: Branched chain amino acid biosynthesis in plants (adapted from (Coruzzi and Last, 2000)

ALS enzymes are widely distributed in nature, having been isolated from bacteria, fungi, algae and plants. ALS enzymes comprise a larger catalytic subunit and a smaller regulatory subunit. The larger catalytic subunit of the soybean ALS was used to derive GM-HRA. The catalytic subunit typically functions as a homotetramer or homodimer, with the active site located at a dimer interface.

Plant *als* genes are encoded in the nucleus, and the ALS enzymes carry an N-terminal transit peptide that targets the enzyme to the chloroplast, the site of branched chain amino acid biosynthesis. The GM-HRA protein is 656 amino acids long with a predicted molecular weight of 71 kDa. Following transport into the chloroplast and cleavage of the transit peptide, the mature protein is 604 amino acids with a predicted molecular weight of 65 kDa.

4.2 Protein Expression Analysis

Study submitted:
Buffington J. (2007) Agronomic Characteristics, Quantitative ELISA, Nutrient Composition Analysis and Magnitude of Glyphosate Residues Analysis of a Soybean Line Containing Event DP-356043-5: U.S. and Canada Locations. Unpublished Pioneer Report PHI-2005-056/000, AU/NZ Final Report (ELISA and Nutrient Composition Analysis Phase Reports).

The levels of the GAT4601 and GM-HRA proteins in seed, forage and root tissue of soybean 356043 and control (Jack) soybean were measured using an enzyme-linked immunosorbent assay (ELISA).

Protein levels were measured in replicated tissue samples collected from T5 generation plants grown at six field locations in North America in 2005. Seed was collected at the R8 stage of development (full maturity), and forage and root tissues were collected at the R3 stage of development (beginning pod) (Gaska, 2006). Three replicated samples per tissue per location were collected for soybean 356043, and one sample per tissue per location for the control Jack soybean.

The results of the ELISA are summarised in Table 5. The mean GAT4601 protein levels in soybean 356043 seed, forage and root were 0.24, 1.6 and 1.6 µg/g of tissue (dry weight), respectively. The mean GM-HRA protein levels in soybean 356043 seed, forage and root were 0.91, 27 and 3.2 µg/g of tissue (dry weight), respectively. Neither GAT4601 nor GM-HRA protein was detected in non-transgenic control (Jack) soybean tissues.

Table 5: Levels of GAT4601 and GM-HRA protein in soybean 356043

Growth Stage/Tissue	**GAT4601 µg/g tissue dry weight**		**GM-HRA µg/g tissue dry weight**	
	Mean ± SD	Range	Mean ± SD	Range
	Soybean 356043			
R8/Seed	0.24 ± 0.072	0.14 – 0.39	0.91 ± 0.17	0.64 – 1.2
R3/Forage	1.6 ± 0.32	1.1 – 2.3	27 ± 8.0	13 – 42
R3/Root	1.6 ± 0.39	1.1 – 2.2	3.2 ± 2.2	0.32 – 7.6
	Control Jack			
R8/Seed	0	0	0	0
R3/Forage	0	0	0	0
R3/Root	0	0	0	0

4.3 Characterisation of the novel proteins in soybean 356043

Studies submitted:
Comstock, B. (2006) Equivalency Assessment of the GAT4601 Protein Derived from a Microbial Expression System with the GAT4601 Protein Derived from Soybeans Containing Event DP-356043-5. Unpublished Pioneer Hi-Bred Report, PHI-2006-014.

Comstock, B. (2006) Equivalency Assessment of the GM-HRA Protein Derived from a Microbial Expression System with the GM-HRA Protein Derived from Soybeans Containing Event DP-356043-5. Unpublished Pioneer Hi-Bred Report, PHI-2006-017.

Studies were conducted to fully characterise the GAT4601 and GM-HRA proteins produced in soybean 356043. A range of analytical techniques was used to determine the identity as well as the physicochemical and functional properties of the plant-produced GAT4601 and GM-HRA proteins isolated from soybean 356043 and to compare them to the reference proteins produced in *E. coli*. These techniques included sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), N-terminal sequencing, and glycosylation analysis.

In addition, characterisation tests were done to confirm the identity and equivalency of GAT4601 and GM-HRA produced in recombinant *E. coli* to those expressed in soybean 356043.

Due to the difficulties in extracting large amounts of individual proteins from plants, larger quantities of the equivalent proteins purified from an *E. coli* expression system were used in some subsequent safety assessment studies.

GAT4601

The predicted GAT4601 protein is 146 amino acids in length and has an approximate molecular weight of 17 kDa.

The GAT4601 protein was extracted from soybean 356043 leaf tissue and partially purified by immunoaffinity chromatography using GAT-specific mouse monoclonal antibodies. The molecular identity and biochemical characteristics of the GAT4601 protein expressed *in planta* were examined using a variety of biochemical techniques.

SDS-PAGE and Western blot analysis of the soybean 356043 produced GAT4601 protein revealed a protein with a molecular weight of approximately 17 kDa. The identity of the plant-produced protein was confirmed by excising the SDS-PAGE band and performing N-terminal sequencing and MALDI-MS. N-terminal sequence analysis indicated that the first 13 amino acids of the GAT4601 protein from plants were consistent with the expected N-terminal sequence for GAT4601. MALDI-MS analysis of the trypsin digested protein from soybean identified five protein fragments that matched the expected mass of the trypsin-digested GAT4601 protein. Two additional peptide matches could be made by allowing for a modification of a cysteine residue by acrylamide (resulting in an observed mass increase of 71.037 Da) and oxidation of a methionine residue (resulting in an observed mass increase of 15.995 Da). The seven identified peptides accounted for 76 of the 146 amino acids, covered 52% of the protein and confirmed the plant-produced GAT4601 as the expected protein.

The isolated plant-produced GAT4601 protein was analysed for post-translational modification through covalently bound carbohydrate moieties. The soybean trypsin inhibitor protein was used as a non-glycosylated negative control and the horseradish peroxidase protein as a positive control. Glycosylation analysis indicated there was no detectable glycosylation of the soybean 356043-derived GAT4601 protein.

A combination of SDS-PAGE and Western blot confirmed the molecular weight and immunoreactivity of plant-derived GAT4601 protein. A combination of N-terminal sequence analysis, MALDI-MS and Western blot confirmed the identity of the plant-produced GAT4601 protein. Glycoprotein staining indicates that the GAT4601 protein is not glycosylated in soybean 356043.

The parallel characterisation of the *E. coli*-produced GAT4601 protein indicated it is equivalent to the plant-produced GAT4601 protein based on comparable electrophoretic mobility, immunoreactivity and absence of detectable glycosylation. Electrospray mass spectroscopy analysis of microbially- produced GAT4601 determined a molecular weight of 16714.72 Da, which is highly consistent with the theoretical value of 16713.9 Da for the expected GAT4601 protein. Based on the similarity of the results from the plant and microbial preparations, the soybean 356043-produced protein is chemically and functionally equivalent to GAT4601 protein expressed in *E. coli*.

GM-HRA

The GM-HRA protein is 656 amino acids long with a predicted molecular weight of 71 kDa. Following transport into the chloroplast and cleavage of the transit peptide, the mature protein is 604 amino acids with a predicted molecular weight of 65 kDa.

The GM-HRA protein was extracted from soybean 356043 leaf tissue and partially purified by immunoaffinity chromatography using GM-HRA-specific mouse monoclonal antibodies. The molecular identity and biochemical characteristics of the GM-HRA protein expressed *in planta* were examined using a variety of biochemical techniques.

SDS-PAGE and Western blot analysis of the soybean 356043 produced GM-HRA protein revealed a protein with the expected molecular weight of approximately 65 kDa. The identity of the plant-produced protein was confirmed by excising the SDS-PAGE band and performing N-terminal sequencing and MALDI-MS. MALDI-MS analysis of the trypsin-digested protein identified 14 peptides that were within 100 ppm of theoretical peptide masses predicted from an *in silico* digestion of the GM-HRA protein. The 14 matched peptides covered 26% or 157 of the 604 amino acids in the GM-HRA amino acid sequence.

The isolated plant-produced GM-HRA protein was analysed for post-translational modification through covalently bound carbohydrate moieties. The soybean trypsin inhibitor protein was used as a non-glycosylated negative control and the horseradish peroxidase protein as a positive control. Glycosylation analysis indicated there was no detectable glycosylation of GM-HRA derived from soybean 356043.

A combination of SDS-PAGE and Western blot confirmed the molecular weight and immunoreactivity of plant-derived GM-HRA protein. A combination of N-terminal sequence analysis, MALDI-MS and Western blot confirmed the identity of the plant-produced GM-HRA protein. Glycoprotein staining indicated that the GM-HRA protein is not glycosylated in soybean 356043.

The parallel characterisation of the *E. coli*-produced GM-HRA protein indicated it is equivalent to the plant-produced GM-HRA protein. The *E. coli*-derived GM-HRA protein was engineered to be the mature form of the protein, excluding the chloroplast transit peptide sequence, which is cleaved following translocation into the chloroplast. The *E. coli*-expressed GM-HRA protein included a peptide tag at the N-terminus to facilitate protein purification. This peptide tag was subsequently cleaved from the GM-HRA protein, resulting in the *E. coli*-derived GM-HRA protein containing one extra N-terminal glycine residue, not found in the plant-derived GM-HRA protein.

MALDI-MS analysis of the trypsin digested protein identified twelve protein fragments that matched the theoretical peptide mass of the trypsin-digested GM-HRA protein. One additional peptide match could be made by allowing for a modification of a cysteine residue by acrylamide (resulting in an observed mass increase of 71.037 Da) and six more matches by allowing for the oxidation of a methionine or tryptophan residue (resulting in an observed mass increase of 15.995 Da). These eighteen identified peptides accounted for 232 of the 605 amino acids and covered 38% of the protein. N-terminal sequence analysis indicated that the first 13 amino acids of the GM-HRA protein were consistent with the expected N-terminal sequence for GM-HRA.

Equivalence of the plant and microbial GM-HRA proteins is based on comparable electrophoretic mobility, immunoreactivity and absence of detectable glycosylation. Also, electrospray mass spectroscopy analysis of microbial GM-HRA determined a molecular weight of 65316 Da, which is highly consistent with the theoretical value of 65312 Da for the mature GM-HRA protein, allowing for the expected extra N-terminal glycine residue. In addition, an ALS activity assay demonstrated that the microbial GM-HRA protein has equivalent ALS biochemical activity both in the presence and absence of an ALS-inhibiting herbicide (chlorosulfuron).

Based on the similarity of the results from the plant and microbial preparations, the soybean 356043-produced protein is chemically and functionally equivalent to GM-HRA protein expressed in *E. coli*.

Conclusion

A large number of studies on the GAT4601 and GM-HRA proteins confirm the identity, and physicochemical and functional properties of the protein, as well as demonstrate equivalence to *E. coli*-produced GAT4601 and GM-HRA proteins respectively. These studies have demonstrated that the two novel proteins expressed in soybean 356043 both conform in size and amino acid sequence to that expected.

The *E. coli*-produced proteins were shown to be equivalent to the plant produced proteins in terms of their size, amino acid sequence and physicochemical properties. In addition, the biochemical activity and herbicide insensitivity of the microbial GM-HRA was demonstrated. The *E. coli*-produced proteins are therefore suitable to use as substitutes for the plant-produced proteins in further safety studies.

4.4 Potential toxicity of novel proteins

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

4.4.1 History of use

GAT4601

The GAT4601 protein sequence was synthesised from the sequence of three GAT enzymes from the common soil bacterium *Bacillus licheniformis*. The GAT4601 amino acid sequence is 84% identical and 94-95% similar to the three GAT proteins from which it is derived. *B. licheniformis* is widespread in the environment and is an approved bacterial source for the production of a number of enzymes used as food processing aids, such as α-amylase, pullulanase (a glucanase) and serine protease. The synthesised GAT4601 enzyme is a member of the GNAT superfamily of N-acetyltransferases, which is present in all organisms, including plants, mammals, fungi, algae and bacteria.

GM-HRA

The GM-HRA protein is derived from the native soybean GM-ALS protein. The herbicide tolerant GM-HRA differs from the native soybean GM-ALS protein at two specific amino acids. ALS proteins are present in many species, including bacteria, fungi, algae and higher plants. Herbicide-tolerant ALS proteins are components of some existing commercial crop varieties, including soybean and canola.

4.4.2 Similarities with known protein toxins

Studies submitted:
Cressman, R.F. (2006) Evaluation of the Amino Acid Sequence Similarity of the GAT4601 Protein to the NCBI Protein Sequence Datasets. Unpublished Pioneer Report PHI-2006-069.

Cressman, R.F. (2006) Evaluation of the Amino Acid Sequence Similarity of the GM-HRA Protein to the NCBI Protein Sequence Datasets. Unpublished Pioneer Report PHI-2006-071.

Bioinformatic analyses are useful for assessing whether the GAT4601 and GM-HRA proteins share any amino acid sequence similarity with known protein toxins.

The GAT4601 (146 amino acids) and GM-HRA (656 amino acids) sequences were compared with the non-redundant ('nr') protein sequence database available from the National Center for Biotechnology Information (NCBI). The Genpept 'nr' dataset incorporates non-redundant entries from all Genbank nucleotide translations along with protein sequences from the SWISS-PROT, PIR, PRF and PDB databases. The NCBI database is a public database containing over 3 million protein sequences, and thus provides a robust source from which to identify any potential protein toxin homologies.

The similarity search used the BLASTP algorithm (Altschul *et al*., 1997), now frequently used for searching for similarities in protein sequences. The BLASTP algorithm searches for short stretches or domains of sequence similarity by performing local alignments. This detects more similarities that would be found using the entire query sequence length.

All database sequences with an Expect value (E-value) of 1 or lower were identified by default by the BLASTP program. The E-value reflects the degree of amino acid similarity between a pair of sequences and can be used to evaluate the significance of the alignment (see Section 3.4.3). Although a statistically significant sequence similarity generally requires a match with an E score of less than 0.01, setting a threshold E score of 1.0 ensures that proteins with even limited similarity will not be excluded.

GAT4601

The BLASTP analysis with the GAT4601 protein sequence returned 192 entries with E-values below 1. The three closest matches were to other synthetic GAT variants produced along with GAT4601 (Castle *et al*., 2004). The next match was to a related acetyltransferase from *B. licheniformis*. Thirty-five matches were to predicted acetyltransferases from other *Bacillus* species. The remaining 153 identified accessions represented known or putative GNAT acetyltransferases from various bacterial, archaebacterial and eukaryotic species. Only six of the alignments were to protein sequences from eukaryotic organisms. Almost all matches with GAT4601 were based on common protein motifs present in the GNAT acetyltransferase superfamily.

None of the protein sequences returned from the BLASTP search with the GAT4601 protein sequence are associated with known toxic or anti-nutritional properties.

GM-HRA

The BLASTP analysis with the GM-HRA protein sequence returned 2000 entries with E-values below 1. Ninety-five of the identified proteins returned E scores of zero and represent closely related ALS proteins from various plant species, including 31 to ALS proteins from various crop plants. Other ALS proteins from various bacterial, archaebacterial and eukaryotic species account for another 922 of the protein matches. The remaining 1078

matches represent a variety of proteins that all possess one or more well characterised, conserved thiamine pyrophosphate (vitamin B1) binding domains.

None of the proteins returned from the BLASTP search with the GM-HRA protein sequence are associated with known toxic or anti-nutritional properties.

4.4.3 Digestibility

See Section 4.5.3.

4.4.4 Thermolability

See Section 4.5.4.

4.4.5 Acute oral toxicity study

Acute oral toxicity studies using mice were conducted to examine the potential toxicity of the GAT4601 and GM-HRA proteins. As it is difficult to extract and purify sufficient quantities of the subject protein from transgenic plants for the acute oral toxicity studies, it has become standard practice to instead use equivalent proteins that have been produced using bacterial expression systems. For these studies, *E. coli*-produced GAT4601 and GM-HRA proteins were used as the test substances. The equivalence of the *E. coli*- and soybean 356043-produced GAT4601 and GM-HRA proteins was established using a range of methods including SDS-PAGE, Western blot analysis, N-terminal sequencing, MALDI-MS, enzyme activity assays (for GM-HRA) and glycosylation analysis (see Section 4.3).

Studies submitted:
Finlay, C. (2006) GAT4601: Acute Oral Toxicity Study in Mice. Unpublished Pioneer Hi-Bred Report PHI-2005-108.

Finlay, C. (2006) GM-HRA: Acute Oral Toxicity Study in Mice. Unpublished Pioneer Hi-Bred Report PHI-2006-008.

GAT4601

Test material	GAT4601 preparation from *E. coli* (84% GAT4601)
Vehicle	Deionised water
Test Species	Crl:CD®-1IKCR)BR mice (five males and five females, fasted)
Dose	2000 mg/kg bw by oral gavage (actual dose 1680 mg/kg)
Control	Bovine serum albumin, 2000 mg/kg, or vehicle alone

Ten mice received a single dose of GAT4601 protein administered by oral gavage at a target dose of 2000 mg/kg. Control groups of ten mice were administered bovine serum albumin at a dose of 2000 mg/kg, or water, once by oral gavage.

Mice were observed for mortality, body weight gain and clinical signs for 14 days. At the end of the study all animals were killed and examined post mortem for organ or tissue damage or dysfunction.

All mice survived through the duration of the study. No clinical signs of systemic toxicity were observed. No gross lesions were present in the mice at necropsy on day 14.

Under the conditions of this study, administration of GAT4601 to male and female mice at a dose of 1680 mg /kg bw produced no test substance-related clinical signs of toxicity, body

weight losses, gross lesions, or mortality. These results support the conclusion that the GAT4601 protein is not acutely toxic.

GM-HRA

Test material	GM-HRA preparation from *E. coli* (29% GM-HRA)
Vehicle	Deionised water
Test Species	Crl:CD®-1IKCR)BR mice (five males and five females, fasted)
Dose	2000 mg/kg bw by oral gavage (actual dose 582 mg/kg)
Control	Bovine serum albumin, 2000 mg/kg, or vehicle alone

Ten mice received a single dose of GM-HRA protein administered by oral gavage at a dose of 582 mg/kg. Control groups of ten mice were administered bovine serum albumin at a dose of 2000 mg/kg, or water, once by oral gavage.

Mice were observed for mortality, body weight gain and clinical signs for 14 days. At the end of the study all animals were killed and examined post mortem for organ or tissue damage or dysfunction. All mice survived through the duration of the study. No clinical signs of systemic toxicity were observed. No gross lesions were present in the mice at necropsy on day 14.

Under the conditions of this study, administration of GM-HRA to male and female mice at a dose of 582 mg /kg bw produced no test substance-related clinical signs of toxicity, body weight losses, gross lesions, or mortality. These results support the conclusion that the GM-HRA protein is not acutely toxic.

4.4.5 Conclusion on potential toxicity

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

4.5 Potential allergenicity of novel proteins

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

4.5.1 Source of protein

GAT4601

The GAT4601 protein in soybean 356043 is derived from *B. licheniformis*, a ubiquitous gram-positive soil bacterium. *B. licheniformis* does not have a history of causing clinical allergy and has not been associated with any adverse allergic effects.

GM-HRA

The GM-HRA protein is derived from the native soybean GM-ALS protein, differing only at two specific amino acids.

Soybean is one of the eight major foods known to cause allergic effects. However, none of the identified soybean allergens is a member of the ALS family (Cordle, 2004). ALS proteins are present in many species, including other plant crop species.

4.5.2 Similarity to known allergens

Studies submitted:
Cressman, R. (2006) Comparison of the Amino Acid Sequence Identity between the GAT4601 Protein and Known Protein Allergens. Unpublished Pioneer Hi-Bred Report PHI-2006-068.

Cressman, R. (2006) Comparison of the Amino Acid Sequence Identity between the GM-HRA Protein and Known Protein Allergens. Unpublished Pioneer Hi-Bred Report PHI-2006-070.

To determine whether the GAT4601 or GM-HRA proteins have significant sequence identity to proteins known or suspected to be allergens, the amino acid sequences of GAT4601 or GM-HRA were compared to the Food Allergy Research and Resource (FARRP, University of Nebraska) Allergen Database (Version 6.0, January 2006) which contains the amino acid sequences of known and putative allergenic proteins (www.allergenonline.com/about.asp) using established criteria (Codex, 2004). Potential similarities between the novel proteins in soybean 356043 and proteins in the allergen database were evaluated using the FASTA sequence alignment tool (Pearson and Lipman, 1988). Alignments were inspected for identities greater than or equal to 35% over 80 or greater residues. The GAT4601 or GM-HRA proteins were also evaluated for any eight or greater contiguous identical amino acid matches to entries in the FARRP Allergen Database. These two approaches aim to identify both short contiguous regions of identity that could potentially correspond to shared IgE binding epitopes, as well as longer stretches of sequence similarity that may infer a potential cross-reactive protein structure.

GAT4601

None of the FASTA alignments between the GAT4601 protein sequence and the sequences in the FARRP Allergen Database exceeded the 35% threshold over 80 or greater amino acids. There were no eight or greater contiguous identical amino acid stretches in common between the GAT4601 protein sequence and any of the protein sequences in the allergen dataset. The results indicate that the GAT4601 protein does not show significant sequence identity with known allergens.

GM-HRA

None of the FASTA alignments between the GM-HRA protein sequence and the sequences in the FARRP Allergen Database exceeded the 35% threshold over 80 or greater amino acids. There were no eight or greater contiguous identical amino acid stretches in common between the GM-HRA protein sequence and any of the protein sequences in the allergen dataset. The results indicate that the GM-HRA protein does not show significant sequence identity with known allergens.

4.5.3 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 (Astwood and Fuchs, 1996; Kimber *et al*., 1999; Metcalfe *et al*., 1996). Therefore a correlation exists between resistance to digestion by pepsin and allergenic potential.

As a consequence, one of the criteria for assessing potential allergenicity is to examine the stability of novel proteins in conditions mimicking human digestion. Proteins that are rapidly degraded in such conditions are considered less likely to be involved in eliciting an allergic response.

A pepsin digestibility assay was conducted to determine the digestive stability of the GAT4601 and GM-HRA proteins. In addition to the pepsin protocol using simulated gastric fluid (SGF), a second digestibility study was done using simulated intestinal fluid (SIF) containing pancreatin, which is a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease. The relevance of the SIF study however is limited because ordinarily an ingested protein would first be exposed to pepsin-mediated hydrolysis in the acidic environment of the stomach before being subject to further digestion in the small intestine.

Simulated gastric fluid study

Studies submitted:
Comstock, B. (2006) Characterization of the *In Vitro* Pepsin Resistance of Glyphosate N-acetyltransferase 4601 Protein (GAT4601). Unpublished Pioneer Hi-Bred Report PHI-2006-028.

Comstock, B. (2006) Characterization of the *In Vitro* Pepsin Resistance of GM-HRA. Unpublished Pioneer Hi-Bred Report PHI-2006-072.

The *in vitro* digestibility of the *E. coli*-derived GAT4601 and GM-HRA proteins in SGF containing pepsin at pH 1.2 was evaluated by SDS-PAGE. Digestibility of the proteins in SGF was measured by incubating samples at 37º for selected times (0.5, 1, 2, 5, 10, 20, 30 and 60 minutes) and subjecting these to SDS-PAGE. Proteins were visualized by staining the gel.

Two control proteins were treated in parallel: bovine serum albumin (BSA) is known to hydrolyse readily in pepsin and served as a positive control; β-lactoglobulin is known to persist in pepsin and was used as a negative control.

Both the GAT4601 and GM-HRA proteins were rapidly hydrolysed in SGF, with no GAT4601 or GM-HRA protein detectable after 30 seconds exposure to SGF. The BSA positive control was also rapidly hydrolysed (< 1 minute) while the β-lactoglobulin negative control persisted for over 60 minutes.

Simulated intestinal fluid study

Studies submitted:
Comstock, B. (2006) Characterization of the *In Vitro* Pancreatin Resistance of Glyphosate N-acetyltransferase 4601 Protein (GAT4601). Unpublished Pioneer Hi-Bred Report PHI-2006-073.

Comstock, B. (2006) Characterization of the *In Vitro* Pancreatin Resistance of GM-HRA. Unpublished Pioneer Hi-Bred Report PHI-2006-074.

The digestibility of *E. coli*-derived GAT4601 and GM-HRA proteins in SIF containing pancreatin was assessed using SDS-PAGE. Digestibility of the proteins in SIF was measured by incubating samples with SIF, for specified time intervals (0, 0.5, 1, 2, 5, 10, 20,

30 and 60 minutes), and analysing by SDS-PAGE with protein staining, and also Western blot analysis.

Two control proteins were treated in parallel: bovine serum albumin (BSA) and β-lactoglobulin. The controls were incubated in SIF for 0, 1 and 60 minutes. Control proteins were detected by protein staining.

No visible GAT4601 protein was present following Western blot analysis at five minutes, indicating that the GAT4601 protein was hydrolysed in less than five minutes in SIF. No visible GM-HRA band was observed following Western blot analysis at one minute.

The β-lactoglobulin positive control was also hydrolysed, with a faint band visible on a protein stained gel after one minute incubation, but no band visible after 60 minutes. The BSA negative control was not completely hydrolysed after 60 minutes.

4.5.4 Thermolability

Studies submitted:
Siehl, D. and Locke, M. (2007) Characterization of the Thermal Stability of Glyphosate Acetyltransferase Enzyme Activity: GAT4601 and GAT4602. Unpublished Pioneer Hi-Bred Report PHI-2006-066/018.

Comstock, B. (2007) Characterization of the Thermal Stability of the GM-HRA Enzyme Activity. Unpublished Pioneer Hi-Bred Report PHI-2006-135.

The heat stability of the microbially produced GAT4601 and GM-HRA proteins was evaluated by examining loss of enzyme activity after exposure to temperatures ranging from 36° to 60º C for 15 minutes.

Following heat treatment, GAT4601 enzyme activity was evaluated using a continuous absorbance spectrophotometric enzyme activity assay using glyphosate as substrate. The results show a loss of 50% enzyme activity when incubated in the range of 49-52 ºC for 15 minutes, and heating at 56º C for 15 minutes completely inhibits GAT4601 enzyme activity.

Heat treated GM-HRA was subjected to the acetolactate synthase activity assay in the presence and absence of a herbicide containing chlorsulfuron. The assay utilises pyruvate as substrate, and indirectly measures abundance of the reaction product, acetolactate, spectrophotometrically. The study indicates that GM-HRA, either in the presence or absence of chlorsulfuron, was inactivated when incubated at 50º C for 15 minutes.

4.5.5 Conclusion on potential allergenicity

A range of information and data have been provided to assess the potential allergenicity of the two novel proteins in soybean 356043. The GAT4601 protein is based on bacterial proteins with a history of human exposure and no association with allergenicity. The GM-HRA protein is a variant of the naturally occurring soybean GM-ALS protein. Although soybean is a major food allergen, the GM-ALS protein is not associated with this allergenicity. Bioinformatic analysis indicates that neither GAT4601 nor GM-HRA shares any significant sequence similarity with known or suspected allergens. Both the GAT4601 and GM-HRA proteins are rapidly degraded in simulated mammalian gastric fluid and both are labile upon heating to temperatures of 56º C and 50º C respectively and above. The weight-of-evidence indicates that the two novel proteins in soybean 356043 are unlikely to be allergenic when present in foods.

4.6 Conclusion from studies on the novel proteins

Soybean 356043 expresses two novel proteins, GAT4601 and GM-HRA, at relatively low levels in the seed. The mean concentration of GAT4601 was 0.24 µg/g dry weight and for GM-HRA, the mean concentration was 0.91 µg/g dry weight.

A large number of studies have been done with the GAT4601 and GM-HRA proteins to confirm their identity and physicochemical and functional properties as well as to examine their potential toxicity and allergenicity.

These studies demonstrate that both proteins conform in size and amino acid sequence to that expected, do not exhibit any post-translational modification including glycosylation, and also demonstrate the expected enzyme activity.

Bioinformatic studies with the GAT4601 and GM-HRA proteins confirmed the absence of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies demonstrated that both proteins would be rapidly degraded in the stomach following ingestion, similar to other dietary proteins. Furthermore, both the GAT4601 and GM-HRA proteins are heat labile. Acute oral toxicity studies in mice with both proteins also confirmed the absence of toxicity in animals. Taken together, these results provide strong evidence that both proteins are unlikely to be toxic or allergenic in humans.

5. COMPOSITIONAL 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 comparator should be the near isogenic parental line grown under identical conditions. In this case, the *gat4601* and *gm-hra* transgenes are the only genetic difference between the two tested varieties. The composition of both herbicide-treated and untreated soybean 356043 was compared to that of the non-transgenic control Jack, the parent soybean line used for the initial transformation. In addition, compositional analyses of four different conventional soybean varieties provide additional comparators to establish reference ranges for compositional constituents. Any statistically significant differences between herbicide-tolerant soybean 356043 and the control Jack can be compared to the reference range to assess whether the differences are likely to be biologically relevant.

5.1 Key components

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 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 nutrients of soybean seed appropriate for a comparative study include the proximates (crude protein, fat, ash, acid detergent fibre and neutral detergent fibre), amino acids and fatty acids. In addition, international guidance suggests levels of the key anti-nutrients phytic acid, trypsin inhibitors, lectins and isoflavones should be determined for new varieties of soybean (OECD, 2001).

Phytic acid chelates mineral nutrients (including calcium, magnesium, potassium, iron and zinc) making them unavailable to monogastric animals, including humans. Protease inhibitors interfere with digestion of protein. Lectins are proteins that bind to carbohydrate-containing molecules. Both protease inhibitors and lectins can inhibit animal growth. The activity of protease inhibitors and lectins is heat-labile and they are inactivated during processing of soybean protein products and soybean meal so that the final edible soybean product should contain minimal levels of these anti-nutrients.

Soybean contains a number of isoflavones reported to possess biochemical activity including estrogenic, anti-estrogenic and hypocholesterolaemic effects that have been implicated in adversely affecting animal reproduction (OECD, 2001). The three basic types of isoflavones in soybeans are daidzein, genistein and glycitein. Soybean also contains two low molecular weight carbohydrates, stachyose and raffinose, that are considered to be anti-nutrients due to the production of intestinal gas and resulting flatulence when they are consumed (OECD, 2001).

5.2 Study design and conduct

Studies submitted:
Buffington J. (2007) Agronomic Characteristics, Quantitative ELISA, Nutrient Composition Analysis and Magnitude of Glyphosate Residues Analysis of a Soybean Line Containing Event DP-356043-5: U.S. and Canada Locations. Unpublished Pioneer Report PHI-2005-056/000, AU/NZ Final Report (ELISA and Nutrient Composition Analysis Phase Reports).

Buffington, J. (2006) Agronomic Characteristics and Nutrient Composition Analysis of Commercial Non-Transgenic Soybean Lines: U.S. and Canada Locations. Unpublished Pioneer Report PHI-2005-055/000.

Compositional analyses were conducted on soybean 356043, both herbicide sprayed and unsprayed, and the nontransgenic parental line Jack, grown at six field locations in soybean growing areas of North America. The integrity of the samples used in the compositional analyses was verified using ELISA testing (sequential 'sandwich') of homogenised tissue from similar forage, root and seed batches from soybean 356043 and control Jack. The results showed no detectable GM-HRA protein present in the Jack samples (see Section 4.2). Therefore, inadvertent cross-contamination of the control samples with GM material had not occurred. Compositional analyses of the soybean forage included proximates (protein, fat, and ash), acid detergent fibre (ADF) and neutral detergent fibre (NDF). Analyses of soybean seed included proximates, ADF, NDF, fatty acids, amino acids, isoflavones, and the anti-nutrients stachyose, raffinose, lectins, phytic acid and trypsin inhibitor, in accordance with the OECD consensus document on compositional considerations for new varieties of soybean (OECD, 2001). In addition, analyses were conducted on mineral and vitamin content of soybean seed.

The T5 generation of soybean 356043 (see Figure 2) and the near-isoline Jack were grown during the 2005 growing season at six field locations in North America representative of the range of environmental conditions in which the soybeans will normally be grown. Sites were located in Wyoming (Illinois), Richland (Iowa), Paynesville (Minnesota), York (Nebraska), Thorndale (Ontario) and Branchton (Ontario).

Plants were grown in a randomised complete block design. In the case of herbicide treatments, plots of soybean 356043 were sprayed with two applications of a herbicide mixture containing the active ingredients glyphosate, clorimuron and thifensulfuron (at 10 to 14 days before flowering and approximately at R2 growth stages) and two applications of herbicide containing glyphosate (at pre-emergence and approximately the R8 growth stage).

For the compositional analyses, three replicate samples (one sample from each of three blocks) per location per treatment were collected for a total of 18 replicates across six locations. Each sample replicate contained approximately 300 grams of seed. Methods of composition analysis were based on internationally recognised procedures (e.g. AOAC International methods) or other published methods.

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

Compositional analyses of four conventional commercial soybean varieties grown in a separate experiment were used to generate a reference range, reflecting the normal variation for the measured analytes. The four reference varieties (92M10, 92B12, 92B63 and 92M72) were grown during the 2005 growing season at six field locations in soybean-growing areas of North America. Sites were located in Bagley (Iowa), York (Nebraska), Glen Allen (Virginia), Germansville (Pennsylvania), Larnet (Kansas) and Branchton (Ontario). The reference varieties were planted, harvested, processed and analysed using the same methods as used for soybean 356043 and the Jack control line.

Data from these commercial varieties were used to calculate population tolerance intervals for each compositional component. Tolerance intervals are expected to contain, with 95% confidence, 99% of the values contained in the population of commercial lines. The population tolerance interval, together with the combined range of values for each analyte available from the published literature (ILSI, 2004; OECD, 2001; Taylor *et al*., 1999), were used to interpret the compositional data for soybean 356043. Mean values for analytes that fell within the tolerance interval and/or the combined literature range were considered to be within the normal variability of commercial soybean varieties.

5.2.1 Statistical analysis

Statistical evaluation of the compositional data compared the seed from the GM soybean population to the non-transgenic control population and tested for statistically significant differences. Data were analysed using a linear mixed model design to account for the design effects of location and blocks within location.

In assessing the significance of any difference between the mean analyte value for soybean 356043 and the non-transgenic control Jack, a P-value of 0.05 was used. This means that approximately 5% of statistically significant differences are expected to occur due to chance alone. In studies comprising multiple comparisons, such as numerous analytes, statistical methods exist to manage the false discovery rate (FDR) by reducing the probability of errors. The Applicant has presented data adjusted according to the method of Benjamini and Hochberg (1995) to account for making multiple comparisons. The method aims to maintain a false positive rate of 5%. The trade-off in using multiple testing correction is that the rate of false negatives (comparisons that are called non-significant when they are) is increased. Data are presented with both FDR-adjusted and non-adjusted P-values. In considering the compositional data provided, this assessment focussed on the non-adjusted P-values.

For those comparisons in which the soybean 356043 test result was statistically different from the control, the test mean 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 commercial soybean varieties.

Statistically significantly different values were also compared to literature ranges (OECD, 2001; Taylor *et al*., 1999) and ranges reported in the International Life Science Institute Crop Composition Database (ILSI 2004).

Although the Applicant provided results for the compositional analyses of forage, the focus of this assessment is necessarily on the food uses of soybean and therefore the forage data are not presented in this report. The results of the comparisons of soybean 356043, sprayed and unsprayed, and the conventional counterpart are presented in Tables 6-10.

5.3 Key nutrients

Proximates

Results of the proximate analysis of soybean 356043 seed are shown in Table 6. Statistically significant differences between unsprayed soybean 356043 and the control Jack (P-value <0.05) were observed for fat and NDF. The mean values for unsprayed soybean 356043 were however within the range of values observed for the non-transgenic control. Additionally, the mean values for all proximates were within the statistical tolerance intervals for commercial soybean varieties and the ranges reported in the literature (ILSI 2004; OECD, 2001).

For sprayed soybean 356043, there was no statistical difference when compared to the non-transgenic control for any analyte measured (P-value >0.05); the mean values were within the statistical tolerance intervals for commercial soybean varieties, and within the range of natural variation reported in the literature(ILSI 2004; OECD, 2001).

Fatty Acids

The levels of a number of fatty acids in soybean seed were measured. No data are shown for ten fatty acids that were below the limit of quantitation. Levels of the quantifiable fatty acids measured for both sprayed and unsprayed soybean 356043 and the control are shown in Table 7.

The mean values for palmitoleic acid (C16:1), stearic acid (C18:0), arachidic acid (C20:0), eicosenoic acid (C20:1) and behenic acid (C22:0) for both unsprayed and sprayed soybean 356043 seed were not statistically significantly different (P-value >0.05) from those of control Jack, and also fell within the statistical tolerance intervals and the published ranges for these fatty acids.

Statistically significant differences (P-value <0.05) between both unsprayed and sprayed soybean 356043 and the control Jack (P-value <0.05) were observed for myristic acid (C14:0), palmitic acid (C16:0), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3). The mean values for myristic acid (C14:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) were, however, within the range of values observed for the non-transgenic control Jack. The mean values for myristic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) were within the statistical tolerance intervals for commercial soybean varieties, and within the range of natural variation reported in the literature (ILSI 2004; OECD, 2001).

The levels of heptadecanoic acid (C17:0) and heptadecenoic acid (C17:1) for both sprayed and unsprayed soybean 356043 are higher than the range of values observed for the non-transgenic control Jack. It is noted that the mean values of these two fatty acids were above the upper limits of the statistical tolerance intervals for commercial soybean varieties, and

also the literature range for soybean varieties (ILSI 2004; OECD, 2001). The Applicant speculates that the observed increase in the levels of C17:0 and C17:1 may result from changes in the availability of the GM-HRA substrates, pyruvate and 2-ketobutyrate. These two compounds are also substrates for the enzyme complex that initiates oil biosynthesis.

Except for C17:0 and C17:1, the fatty acid content of seed from soybean 356043 is comparable to near isogenic and reference soybean lines. The unexpected increase in the levels of C17:0 and C17:1 in soybean 356043 is discussed below in Section 5.5.

Amino acids

Levels of total amino acids in seed from soybean 356043, sprayed and unsprayed, and control line Jack, were analysed and the results are shown in Table 8. As the ALS enzyme is involved in branched chain amino acid biosynthesis, particular attention was paid to levels of leucine, isoleucine and valine.

Total levels of 18 amino acids were measured. As asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, listed aspartate levels include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine. For 17 of the measured amino acids, there were no statistically significant differences in total levels between soybean 356043, sprayed or unsprayed, and the control line Jack. The exception was alanine, with total levels in unsprayed soybean 356043 statistically significantly higher than those of the control line, while levels in sprayed soybean 356043 were not significantly different from the control. The mean values for total alanine were however within the ranges observed for the non-transgenic control line and were also within the statistical tolerance intervals for commercial soybean varieties, and within the range of natural variation reported in the literature (ILSI 2004; OECD, 2001).

Levels of the branched chain amino acids, leucine, isoleucine and valine, are not altered in soybean 356043. Levels of aspartate and glutamate are also not altered in soybean 356043 in comparison to the control line Jack. In summary, total amino acid analysis of soybean seed support the conclusion that soybean 356043, under both sprayed and unsprayed conditions, is comparable to the near isogenic control line and reference soybean varieties.

Minerals

Mineral analysis included calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc (data not shown). Statistically significant differences (P-value <0.05) were noted for the minerals calcium and magnesium for unsprayed soybean 356043, and for magnesium for sprayed soybean 356043. The mean values were within the ranges observed for the non-transgenic control line and were also within the statistical tolerance intervals for commercial soybean varieties, and within the range of natural variation reported in the literature (ILSI 2004; OECD, 2001).

Vitamins

Vitamin analysis included vitamins thiamin (B_1) riboflavin (B_2), folic acid, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and total tocopherols (data not shown). Mean thiamin, folic acid and α-tocopherol values for both unsprayed and sprayed soybean 356043 were statistically significantly different (P-value <0.05) from the control line Jack. For all three vitamins, the mean values were however within the ranges observed for the non-transgenic control line and were also within the statistical tolerance intervals for commercial soybean varieties. The mean values for vitamin B_1 and folic acid also fell within the range of natural variation reported in the literature (ILSI 2004; OECD, 2001).

Although the levels of α-tocopherol in soybean 356043 were higher than the ranges reported in the literature, this was also true for the control line Jack. These differences are not considered to be biologically significant.

Table 6: Proximates in Seed for Unsprayed and Sprayed Soybean 356043

Analyte (%Dry Weight)		Control (Jack)	356043 (Unsprayed‡)	356043 (Sprayed‡)	Tolerance Interval	Combined Literature Ranges[1]
Protein	Mean[2]	40.0	40.2	40.2	29.9 – 48.7	33.2 – 47.4
	Range[3]	38.0 – 41.9	38.7 – 42.1	38.4 – 41.8		
	SD[6]	0.996	1.07	0.953		
	Adjusted P-value[4]		0.844	0.799		
	P-value[5]		0.625	0.622		
Fat	Mean	16.0	15.3	15.6	7.01 – 24.2	8.10 – 23.6
	Range	12.7 – 18.8	11.6 – 18.2	12.4 – 19.0		
	SD	2.02	1.80	2.01		
	Adjusted P-value		0.0779	0.480		
	P-value		0.0125	0.160		
ADF	Mean	17.6	18.6	18.2	8.51 – 22.1	7.81 – 18.6
	Range	7.65 – 28.4	8.95 – 24.2	9.83 – 26.9		
	SD	5.74	4.10	5.45		
	Adjusted P-value		0.672	0.778		
	P-value		0.423	0.583		
NDF	Mean	16.1	18.2	17.1	8.07 – 21.9	8.53 – 21.3
	Range	10.6 – 21.4	12.0 – 24.8	10.4 – 24.1		
	SD	3.06	3.09	3.51		
	Adjusted P-value		0.166	0.641		
	P-value		0.0417	0.324		
Ash	Mean	5.12	5.08	5.16	3.19 – 7.67	3.89 – 6.54
	Range	4.68 – 5.54	4.61 – 5.58	4.63 – 5.85		
	SD	0.295	0.307	0.307		
	Adjusted P-value		0.672	0.718		
	P-value		0.419	0.408		

[1]Combined ranges are taken from published literature for soybeans (OECD, 2001; ILSI 2004; Taylor *et al*., 1999).
[2]Least Square Mean (same as raw mean)
[3]Range denotes the lowest and highest individual values across locations.
[4]False Discovery Rate (FDR) adjusted P-value
[5]Non-adjusted P-value
[6]Standard Deviation

† = Three replicate samples (1 sample per block) per location per treatment were collected for a total of 18 replicates across 6 locations. Each sample replicate contained approximately 300 grams of seed.
‡ = The 356043 soybeans unsprayed and sprayed were statistically analyzed separately using two analysis of variance (ANOVA) comparisons: i) soybean 356043 (unsprayed) with non GM (Jack) control, and ii) soybean 356043 (sprayed) with non GM (Jack) control.

Table 7: Major Fatty Acids in Seed for Unsprayed and Sprayed Soybean 356043

Fatty Acids (% Total)		Control (Jack)	356043 (Unsprayed[2])	356043 (Sprayed[2])	Tolerance Intervals[1]	Combined Lit. Ranges[2]
Myristic acid (C14:0)	Mean[3]	0.0799	0.0764	0.0770	0 – 0.174	0.0710 – 0.238
	Range[4]	0.0727 – 0.0889	0.0696 – 0.0822	0.0729 – 0.0811		
	SD[9]	0.00428	0.00358	0.00284		
	Adjusted P-value[5]		0.0437[7]	0.0908		
	P-value[6]		0.00490	0.0157[8]		
Palmitic acid (C16:0)	Mean	9.98	9.30	9.36	2.93 – 19.6	7.00 – 12.7
	Range	9.59 – 10.2	8.86 – 9.65	9.04 – 9.72		
	SD	0.223	0.23	0.174		
	Adjusted P-value		0.00270[7]	0.00270[7]		
	P-value		0.000100	0.000100[8]		
Palmitoleic acid (C16:1)	Mean	0.0967	0.0938	0.0941	0.0110 – 0.177	0.0860 – 0.159
	Range	0.0879 – 0.106	0.0850 – 0.102	0.0829 – 0.106		
	SD	0.00597	0.00466	0.00599		
	Adjusted P-value		0.277	0.469		
	P-value		0.0959	0.138		
Heptadecanoic acid (C17:0)	Mean	0.110	0.332	0.337	0.0722 – 0.131	0.0850 – 0.138
	Range	0.0896 – 0.130	0.280 – 0.391	0.274 – 0.392		
	SD	0.013	0.0356	0.0368		
	Adjusted P-value		0.00270[7]	0.00270[7]		
	P-value		0.000100	0.000100[8]		
Heptadecenoic acid (C17:1)	Mean	0.0667	0.191	0.189	0.0351 – 0.0732	0.0730 – 0.0870
	Range	0.0571 – 0.0772	0.153 – 0.243	0.155 – 0.231		
	SD	0.00694	0.0279	0.0258		
	Adjusted P-value		0.00270[7]	0.00270[7]		
	P-value		0.000100	0.000100[8]		
Stearic acid (C18:0)	Mean	4.44	4.53	4.51	0.852 – 8.34	2.00 – 5.71
	Range	3.77 – 4.97	4.00 – 5.21	4.13 – 4.98		
	SD	0.33	0.336	0.275		
	Adjusted P-value		0.381	0.557		
	P-value		0.174	0.253		
Oleic acid (C18:1)	Mean	21.1	22.2	21.9	11.3 – 32.6	14.3 – 34.0
	Range	18.4 – 23.6	19.5 – 24.9	18.2 – 24.7		
	SD	1.83	1.94	2.22		
	Adjusted P-value		0.0135[7]	0.0459[7]		
	P-value		0.00100	0.00510[8]		
Linoleic acid (C18:2)	Mean	54.7	53.7	54.0	41.7 – 64.3	48.0 – 60.0
	Range	53.1 – 56.1	52.0 – 55.7	51.9 – 56.1		
	SD	1.04	1.17	1.43		
	Adjusted P-value		0.0135[7]	0.0459[7]		
	P-value		0.000700	0.00460[8]		
Linolenic acid (C18:3)	Mean	8.35	8.54	8.49	1.15 – 14.7	2.00 – 12.5
	Range	6.85 – 10.2	7.01 – 10.4	7.10 – 10.5		
	SD	1.22	1.24	1.27		
	Adjusted P-value		0.0415[7]	0.103		
	P-value		0.00410	0.0199[8]		

Table 7 (continued): Major Fatty Acids in Seed for Unsprayed and Sprayed Soybean 356043

Fatty Acids (% Total)		Control (Jack)	356043 Unsprayed‡	356043 Sprayed‡	Tolerance Intervals[1]	Combined Lit. Ranges[2]
Arachidic acid (C20:0)	Mean	0.341	0.342	0.335	0.103 – 0.619	0 – 1.00
	Range	0.305 – 0.390	0.305 – 0.400	0.310 – 0.381		
	SD	0.0246	0.0262	0.0228		
	Adjusted P-value		0.959	0.717		
	P-value		0.913	0.379		
Eicosenoic acid (C20:1)	Mean	0.154	0.151	0.160	0.0549 – 0.319	0.140 – 0.316
	Range	0.122 – 0.191	0.115 – 0.186	0.127 – 0.195		
	SD	0.0183	0.0165	0.0208		
	Adjusted P-value		0.799	0.476		
	P-value		0.582	0.147		
Behenic acid (C22:0)	Mean	0.346	0.343	0.346	0.188 – 0.458	0.277 – 0.571
	Range	0.322 – 0.383	0.316 – 0.388	0.309 – 0.384		
	SD	0.0172	0.0211	0.0205		
	Adjusted P-value		0.784	0.948		
	P-value		0.561	0.913		

[1]Negative tolerance limits have been set to zero.
[2]Combined ranges are taken from published literature for soybeans (OECD, 2001 ILSI 2004; Taylor *et al*., 1999)
[3]Least Square Mean (also the raw mean).
[4]Range denotes the lowest and highest individual values across locations.
[5]False Discovery Rate (FDR) adjusted P-value
[6]Non-adjusted P-value
[7]Statistically significant difference; adjusted P-value< 0.05
[8]Statistically significant difference, non-adjusted P-value< 0.05
[9]Standard Deviation

† = Three replicate samples (1 sample per block) per location per treatment were collected for a total of 18 replicates across 6 locations. Each sample replicate contained approximately 300 grams of seed.
‡ = The 356043 soybeans unsprayed and sprayed were statistically analyzed separately using two analysis of variance (ANOVA) comparisons: i) soybean 356043 (unsprayed) with non GM (Jack) control, and ii) soybean 356043 (sprayed) with non GM (Jack) control.

Table 8: Total Amino Acids in Seed for Unsprayed and Sprayed Soybean 356043

% Dry Weight Amino Acids		Control (Jack)	356043 Unsprayed‡	356043 Sprayed‡	Tolerance Interval	Combined Ranges[1]
Methionine	Mean[2]	0.709	0.732	0.720	0.488 – 0.852	0.431 – 0.681
	Range[3]	0.664 – 0.745	0.620 – 0.978	0.602 – 0.837		
	SD[6]	0.0261	0.0932	0.0539		
	Adjusted P-value[4]		0.487	0.778		
	P-value[5]		0.228	0.550		
Cystine	Mean	0.640	0.644	0.647	0.378 – 0.869	0.370 – 0.808
	Range	0.574 – 0.699	0.531 – 0.795	0.588 – 0.700		
	SD	0.0329	0.0648	0.0324		
	Adjusted P-value		0.909	0.799		
	P-value		0.797	0.621		
Lysine	Mean	3.07	3.13	3.14	1.98 – 3.10	2.29 – 2.86
	Range	2.79 – 3.38	2.88 – 3.55	2.83 – 3.49		
	SD	0.174	0.177	0.169		
	Adjusted P-value		0.668	0.641		
	P-value		0.396	0.315		
Tryptophan	Mean	0.497	0.492	0.490	0.359 – 0.632	0.356 – 0.540
	Range	0.440 – 0.562	0.416 – 0.546	0.413 – 0.544		
	SD	0.0317	0.0362	0.0331		
	Adjusted P-value		0.863	0.778		
	P-value		0.708	0.556		
Threonine	Mean	1.91	1.94	1.91	1.57 – 2.21	1.25 – 1.89
	Range	1.69 – 2.09	1.76 – 2.09	1.73 – 2.13		
	SD	0.125	0.117	0.122		
	Adjusted P-value		0.580	0.948		
	P-value		0.315	0.896		
Isoleucine	Mean	1.86	1.88	1.87	1.56 – 2.09	1.46 – 2.12
	Range	1.67 – 2.01	1.79 – 1.97	1.67 – 1.96		
	SD	0.0994	0.0598	0.0904		
	Adjusted P-value		0.780	0.948		
	P-value		0.539	0.937		
Histidine	Mean	1.28	1.33	1.30	0.897 – 1.41	0.878 – 1.22
	Range	1.14 – 1.43	1.13 – 1.42	1.09 – 1.47		
	SD	0.086	0.0889	0.0997		
	Adjusted P-value		0.202	0.718		
	P-value		0.0622	0.404		
Valine	Mean	1.95	1.99	1.97	1.58 – 2.18	1.50 – 2.44
	Range	1.76 – 2.11	1.91 – 2.08	1.79 – 2.06		
	SD	0.102	0.0501	0.0825		
	Adjusted P-value		0.356	0.778		
	P-value		0.150	0.505		
Leucine	Mean	3.12	3.16	3.14	2.53 – 3.52	2.20 – 4.00
	Range	2.87 – 3.38	3.05 – 3.35	2.86 – 3.31		
	SD	0.142	0.0853	0.132		
	Adjusted P-value		0.566	0.778		
	P-value		0.285	0.564		

Table 8 (continued): Total Amino Acids in Seed for Unsprayed and Sprayed Soybean 356043

% Dry Weight Amino Acids		Control (Jack)	356043 Unsprayed‡	356043 Sprayed‡	Tolerance Interval	Combined Ranges[1]
Arginine	Mean	2.76	2.83	2.79	2.01 – 3.60	2.29 – 3.36
	Range	2.36 – 3.04	2.64 – 3.12	2.50 – 2.99		
	SD	0.167	0.15	0.125		
	Adjusted P-value		0.176	0.752		
	P-value		0.0499	0.439		
Phenylalanin	Mean	2.10	2.11	2.10	1.74 – 2.43	1.60 – 2.24
	Range	1.86 – 2.30	1.98 – 2.29	1.85 – 2.33		
	SD	0.133	0.0826	0.125		
	Adjusted P-value		0.953	0.948		
	P-value		0.871	0.911		
Glycine	Mean	1.94	1.95	1.91	1.54 – 2.18	1.46 – 2.02
	Range	1.74 – 2.13	1.73 – 2.14	1.73 – 2.16		
	SD	0.146	0.132	0.142		
	Adjusted P-value		0.784	0.557		
	P-value		0.555	0.255		
Alanine	Mean	1.67	1.73	1.71	1.35 – 2.07	1.51 – 1.87
	Range	1.50 – 1.84	1.61 – 1.96	1.61 – 1.91		
	SD	0.0863	0.0903	0.0813		
	Adjusted P-value		0.152	0.480		
	P-value		0.0356	0.157		
Aspartic Acid	Mean	5.23	5.36	5.30	3.67 – 6.33	3.81 – 5.12
	Range	4.57 – 6.09	4.99 – 5.88	4.56 – 5.93		
	SD	0.435	0.271	0.416		
	Adjusted P-value		0.565	0.778		
	P-value		0.272	0.573		
Glutamic Acid	Mean	7.92	8.00	7.92	6.04 – 9.54	5.84 – 8.72
	Range	7.21 – 8.73	7.54 – 8.59	7.32 – 8.72		
	SD	0.417	0.289	0.41		
	Adjusted P-value		0.751	0.995		
	P-value		0.510	0.995		
Proline	Mean	2.55	2.55	2.57	1.85 – 2.70	1.69 – 2.61
	Range	2.26 – 2.75	2.42 – 2.71	2.42 – 2.81		
	SD	0.143	0.0986	0.124		
	Adjusted P-value		0.986	0.825		
	P-value		0.962	0.707		
Serine	Mean	2.24	2.29	2.26	1.85 – 2.71	1.63 – 2.48
	Range	2.07 – 2.47	2.14 – 2.43	2.11 – 2.42		
	SD	0.111	0.094	0.0996		
	Adjusted P-value		0.262	0.778		
	P-value		0.0873	0.537		
Tyrosine	Mean	1.49	1.50	1.49	0.908 – 1.69	1.02 – 1.62
	Range	1.30 – 1.66	1.36 – 1.69	1.32 – 1.77		
	SD	0.113	0.106	0.122		
	Adjusted P-value		0.751	0.948		
	P-value		0.509	0.886		

[1]Combined ranges are taken from published literature for soybeans (OECD, 2001 ILSI 2004; Taylor *et al*., 1999)
[2]Least Square Mean (also the raw mean).
[3]Range denotes the lowest and highest individual values across locations.
[4]False Discovery Rate (FDR) adjusted P-value
[5]Non-adjusted P-value
[6]Standard Deviation
† = Three replicate samples (1 sample per block) per location per treatment were collected for a total of 18 replicates across 6 locations. Each sample replicate contained approximately 300 grams of seed.
‡ = The 356043 soybeans unsprayed and sprayed were statistically analyzed separately using two analysis of variance (ANOVA) comparisons: i) soybean 356043 (unsprayed) with non GM (Jack) control, and ii) soybean 356043 (sprayed) with non GM (Jack) control.

5.4 Anti-nutrients and secondary plant metabolites

The levels of the key anti-nutrients phytic acid, trypsin inhibitors, lectins and isoflavones should be determined for new varieties of soybean (OECD, 2001).

Isoflavones

The three basic types of isoflavones in soybeans are daidzein, genistein and glycitein. Each of these can exist in three conjugate forms: glucoside, acetylglucoside or malonylglucoside. The levels of the 12 forms of isoflavones in soybean 356043 and the control line Jack were analysed. Levels of four of these forms, acetylgenistin, acetyldaidzin, glycitein and acetylglycitin, were below the limit of quantitation. Levels of the remaining isoflavones analysed are shown in Table 9. The mean levels of daidzin (soybean 356043 unsprayed), malonyldaidzin, glycitin and malonylglycitin were statistically significantly different from those of the control line Jack. The mean values were however well within the range of values observed for the non-transgenic control Jack, and were also within the statistical tolerance intervals for commercial soybean varieties. The data for the non-transgenic control Jack indicate that these analytes are typically present over a broad natural range and therefore differences in the mean values are not considered to be biologically significant.

Other Antinutrients

The levels of key antinutrients measured in soybean 356043 seed, both sprayed and unsprayed, and the control line Jack are presented in Table 10. No differences were observed in the levels of stachyose, raffinose, lectins or phytic acid, and the mean values observed in soybean 356043 were within the statistical tolerance interval for commercial varieties of soybean and within the range of natural variation reported in the literature (ILSI 2004; OECD, 2001).

The levels of trypsin inhibitor in soybean 356043 (both sprayed and unsprayed) were statistically significantly different from those in the control line Jack. However, the mean values were within the range observed for the non-transgenic control line and were also within the statistical tolerance intervals for commercial soybean varieties. The mean values were also found within the range of natural variation reported in the literature (ILSI 2004; OECD, 2001). Therefore, the differences in trypsin inhibitor levels are not considered to be biologically significant.

Based on these results, the levels of anti-nutrients and secondary plant metabolites in soybean 356043 are comparable to those found in conventional soybean.

5.5 Compositional differences

The levels of two fatty acids, heptadecanoic acid (C17:0) and heptadecenoic acid (C17:1), are increased in soybean 356043. Together, these two fatty acids constitute around 0.5% of the total fatty acid content in soybean 356043, compared to 0.2% in the control line Jack.

The Applicant suggests this increase may result from shifts in the availability of the GM-HRA enzyme substrates, pyruvate and 2-ketobutyrate. These two compounds are also substrates for the enzyme complex that initiates oil biosynthesis.

The nutritional significance of higher amounts of C17:0 and C17:1 from soybean consumption in terms of food safety was evaluated by considering current sources of dietary exposure and the normal mode of metabolism of 17-carbon fatty acids in humans.

Table 9: Isoflavones in Seed for Unsprayed and Sprayed Soybean 356043

Analyte (ug/g Dry Weight)		Control (Jack)	356043 (Unsprayed‡)	356043 (Sprayed‡)	Tolerance Interval[1]	Combined Literature Range[2]
Genistin	Mean[3]	139	144	148	0 – 402	11.7 – 143
	Range[4]	71.0 – 223	<4.00 – 240	85.2 – 229		
	SD[8]	47	58.2	43.4		
	Adjusted P-value[5]		0.873	0.778		
	P-value[6]		0.733	0.573		
Malonyl-genistin	Mean	1070	1120	1110	0 – 2810	6.0 – 603
	Range	499 – 1560	652 – 1650	617 – 1590		
	SD	344	318	342		
	Adjusted P-value		0.331	0.493		
	P-value		0.128	0.197		
Genistein	Mean	10.4	12.3	13.4	0 – 32.3	0.1 – 22.6
	Range	<4.00 – 20.8	<4.00 – 31.2	<4.00 – 51.2		
	SD	5.07	6.55	11		
	Adjusted P-value		0.566	0.427		
	P-value		0.295	0.116		
Daidzin	Mean	58.6	67.3	63.9	0 – 343	0.7 – 83.6
	Range	34.5 – 86.3	51.4 – 92.3	48.6 – 86.7		
	SD	17	12.6	12.2		
	Adjusted P-value		0.0779	0.386		
	P-value		0.0121	0.0906		
Malonyl-daidzin	Mean	703	790	771	0 – 2880	0.9 – 558
	Range	349 – 977	511 – 1190	503 – 1060		
	SD	188	170	165		
	Adjusted P-value		0.0437[6]	0.103		
	P-value		0.00540	0.0203		
Daidzein	Mean	9.32	9.94	12.0	0 – 47.1	0.1 – 21.2
	Range	<4.00 – 18.3	<4.00 – 24.2	<4.00 – 49.5		
	SD	4.7	5.85	10.2		
	Adjusted P-value		0.863	0.427		
	P-value		0.711	0.110		
Glycitin	Mean	65.7	87.6	93.6	0 – 115	0.6 – 33.5
	Range	28.5 – 132	34.9 – 137	42.2 – 148		
	SD	26.3	30.4	31.9		
	Adjusted P-value		0.141	0.0611		
	P-value		0.0278	0.00830		
Malonyl-glycitin	Mean	189	233	243	0 – 295	0.3 – 71.2
	Range	87.1 – 332	121 – 338	110 – 345		
	SD	59.6	60.3	67.2		
	Adjusted P-value		0.0604	0.0324[7]		
	P-value		0.00820	0.00240		

[1]Negative tolerance limits have been set to zero.
[2]Combined ranges are taken from published literature for soybeans (ILSI 2004; Kim *et al*., 2005; OECD, 2001; Taylor *et al*., 1999)
[3]Least Square Mean (also the raw mean).
[4]Range denotes the lowest and highest individual values across locations.
[5]False Discovery Rate (FDR) adjusted P-value
[6]Non-adjusted P-value
[7]Statistically significant difference; adjusted P-value< 0.05
[8]Standard Deviation

† = Three replicate samples (1 sample per block) per location per treatment were collected for a total of 18 replicates across 6 locations. Each sample replicate contained approximately 300 grams of seed.
‡ = The 356043 soybeans unsprayed and sprayed were statistically analyzed separately using two analysis of variance (ANOVA) comparisons: i) soybean 356043 (unsprayed) with non GM (Jack) control, and ii) soybean 356043 (sprayed) with non GM (Jack) control.

Table 10: Oligosaccharides and Antinutrients in Seed for Unsprayed and Sprayed Soybean 356043

Analyte		Control (Jack)	356043 (Unsprayed‡)	356043 (Sprayed‡)	Tolerance Interval[1]	Combined Ranges[2]
Stachyose (% dry weight)	Mean[3]	3.14	3.12	3.33	2.65 – 4.78	1.21 – 3.50
	Range[4]	2.23 – 3.96	2.27 – 3.89	2.56 – 3.80		
	SD[8]	0.484	0.489	0.327		
	Adjusted P-value[5]		0.935	0.272		
	P-value[6]		0.842	0.0605		
Raffinose (% dry weight)	Mean	0.619	0.637	0.631	0 – 1.99	0.212 – 0.661
	Range	0.344 – 0.986	0.351 – 1.11	0.282 – 0.981		
	SD	0.185	0.207	0.192		
	Adjusted P-value		0.727	0.815		
	P-value		0.476	0.656		
Lectins (hemagglutinating units/mg)	Mean	5.80	5.97	3.85	0 – 11.4	0.105 – 9.04
	Range	1.95 – 12.4	0.615 – 13.7	0.822 – 8.34		
	SD	3.45	4.18	2.21		
	Adjusted P-value		0.958	0.488		
	P-value		0.899	0.187		
Phytic acid (% dry weight)	Mean	1.22	1.20	1.18	0.459 – 1.78	0.634 – 1.96
	Range	0.924 – 1.80	0.830 – 1.57	0.842 – 1.52		
	SD	0.239	0.25	0.181		
	Adjusted P-value		0.863	0.752		
	P-value		0.695	0.455		
Trypsin Inhibitor (trypsin inhibitor units/mg)	Mean	48.8	43.2	44.8	8.71 – 80.4	19.6 – 119
	Range	41.1 – 65.9	31.0 – 65.5	33.7 – 55.3		
	SD	8.45	8.52	6.58		
	Adjusted P-value		0.0135[7]	0.0908		
	P-value		0.000900	0.0157		

[1]Negative tolerance limits have been set to zero.
[2]Combined ranges are taken from published literature for soybeans (ILSI 2004; OECD, 2001; Taylor *et al*., 1999)

[3]Least Square Mean (also the raw mean).
[4]Range denotes the lowest and highest individual values across locations.
[5]False Discovery Rate (FDR) adjusted P-value
[6]Non-adjusted P-value
[7]Statistically significant difference; adjusted P-value< 0.05
[8]Standard Deviation

† = Three replicate samples (1 sample per block) per location per treatment were collected for a total of 18 replicates across 6 locations. Each sample replicate contained approximately 300 grams of seed.
‡ = The 356043 soybeans unsprayed and sprayed were statistically analyzed separately using two analysis of variance (ANOVA) comparisons: i) soybean 356043 (unsprayed) with non GM (Jack) control, and ii) soybean 356043 (sprayed) with non GM (Jack) control.

Metabolism of Fatty Acids

The degradation of fatty acids in humans and animals occurs in the mitochondria of cells in a cyclic process called β-oxidation. In this process, two carbon units are cleaved from the carboxy-terminus as acetyl-CoA subunits. Acetyl-CoA units are able to directly enter the tricarboxylic acid (TCA cycle) to generate usable energy. In the case of fatty acids with an odd number of carbon atoms, such as C17:0 and C17:1, following the stepwise cleavage of acetyl-CoA units, the terminal metabolic substrate is a three carbon substance, propionyl-

CoA. Propionyl-CoA is further metabolised to succinyl-CoA, a four carbon molecule, which then directly enters the TCA cycle.

Fatty acids such as C17:0 and C17:1 are therefore readily metabolised through normal biochemical pathways and used in energy production.

C17:0 and C17:1 in other foods

The levels of C17:0 and C17:1 in soybean 356043 oil and other commonly consumed foods may be compared using the information presented in Table 11. C17:0 and C17:1 are found naturally in many foods, particularly in animal-based foods, at very low levels relative to other fatty acids, generally representing considerably less than 1% of total fat in any food. The contribution of these fatty acids to total fat intake in a typical diet is therefore minor.

The information in Table 11 shows that although C17:0 levels in soybean 356043 are higher than in the control soybean, the level is relatively low compared with that found in a typical serving of a range of commonly consumed foods such as shortbread biscuits, pastry, doughnuts, butter, chocolate, cream cheeses, lamb, beef and egg yolk. For C17:1, the level in soybean 356043 oil is comparable with that found in canola oil, and a typical serve of this oil would contain less C17:1 (15 mg per 10 g serve) than a serve of grilled salmon (96 mg per 120 g serve).

Fats and oils are not the major sources of dietary fat in Australia and New Zealand. According to the National Nutrition Survey conducted in 1995 in Australia, dairy fats and meat and meat products each contributed around 20% of total fat intake, compared to around 10% of total fat from edible oils and margarine (ABS, 1995). In addition, soybean oil is likely to represent a fairly small part of the total Australian and New Zealand edible oils market and therefore minor changes in levels of C17:0 and C17:1 in soybean 356043 oil are unlikely to make any impact at all to overall intakes of these fatty acids.

Table 11: Levels of C17:0 and C17:1 in soybean 356043 and some commonly consumed foods

Food	Average Serve	C17:0 (mg/serve)	C17:1 (mg/serve)
Soybean oil DP-356043-5	10 grams	26	15
Soybean oil Control Jack	10 grams	8	0
Beef, rump steak, lean, grilled	120 grams	60	0
Lamb, loin chop, grilled	120 grams	204	0
Beef, mince, premium, dry fried	120 grams	169	0
Atlantic salmon, grilled	120 grams	60	96
Chocolate, milk	1 row = 28 grams	27	8
Milk, cow, 3.2% fat	1 cup = 250 grams	50	0
Canola oil	10 grams	10	19
Egg yolk, hard boiled	1 large = 14 grams	13	0

5.5.1 Summary and conclusion

The fatty acids C17:0 and C17:1 are normal constituents of the human diet and are readily metabolised. While C17:0 is naturally present in foods such as vegetable oils, butter and meat, and C17:1 is naturally present in foods such as beef, cheese and olive oil, these fatty acids are regarded as minor components as they typically amount to less than 1% of the total fatty acids in any of these foods. In soybean 356043 oil, C17:0 is 0.3% of total fatty acids and C17:1 is 0.2% of total fatty acid content. Taken together, these fatty acids comprise approximately 0.5% of the total fatty acid content of oil derived from soybean 356043, and are therefore also considered as minor components. In terms of the overall diet, the commercialisation of soybean 356043 would have minimal impact on dietary exposures to C17:0 and C17:1 fatty acids. No safety or nutritional issues are raised by their presence at elevated levels relative to the control soybean Jack.

5.6 Characterisation of metabolites

Studies submitted:
Siehl, D. and Locke, M. (2007) Characterization of Substrate Specificity of a Microbial Acetyltransferase Optimized for Activity with Glyphosate: GAT4601 and GAT4602. Unpublished Pioneer Report PHI-2006-066/017.

Buffington, J. (2006) Characterization of Free Amino Acid Content from Soybean Line DP-356043-5: U.S. and Canada Locations. Unpublished Pioneer Report PHI-2006-082 AU/NZ Report.

Buffington, J. (2006) Analysis of N-Acetyl-L-Glutamate and N-Acetyl-L-Aspartate Levels in Soybean Line DP-356043-5: U.S. and Canada Locations. Unpublished Pioneer Report PHI-2006-125 AU/NZ Report.

The expression of the enzyme GAT4601, with optimised acetylation activity, also raises the possibility of new or altered levels of metabolites in soybean 356043 resulting from the acetylation of substrates other than glyphosate. This assessment has therefore considered whether the accumulation of any novel metabolites in soybean 356043 raises any food safety concerns.

Alternative substrates for the GAT4601-catalysed acetylation reaction were investigated. Purified GAT4601 protein was tested for enzyme activity in a survey of substrates including a variety of agrochemicals, antibiotics, and amino acids. The only quantifiable enzyme activity with this range of chemicals was with L-aspartate and L-glutamate, with a catalytic efficiency about 3% of that with glyphosate. The activity of GAT4601 was also tested with a panel of glyphosate analogs (aminophosphonate compounds). The GAT4601 enzyme acetylates D-2-amino-3-phosphonopropionate with a catalytic efficiency about 5% of that with glyphosate.

Despite the low reaction efficiency, the levels of the acetylated amino acids N-acetylglutamate (NAGlu) and N-acetylaspartate (NAAsp) in soybean 356043 were compared to those in the control line under both sprayed and unsprayed conditions. The results are shown in Table 12. The mean values for both NAAsp and NAGlu are significantly higher than those of the control line Jack.

Table 12: N-Acetylaspartate and N-Acetylglutamate in Seed for Unsprayed and Sprayed Soybean 356043*

Analyte (µ g/g dry weight)		Control (Jack)	356043 Soybean (Unsprayed†)	356043 Soybean (Sprayed†)	Tolerance Interval[1]
NAA	Mean[2]	2.52	580	584	0 – 2.27
	Range[3]	1.06-12.6	434-958	449-860	
	SD[8]	2.72	129	103	
	Adjusted P-value[4]		0.0001[6]	0.0001[6]	
	P-value[5]		0.0001[7]	0.0001[7]	
NAG	Mean	1.53	11.6	10.8	0 – 0.00317
	Range	0.876-2.35	4.84-21.2	3.83-16.3	
	SD	0.437	5.18	3.95	
	Adjusted P-value		0.0001[6]	0.0001[6]	
	P-value		0.0001[7]	0.0001[7]	

[1]Negative tolerance limits have been set to zero.
[2]Least Square Mean (also the raw mean).
[3]Range denotes the lowest and highest individual values across locations.
[4]False Discovery Rate (FDR) adjusted P-value
[5]Non-adjusted P-value
[6]Statistically significant difference; FDR adjusted P-value< 0.05
[7]Statistically significant difference, non-adjusted P-value< 0.05
[8]Standard Deviation
*Samples combined from six field sites in soybean growing regions of North America and Canada.

† = Three replicate samples (1 sample per block) per location per treatment were collected for a total of 18 replicates across 6 locations. Each sample replicate contained approximately 300 grams of seed.
‡ = The 356043 soybeans unsprayed and sprayed were statistically analyzed separately using two analysis of variance (ANOVA) comparisons: i) soybean 356043 (unsprayed) with non GM (Jack) control, and ii) soybean 356043 (sprayed) with non GM (Jack) control.

To assess whether the acetylation of aspartate and glutamate in soybean 356043 affected the overall amount or composition of the free amino acid pool, individual free amino acid levels were analysed in soybean 356043 and the control line Jack. The mean level of 21 individual free amino acids in soybean 356043, sprayed and unsprayed, and the control line Jack are presented in Table 13. Statistically significant differences were found in the levels of L-aspartic acid (356043 sprayed), L-proline, L-glycine (356043 sprayed), L-valine, L-ornithine (356043 sprayed) and L-histidine. In all cases, the mean values in soybean 356043 fell within the range of values measured for the control line Jack, and within the statistical tolerance intervals for commercial soybeans.

The overall distribution of amino acids into those incorporated into proteins and free amino acids is similar between soybean 356043 and the control line Jack, with >99% of amino acids incorporated into proteins and <1% in the free amino acid pool (see Table 14). These data indicate that the higher levels of acetylated-aspartate and acetylated-glutamate in soybean 356043 are not affecting the incorporation of amino acids into proteins, or the overall level or composition of the free amino acid pool. The data in Table 14 show that NAAsp and NAGlu account for only a small percentage (0.14%) of the total amino acid content in soybean 356043 seed.

Table 13: Free Amino Acids in Grain for Unsprayed and Sprayed Soybean 356043

Analyte (mg/g dry weight)		Control	356043 (Unsprayed)†	356043 (Sprayed)‡	Tolerance Interval[1]
L-Aspartic Acid	Mean[2]	0.390	0.385	0.335	0-1.07
	Range[3]	0.158-0.780	0.149-0.635	0.172-0.599	
	SD	0.179	0.148	0.133	
	Adjusted P-Value[4]		0.567	0.241	
	P-Value[5]		0.156	0.0265	
L-Threonine	Mean	0.0307	0.0316	0.0329	0-0.0553
	Range	0.0217-0.0495	0.0238-0.0431	0.0255-0.0520	
	SD	0.00798	0.00536	0.00819	
	Adjusted P-Value		0.886	0.571	
	P-Value		0.690	0.347	
L-Serine	Mean	0.0306	0.0333	0.0317	0-0.0808
	Range	0.0217-0.0461	0.0246-0.0456	0.0246-0.0436	
	SD	0.00619	0.00639	0.00549	
	Adjusted P-Value		0.712	0.824	
	P-Value		0.334	0.694	
L-Asparagine	Mean	0.180	0.202	0.183	0-3.11
	Range	0.0837-0.322	0.0831-0.467	0.0647-0.372	
	SD	0.0871	0.0903	0.0846	
	Adjusted P-Value		0.752	0.911	
	P-Value		0.383	0.893	
L-Glutamate	Mean	0.200	0.216	0.241	0-0.486
	Range	0.136-0.336	0.143-0.414	0.137-0.379	
	SD	0.0585	0.0704	0.0643	
	Adjusted P-Value		0.567	0.480	
	P-Value		0.133	0.198	
L-Glutamine	Mean	0.00940	0.0118	0.0153	0-0.0724
	Range	0.000-0.0364	0.000-0.0448	0.000-0.0701	
	SD	0.0107	0.011	0.0191	
	Adjusted P-Value		0.841	0.349	
	P-Value		0.490	0.110	
L-Proline	Mean	0.0330	0.0407	0.0408	0.00258-0.0762
	Range	0.0225-0.0486	0.0277-0.0662	0.0284-0.0627	
	SD	0.00656	0.0104	0.00947	
	Adjusted P-Value		0.0765	0.0663	
	P-Value		0.00280	0.00260	
L-Glycine	Mean	0.0249	0.0272	0.0283	0-0.0546
	Range	0.0212-0.0331	0.0203-0.0382	0.0190-0.0399	
	SD	0.00311	0.00503	0.00566	
	Adjusted P-Value		0.567	0.282	
	P-Value		0.151	0.0422	
L-Alanine	Mean	0.08 16	0.0819	0.0862	0-0.301
	Range	0.0579-0.152	0.0515-0.155	0.0448-0.152	
	SD	0.0239	0.0286	0.0313	
	Adjusted P-Value		0.967	0.649	
	P-Value		0.967	0.458	
L-Valine	Mean	0.169	0.190	0.188	0.0919-0.275
	Range	0.133-0.213	0.159-0.226	0.143-0.218	
	SD	0.0225	0.0206	0.0213	
	Adjusted P-Value		0.112	0.152	
	P-Value		0.00650	0.0119	
L-Methionine	Mean	0.0174	0.0168	0.0168	0-0.0891
	Range	0.00887-0.0299	0.00964-0.0249	0.00942-0.0325	
	SD	0.0052	0.0052	0.00582	
	Adjusted P-Value		0.886	0.831	
	P-Value		0.698	0.717	
L-Isoleucine	Mean	0.0249	0.0271	0.0294	0-0.108
	Range	0.0156-0.0393	0.0166-0.0521	0.0159-0.0907	
	SD	0.00598	0.00919	0.017	
	Adjusted P-Value		0.841	0.484	
	P-Value		0.538	0.228	

Table 13 (Continued): Free Amino Acids in Grain for Unsprayed and Sprayed Soybean 356043

Analyte (mg/g dry weight)		Control	356043 (Unsprayed)†	356043 (Sprayed)‡	Tolerance Interval[1]
L-Leucine	Mean	0.0223	0.0289	0.0284	0-0.117
	Range	0.0106-0.0487	0.0120-0.0779	0.0113-0.0782	
	SD	0.0107	0.02	0.0186	
	Adjusted P-Value		0.525	0.349	
	P-Value		0.0742	0.0927	
L-Tyrosine	Mean	0.0296	0.0285	0.0363	0-0.0526
	Range	0.0187-0.0487	0.0142-0.0484	0.0154-0.135	
	SD	0.00742	0.0104	0.0275	
	Adjusted P-Value		0.941	0.484	
	P-Value		0.842	0.237	
L-Phenylalanine	Mean	0.0416	0.0413	0.0489	0.0236-0.0900
	Range	0.0263-0.0747	0.0206-0.0799	0.0205-0.156	
	SD	0.0133	0.0161	0.0308	
	Adjusted P-Value		0.967	0.349	
	P-Value		0.951	0.123	
γ-Amino-n-Butyric Acid†	Mean	0.880	0.0904	0.107	0-0.243
	Range	0.0497-0.148	0.0455-0.158	0.0518-0.379	
	SD	0.0306	0.0295	0.0737	
	Adjusted P-Value		0.959	0.495	
	P-Value		0.884	0.259	
Ethanolamine†	Mean	0.0880	0.0732	0.0735	0-0.267
	Range	0.0188-0.206	0.0127-0.196	0.00719-0.200	
	SD	0.0715	0.0729	0.0769	
	Adjusted P-Value		0.525	0.349	
	P-Value		0.0918	0.0959	
Ammonia†	Mean	0.0200	0.0202	0.0204	0.000726-0.0343
	Range	0.0147-0.0284	0.0147-0.0286	0.00963-0.0371	
	SD	0.00442	0.00329	0.00623	
	Adjusted P-Value		0.967	0.845	
	P-Value		0.913	0.746	
L-Ornithine†	Mean	0.00210	0.00329	0.00355	NC[6]
	Range	0.000916-0.00533	0.00159-0.00701	0.00124-0.0128	
	SD	0.00115	0.00176	0.00275	
	Adjusted P-Value		0.525	0.241	
	P-Value		0.0620	0.0283	
L-Tryptophan	Mean	0.225	0.201	0.190	0-1.80
	Range	0.0744-0.454	0.0770-0.318	0.773-0.266	
	SD	0.11	0.0779	0.0495	
	Adjusted P-Value		0.791	0.495	
	P-Value		0.430	0.262	
L-Lysine	Mean	0.0398	0.0427	0.0453	0.00838-0.0654
	Range	0.0301-0.0498	0.0303-0.0520	0.0322-0.0766	
	SD	0.0049	0.00535	0.0105	
	Adjusted P-Value		0.683	0.346	
	P-Value		0.295	0.0610	
L-Histidine	Mean	0.0532	0.0698	0.0689	0-0.447
	Range	0.0209-0.108	0.0256-0.161	0.0239-0.165	
	SD	0.0274	0.0355	0.0378	
	Adjusted P-Value		0.432	0.282	
	P-Value		0.0339	0.0443	
L-Arginine	Mean	0.537	0.646	0.552	0-2.19
	Range	0.297-1.24	0.280-1.59	0.283-1.27	
	SD	0.311	0.335	0.234	
	Adjusted P-Value		0.525	0.880	
	P-Value		0.104	0.811	
Total Free Amino Acids	Mean	2.34	2.48	2.38	0-8.75
	Range	1.76-3.74	1.80-4.02	1.92-3.44	
	SD	0.556	0.562	0.446	
	Adjusted P-Value		0.712	0.880	
	P-Value		0.335	0.795	

Table 13 (continued): Free Amino Acids in Seed for Unsprayed and Sprayed Soybean 356043

[1]Negative tolerance limits have been set to zero.
[2]Least Square Mean (also the raw mean).
[3]Range denotes the lowest and highest individual values across locations.
[4]False Discovery Rate (FDR) adjusted P-value
[5]Non-adjusted P-value
[6]A tolerance interval could not be calculated because there was no variation in the sample replicates
†Ammonia and ethanolamine are not amino acids but are typically measured as part of a free amino acid analysis. γ-amino-n-butyric acid and ornithine are amino acids but are not incorporated into proteins. All four compounds are included in the total free amino acid calculation.

† = Three replicate samples (1 sample per block) per location per treatment were collected for a total of 18 replicates across 6 locations. Each sample replicate contained approximately 300 grams of seed.
‡ = The 356043 soybeans unsprayed and sprayed were statistically analyzed separately using two analysis of variance (ANOVA) comparisons: i) soybean 356043 (unsprayed) with non GM (Jack) control, and ii) soybean 356043 (sprayed) with non GM (Jack) control.

Table 14: Distribution of Amino Acids in Seed for Soybean 356043

	mg/g dry weight	Control (Jack)	356043 (Unsprayed‡)	356043 (Sprayed‡)
Total amino acids[1]	Mean (range)	429.36 (384.68 – 474.16)	436.18 (404.97 – 475.89)	432.37 (389.93-475.41)
	% of total amino acids	(100%)	(100%)	(100%)
Free amino acids	Mean (range)	2.340 (1.760 – 3.740)	2.480 (1.800 – 4.020)	2.380 (1.920 – 3.440)
	% of total amino acids	(0.54%)	(0.57%)	(0.55%)
Acetylated amino acids NAA + NAG	Mean (range)	0.00405 (0.00194 – 0.01495)	0.592 (0.439 – 0.979)	0.595 (0.453-0.876)
	% of total amino acids	(0.0009%)	(0.14%)	(0.14%)
Incorporated amino acids (by subtraction[2])	Mean	427.04	433.08	429.39
	% of total amino acids	(99.46%)	(99.29%)	(99.31%)

[1]Individual amino acids from Table 13 (% dry weight of tissue) were totalled and converted to mg/g to obtain total amino acid weight.
[2]The amount of incorporated amino acids was calculated by subtracting total free amino acids and acetylated amino acids from the total amino acid amount.

‡ = The 356043 soybeans unsprayed and sprayed were statistically analyzed separately using two analysis of variance (ANOVA) comparisons: i) soybean 356043 (unsprayed) with non GM (Jack) control, and ii) soybean 356043 (sprayed) with non GM (Jack) control.

5.6.1 Safety of acetylated amino acids NAGlu and NAAsp

As the levels of NAGlu and NAAsp are increased in soybean 356043, the available data on the metabolism of acetylated amino acids has been reviewed, together with acute and short term (28 days) toxicity studies.

5.6.1.1 Biochemistry of NAGlu and NAAsp

N-terminal acetylation is a common post-translational modification of cytosolic proteins (Polevoda and Sherman, 2002). It has been suggested that the biological role of acetylation of N-terminal amino acids of cytosolic proteins may be protection from proteolysis by intracellular aminopeptidases (Berger *et al*., 1981; Brown, 1979) and also possibly regulation of a wide range of cellular processes (Polevoda and Sherman, 2002). A number of acylases (enzymes responsible for deacetylation of N-acetylated amino acids) have been described, with acylation reported in a number of mammalian organs including kidney and liver (Endo, 1980; Gade and Brown, 1981; Kaul *et al*., 1993).

In prokaryotes, lower eukaryotes and plants, NAGlu is the first intermediate in the biosynthesis of arginine. In mammalian liver, NAGlu is an essential cofactor of mitochondrial carbamylphosphate synthetase I, the first enzyme of the urea cycle (Caldovic and Tuchman, 2003). NAGlu is synthesised endogenously from acetyl-CoA and glutamate by the mitochondrial enzyme, N-acetylglutamate synthetase, present in a variety of organs, including liver, intestine and lung (Caldovic and Tuchman, 2003).

NAAsp has also been shown to have an important role in the development of the central nervous system by processes ranging from postnatal myelination of nerve fibres to neuronal osmoregulation and brain nitrogen balance (Moffett *et al*., 2007). It has been shown to be synthesized and stored in neurons where its concentration is one of the highest of all free amino acids (Baslow, 2003) with levels as high as 12 mM being reported in certain parts of the brain (Pan and Takahashi, 2005).

5.6.1.1 Metabolism

Aminoacylases catalyse the conversion of N-acyl α-amino acids to amino acids and fatty acid residues. Several aminoacylases have so far been described. These are acylase 1 (EC 3.5.1.14; N-acylamino acid amidohydrolase), aspartoacylase or acylcylase II (EC 3.5.1.15); N-acyl –L-aspartate amidohydrolase), acyllysine deacylase (EC 3.1.5.17; N-acyl-L-lysine amidohydrolase) and acylase III, which preferentially catalyses the N-deacetylation of N-acyl aromatic amino acids (Birnbaum *et al*., 1952).

In humans mutations in the genes which encode for acylase I (*acy1*) and acylase II (*acy2)* are rare but have been described (Sass et al, 2006, Kaul et al, 1993). In the case of acylase I deficiency, the clinical indicators are delays in psychomotor activity coupled with increased urinary excretion of N-acetyl amino acids, including methionine, glutamic acid, alanine, leucine, glycine, valine and isoleucine. Deficiency in acylase II results in Canavan disease (CD), a fatal neurodegenerative disease (Kaul et al, 1993). Diagnosis is based on an unusually high excretion of NAAsp.

The high concentrations of NAAsp *per se* do not appear to mediate the pathological characteristics of CD, rather it is a lack of deacetylation of NAAsp that results in a deficiency of acetyl groups which in turn leads to spongy degeneration of the brain and eventually death in CD sufferers (Hershfield *et al*., 2006; Madhavarao *et al*., 2005; Moffett *et al*., 2007).

Human sequences of the *acy2* gene cross-hybridize with genomic DNA from yeast, chicken, rabbit, cow, dog, mouse, rat and monkey indicating evolutionary conservation (Kaul *et al*., 1994). The protein sequence of ACY1 is also evolutionarily conserved and, for example human ACY1 shares similarity with ACY1 from fish, frog, mouse and rat (Sass *et al*., 2006). Given the highly conserved nature of the ACY1 and ACY2 enzymes, their activity would be similar across mammalian systems in general.

5.6.1.2 Toxicity studies

Published studies:
Delaney, B., Shen, Z.A., Powley, C.R., Gannon, S., Munley, S.A., Maxwell, C. and Barett, J.F. Jr. (2008). Acute and repeated dose oral toxicity of N-acetyl-L-aspartic acid in Sprague-Dawley rats. *Food Chem.Toxicol*. **46**:2023-2034.

Karaman, S., Myhre, A., Donner, E.M., Munley, S.M. and Delaney, B. (2009). Mutagenicity Studies with N-acetyl-L-aspartic acid. *Food Chem.Toxicol*. **47**(**8**):1936-1940.

Delaney *et al.*

The acute oral toxicity of NAAsp in SD rats was tested at a limit gavage dose of 2000 mg/kg bw. The animals were observed on multiple occasions for clinical signs in the first 4 h after dosing and daily thereafter. Observations for mortality and signs of illness, injury or abnormal behaviour were conducted twice daily. All animals were killed after 14 days and complete gross pathology examinations were performed. The results showed that all animals gained weight over the course of the study and no adverse clinical signs were recorded. No gross lesions were found on any organs at necropsy. The authors concluded that NAAsp was not acutely toxic in rats at a limit dose of 2000 mg/kg bw.

In a separate study, NAAsp was fed to rats (10/sex/group) in their diet for 28 days, at concentrations of 10, 100 or 1000 mg/kg bw/day for 14 consecutive days and 100, 500 and 1000 mg/kg bw/day for another 14 days. Using analytically measured NAAsp and feed consumption values, the amount of NAAsp consumed daily by each of the dose groups was determined between days 15-28 of the study. Based on these calculations, the actual consumed doses in male rats were 89, 439 and 852 mg/kg bw/day, and 95, 501 and 890 mg /kg bw/day for female rats.

Clinical signs and the general appearance and behaviour of the rats were monitored at least twice daily. Detailed clinical observations were recorded on days 8, 15 and 22. These included conducting a functional observational battery and monitoring motor activity. Ophthalmology examinations were performed on all animals prior to dosing and at the end of the study. Body weights and feed consumption were measured at regular intervals up to day 29 (prior to sacrifice). At necropsy, a suite of toxicological parameters including haematology, coagulation, serum chemistry and anatomical pathology were investigated.

There were no adverse clinical effects observed in any of the dosage groups, and all rats survived until the end of the study. There were no biologically significant differences between groups in haematology or clinical chemistry parameters that could be related to treatment. No differences in terminal body weights or organ-to-body weight ratios were observed in any of the treatment groups. There were no microscopic changes observed in any of the organs and tissues evaluated that would indicate any systemic toxicity as a result of the dietary exposure to the test substance. Similarly, there were no adverse neurobehavioural or histopathological changes observed in male or female rats. Based on the findings in this study, the NOAEL for *N*-acetyl-L-aspartate in rats to be 1000 mg/kg bw, the highest tested dose.

Karaman *et al*.

The potential mutagenicity of NAAsp was assessed using standard OECD (OECD, 1998a; OECD, 1998b) guidelines for the *in vitro* bacterial reverse mutation (ie. Ames) assay, and the *in vivo* mouse bone marrow micronucleus assay. In these systems, L-aspartic acid (Asp) as the constituent amino acid was used for comparison and for control data. No evidence of mutagenicity was observed in either test system with either NAAsp or Asp.

5.6.1.3 NAGlu and NAAsp concentrations in foods

NAAsp and NAGlu occur naturally in foods as minor constituents relative to other amino acids. A small study measuring the levels of NAAsp and NAGlu in a variety of foods was provided by the Applicant. These data identified that NAAsp was present in significant amounts in foods such as autolysed yeast (12.6 µg/g), chicken bouillon-vegan (12.1 µg/g), eggs (1.4 µg/g), ground turkey (4.0 µg/g), ground chicken (1.5 µg/g) and ground beef (1.1 µg/g). NAGlu was present in autolysed yeast (159.8 µg/g), ground beef (1.5 µg/g), ground turkey (0.8 µg/g) and dried egg powder (0.7 µg/g).

More comprehensive information, documenting and quantifying the levels in a wide range of foods, has only recently become available.

Published study:
Hession, A.O., Esrey, E.G., Croes, R.A. and Maxwell, C.A. (2008). N-Acetylglutamate and N-Acetylaspartate in Soybeans (*Glycine max*. L.), Maize (*Zea* maize L.), and Other Foodstuffs. *J. Agric. Food Chem*. **56**:9121-9126.

A sensitive method using ultra-performance liquid chromatography – electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) was developed for extracting and quantifying NAAsp and NAGlu naturally present in a range of foods including fruits, vegetables, meats, fish, grains, milk, coffee, tea and cocoa. In some cases, the foods were freeze-dried in order to concentrate the NAAsp and NAGlu to improve chances of detection. A shortened list of foodstuffs (selected for the highest levels of NAAsp or NAGlu) is presented in Table 15. For comparative purposes, the levels in soybean 356043 and the control Jack are also presented from data provided by the Applicant (using the triple quad mass spectrometer method of analysis).

These results demonstrate that NAGlu and NAAsp are normal components of human diets as they are present in a wide range of common foods. The highest concentration of NAAsp was found in roasted coffee beans (62.8 µg/g), approximately 10x lower than the concentration in seeds of soybean 356043, and the highest concentration of NAGlu occurred in cocoa powder (62.3 µg/g), which was approximately 6x higher than in soybean 356043 seeds.

Acetylated derivatives of certain amino acids are sometimes used in foods for special medical purposes (EFSA, 2003a; EFSA, 2003b), and to supplement animal feed, where the acetylated form has higher water solubility, and provides a stable, palatable and bioavailable source of the amino acid. In these cases, addition of the non-acetylated form of the amino acid is not favoured, as they may be subject to chemical interaction and breakdown. For example methionine interacts with reducing sugars yielding methional, which imparts undesirable sulphur odours and flavours to the food, while threonine and lysine are susceptible to interaction with reducing sugars rendering them nutritionally unavailable (Boggs, 1978).

Table 15: Levels of NAAsp and NAGlu in some commonly consumed foods. The data on four commercial soybean lines and other foods are derived from Hession *et al.* (2008)* and these values are not adjusted for moisture content of the sample.

Food	NAAsp (µg/g calculated fresh weight)	NAGlu (µg/g calculated fresh weight)
Unprocessed soybean 356043 seeds (sprayed)[a]	530.7 (584 µg/g dry weight)	9.81 (10.8 µg/g dry weight)
Unprocessed soybean Jack seeds[a]	2.29 (2.52 µg/g dry weight)	1.39 (1.53 µg/g dry weight)
Soybean seeds (mean of 4 commercial lines)[b]	0.60 (range 0.32 – 0.71)	0.953 (range 0.68 – 1.20)
Roasted coffee beans (Arabica)	62.78	4.97
Brewed espresso (Arabica)	15.37	1.84
Cocoa powder	26.80	62.25
Dark chocolate	4.55	10.25
Sardine with bone[c]	10.24	0.21
Ground turkey[c]	7.41	0.09
Ground chicken[c]	4.70	0.07
Egg yolk[c]	2.30	0.10
Whole egg[c]	1.48	0.04
Black tea leaves (dried)	0.57	3.62
Green tea leaves (dried)	0.45	5.30

[a] Values taken from compositional data in Table 12. The soybean seed moisture content reported by the Applicant ranged from approximately 9-20%. OECD (2001) reports a soybean moisture content of 5.6-11.5%. Therefore, moisture was assumed to be 10% for calculation of fresh weight.
[b] Pioneer varieties 93B87, 93B86, 93B15, 93M40
[c] Fresh weight value calculated from a freeze dried sample
*Limit of detection 0.3 ng/ml; limit of quantification 1 ng/ml

5.6.1.4 NAGlu and NAAsp concentrations in processed soybean

The concentration of NAAsp and NAGlu reported in the compositional analyses (Section 5.6 and Table 12), relate to the raw agricultural commodity (unprocessed, whole soybean seeds). Human food uses of soybean are typically various processed fractions of soybean. A processing study was therefore conducted to determine the concentration of NAAsp and NAGlu in various soybean fractions. The soybean 356043 seed used in the processing study was from the T7 generation from the 2005/06 growing season in South America. Processing of whole soybeans resulted in significant reductions in NAAsp and NAGlu concentration in the majority of food fractions (Table 16). In particular, it is noteworthy that concentration of NAAsp and NAGlu in soybean oil, the principal processed fraction used by the food industry, are below the limit of quantitation. Values for soybean hulls are more relevant to animal feed.

Table 16: Levels of NAAsp and NAGlu in processed fractions of soybean 356043

Fraction	NAAsp (µg/g)	NAAsp processing factor	NAGlu (µg/g)	NAGlu processing factor
Whole soybeans (raw agricultural commodity grown in South America)	636.05		19.97	
Hull material	1766.54	2.78	34.66	1.74
Defatted raw flakes	595.87	0.94	22.67	1.14
Defatted toasted meal	550.05	0.87	24.84	1.24
Defatted flour	479.96	0.76	21.68	1.09
Refined, bleached and deodorised oil[1]	<4	0.003	<4	0.10
Protein isolate	<4	0.003	<4	0.10
Protein concentrate	23.39	0.037	<4	0.10
Soy milk (mg/l)[2]	30.71	NC	1.58	NC

[1] The lower limit of quantitation for NAAsp and NAGlu was 4 µg/g in this assay system
[2] NC - processing factors for soy milk were not calculated

5.6.1.5 Dietary intake estimates

Based on the likely food uses of soybean 356043, increased intakes of NAAsp and NAGlu would not be expected to be nutritionally significant, particularly as these constituents are quantitatively only minor, relative to the total amino acid pool (see Table 14). Nevertheless, dietary modelling was used to estimate intakes of NAAsp and NAGlu for the Australian and New Zealand populations. For these calculations, it was assumed that 45% of soybean products would be replaced with soybean 356043 (considered to be a conservative over-estimate). In this case, it was considered commercially unrealistic to assume a 100% market share for one GM soybean line.

Assuming that soybean 356043 occupied a 45% share of the total soybean market, for all population groups/sub-groups, the estimated mean dietary intake of NAAsp was predicted to increase by no more than 3 fold (1.8 - 2.9x), while intake of NAGlu was estimated to increase only marginally (1.1x) (see Table 17). Additionally, assuming that soy infant formula currently contains no NAAsp, mean intakes for infants aged three months (exclusively soy formula-fed infants) were estimated to increase to approximately 32 µg/day, based on a model diet (data not presented). Based on the same assumptions, intake of NAGlu in exclusively soy-fed infants also increased to 32 µg/day. This corresponds to an estimated intake for both NAAsp and NAGlu in infants of approximately 5 µg/kg bw/day in this scenario. It is possible that soy infant formula naturally contains NAAsp and NAGlu, however no current data are available. Calculations were based on the measured levels of NAAsp and NAGlu in soy protein isolate derived from soybean 356043 presented in Table 16.

Table 17: A comparison of estimated dietary intakes for consumers of NAAsp and NAGlu at baseline (no soybean 356043) and if soybean 356043 occupied a 45% market share.

Country	Population Group	Scenario	Mean Estimated Dietary Intake (µg/kg bw/day)	
			NAAsp	NAGlu
Australia	2 – 6 years	Baseline exposure	12.1	9.6
		45% soybean 356043	35.5	10.2
	2 years & above	Baseline exposure	10.7	4.5
		45% soybean 356043	20.2	4.8
NZ	15 years & above	Baseline exposure	9.8	3.7
		45% soybean 356043	17.7	3.9

5.6.1.5 Discussion and conclusion

NAAsp and NAGlu are typical constituents of the human diet, being present in a wide range of foods. Both compounds are also produced endogenously, with high levels of NAAsp in the central nervous system in humans and other species. Both acetylated amino acids are readily metabolisable by acylase enzymes present in the gastrointestinal tract and in numerous organs including the liver and kidney. In normal individuals, NAAsp and NAGlu would be expected to be metabolised to constituent amino acid and acetate. Toxicity studies in rats have shown that NAAsp is not acutely toxic and causes no adverse effects when present in the diet at high doses for 28 days.

The biochemical and toxicological evidence indicates that the mammalian system can adequately cope with an increase in the levels of NAAsp and NAGlu of the magnitude expected if soybean 356043 were to be introduced into the Australian and New Zealand food supplies. Dietary modelling indicates that there could be a small increase in intake of NAAsp and a very minor increase in NAGlu if soybean 356043 were to be introduced into the Australian and New Zealand food supplies. Overall, the weight of evidence indicates that the presence of NAAsp and NAGlu in food derived from soybean 356043 is unlikely to result in any adverse effects.

5.7 Assessment of endogenous allergenic potential

Study Submitted:
Sampson, H. (2007) Evaluation of the IgE Binding of Conventional and 356043 Soybean Seeds Using Sera from Soy Allergic Subjects. unpublished Pioneer Report PHI-2007-003.

Soybean naturally contains allergenic proteins and is one of a group of known allergenic foods including milk, eggs, fish, shellfish, wheat, peanuts, tree nuts and sesame. This group of foods accounts for approximately 90% of all food allergies. The presence of allergenic proteins in the diet of hypersensitive individuals can cause severe adverse reactions. The allergenic effect of soybeans is attributed to the globulin fraction of soybean proteins that comprise about 85% of total protein (OECD, 2001). Soybean-allergic individuals will also be allergic to soybean 356043.

In order to assess whether soybean 356043 has altered endogenous allergenic potential, a study was conducted to determine binding levels of IgE antibody to protein extracts prepared from soybean 356043 and the parental soybean line Jack. The study involved comparing soybean 356043 to the control line Jack by both one dimensional (1D) IgE immunoblot and by enzyme linked immuno-sorbent assay (ELISA) inhibition. The 1D IgE immunoblot analysis is a visual, qualitative comparison that compares specific proteins in the two soybean lines that bind IgE. The ELISA inhibition assay is a quantitative comparison that compares the relative reactivity of the two soybean extracts. These immunoassays are routinely used to identify protein-specific IgE binding by sera of individuals allergic to a particular food.

Serum from five soy-sensitive subjects with known clinical sensitivity to soybean was pooled and used in the study. Non-allergic human serum was used as a negative control.

Protein extracts from soybean 356043 and Jack flour were separated by SDS-PAGE and transferred to a membrane, then incubated with the pool of sera from documented soy allergic subjects and subsequently labelled with I^{125}-goat anti-human IgE. The 1D immunoblot data with soy allergic sera indicate that soybean 356043 and Jack have very similar IgE binding profiles, while a parallel negative control blot incubated with normal (non-allergic) sera did not produce any IgE binding with either soy extract.

For the ELISA inhibition assay, serum from a pool of known soy-allergic subjects was pre-incubated with protein extract from either soybean 356043 or non-transgenic Jack. These samples were then added to protein extract from non-transgenic Jack previously coated onto a 96-well plate. Following incubation and washing, plates were further incubated with labelled goat anti-human IgE. The level of binding in wells containing no protein extract inhibitor was set as 100%, and the percent inhibition values were calculated by the reduction in binding relative to that. The ELISA inhibition data showed similar inhibition patterns for soybean 356043 and Jack extracts.

These data indicate that soybean 356043 and control Jack have similar protein/allergen profiles when tested with soy-allergic material. Thus, soybean 356043 appears to be equivalent to the non-transgenic counterpart in terms of its endogenous allergenicity.

5.8 Conclusion from compositional studies

The levels of key nutrients and anti-nutrients in soybean 356043 were compared to levels in the non-transgenic parental line Jack and to a range of conventional soybean varieties. The compositional analyses indicate that, for the majority of components, there are no compositional differences of biological significance in forage or seed from transgenic soybean 356043, compared to the non-GM control. Several minor differences in key nutrients and other constituents were noted, however, the mean levels observed were within the range of values observed for the non-transgenic comparator and within the range of natural variation.

An unintended change in the levels of two fatty acids, heptadecanoic acid (C17:0) and heptadecenoic acid (C17:1) was observed. As C17:0 and C17:1 are typical constituents of the human diet and are readily metabolised, consideration of the safety and nutritional issues did not raise any concerns.

With the exception of these two fatty acids, the compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key components in seed from soybean 356043 when compared with the nontransgenic counterpart and to conventional soybean varieties currently on the market.

The GAT4601 enzyme was shown to also acetylate the amino acids glutamate and aspartate. Consequently, levels of NAGlu and NAAsp in soybean 356043 are significantly higher than those of the non-GM parent and are also outside the established tolerance interval for soybean. Acetylated amino acids occur naturally, and the deacetylation and bioavailability of N-acetylated amino acids appears to be a general phenomenon, so it is likely that NAGlu and NAAsp will be similarly metabolised. Both NAGlu and NAAsp were found to be present in common foods, indicating that they are normal components of human diets. These analyses demonstrate a safe history of exposure to these metabolites, and therefore no food safety concerns were identified.

In addition, no difference between soybean 356043 and the nontransgenic parent were found in allergenicity studies using sera from soybean-allergic individuals.

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 compositional 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. In the case of soybean 356043, there are unintended changes in the levels of two fatty acids, (C17:0 and C17:1), and two-acetylated amino acids (NAG and NAAsp), that are outside the range of natural variation for soybean. As discussed in section 5, all of these components are typical constituents of the human diet, and are readily metabolisable, and thus raise no safety issues resulting from their presence in food derived from soybean 356043.

The extent of the compositional and other available data is considered sufficient to establish the nutritional adequacy of soybean 356043. However, the Applicant submitted the results of a feeding study with soybean 356043 using chickens. This has been evaluated by FSANZ as additional supporting information.

6.1 Feeding study in chickens

Company Study PHI-2006-063:
Delaney, B. and Smith, B. (2006) Nutritional Equivalence Study of Transgenic Soybean Line DP-356043-5: Poultry Feeding Study.

Published as:
McNaughton, J., Roberts, M., Smith, B., Rice, D., Hinds, M., Schmidt, J., Locke, M., Brink, K., Bryant, A., Rood, T., Layton, R., Lamb, I. and Delaney, B. (2007). Comparison of Broiler Performance when Fed Diets Containing Event DP-356043-5 (Optimum™GAT™), Nontransgenic Near-Isoline Control, or Commercial Reference Soybean Meal, Hulls and Oil. *Poultry Science* **86**:2569-2581.

Study aim

To assess the nutritional performance in chickens of diets containing soybean meal produced from soybean 356043 in comparison to conventional non-GM soybean meal (Control Jack, and three reference soybean varieties).

Study conduct

Six groups of broilers (Ross x Cobb) consisting of 120 birds per group (equal numbers of males and females) were used in a 42-day study. As well as the four non-GM varieties, two lots of soybean 356043 were used. One lot was not herbicide treated, and a second lot was obtained from plants treated twice with a mixture of glyphosate and the ALS-inhibiting herbicides, chlorimuron and thifensulfuron. Feed and drinking water were available *ad libitum* throughout the study.

Diets were formulated to meet nutrient requirements of a typical commercial broiler diet (National Research Council, 1994). Diets were fed in three phases according to standard commercial poultry farming practice, with soybean fractions incorporated at 30% meal for

starter diets (days 0-21), 26% meal for grower (days 22-35) and 21.5% meal for finisher diets (days 36-42). Hulls and oil were added at 1.0% and 0.5% respectively to all diets.

Birds were observed three times daily for overall health, behaviour and/or evidence of toxicity. Body weights and feed weights were determined every seven days. Body weight gain, feed intake and mortality-corrected feed:gain ratio (feed efficiency[11]) were calculated daily for the duration of the study. At study termination, all surviving birds were processed to collect carcass and carcass part yield data. Carcass yield, thighs, breasts, wings, legs, abdominal fat, kidneys and whole liver were harvested for 576 broilers (four males and four females per pen).

Results

Performance measures were not different between the broilers fed diets containing soybean 356043, unsprayed or sprayed, and those fed control soybean meal. The measurements included final body weights, weight gain, mortality and mortality-adjusted feed efficiency. In addition, all growth performance measures for broilers fed the two soybean 356043 test diets and the control diet were within the tolerance intervals established for broilers fed the non-GM conventional soybean varieties.

Likewise, carcass measurements were not different between birds fed soybean 356043, unsprayed or sprayed, and those fed control soybean meal. These measurements included post-chill carcass weight, and weights of breast, thigh, leg, wing and abdominal fat. In addition, all carcass and individual parts yields for broilers fed the two soybean 356043 test diets and the control diet were within the tolerance intervals established for broilers fed conventional soybean varieties.

Kidney yields were not significantly different between birds fed soybean 356043, unsprayed or sprayed, and those fed control soybean meal, and values for all three groups fell within the tolerance intervals established for broilers fed conventional soybean varieties. Overall liver yields and liver yields for female broilers were not significantly different between birds fed soybean 356043, unsprayed or sprayed, and those fed control soybean meal. Within males, liver yield was higher ($p<0.05$) for the herbicide-treated soybean 356043 diet group compared to the near-isogenic control diet group. However, values were within the tolerance intervals established for broilers fed conventional soybean varieties.

Conclusion

No differences were detected between the test diets used in this study in terms of bird health, growth performance and carcass measurements. The soybean 356043 diet was comparable to conventional soybean diets in terms of its nutritional qualities and wholesomeness.

7. OTHER STUDIES

In the case of herbicide-tolerant soybean 356043, the extent of the molecular, compositional and other available data is considered sufficient to establish the safety of the food. However, the Applicant also conducted a sub-chronic feeding study, in which rats were fed a diet containing soybean 356043 for a period of 13 weeks. While FSANZ does not routinely require animal toxicity studies to be undertaken, where such studies already exist, FSANZ evaluates them as additional supporting information.

[11] Calculated as g of feed intake per g of body weight gain.

This approach is consistent with the recommendations of an expert panel FSANZ convened to consider the role of animal feeding studies in the safety assessment of genetically modified foods[12]. The panel noted that whole-food animal feeding studies may be informative in some limited circumstances, but that any potential adverse health effects can generally be identified by a scientifically informed comparative assessment of the GM food against its conventional counterpart. The panel also recommended that, where the results of relevant animal feeding studies are available, FSANZ evaluate them with critical attention to the methodology and potential limitations in interpretation of the results.

Company Studies
PHI-2006-030:
Munley,S.M. (2007). Thirteen-week Rat Feeding Study with Soybean Containing Event DP-356043-5.
PHI-2006-163:
Maxwell, C. (2008). Concentration of N-Acetylglutamate and N-Acetylaspartate in Rodent Diets.

Published as:
Appenzeller, L.M., Munley, S.M., Hoban, D., Sykes, G.P., Malley, L.A. and Delaney, B. (2008) Subchronic Feeding Study of Herbicide-Tolerant Soybean DP-356043-5 in Sprague-Dawley Rats. *Food Chem Toxicol* **46(6)**:2201-2213.

Study aim

To compare the nutritional quality of soybean 356043 with that of non-GM soybeans when administered to rats through their diet for at least 90 days.

Study conduct

The study comprised six groups of 7-8 week old Sprague-Dawley rats, each consisting of 12 animals/sex/group. The diets were formulated to contain approximately 20% (w/w) of soybean meal and 1.5% (w/w) toasted ground hulls, and conformed to the specifications for PMI Certified Rodent LabDiet #5002 for protein and calorie content. One test group was administered a diet containing soybean 356043 from unsprayed plants; the second test group received a diet containing soybean 356043 from plants treated twice with a herbicide mix containing glyphosate, chlorimuron and thifensulfuron. The control group received a diet containing Jack, the near-isogenic line. Three remaining groups each received a diet containing a non-GM, commercially-available, reference soybean variety (93B86, 93B15 or 93M40).

Parameters Evaluated

All animals were observed twice daily for mortality, moribundity, abnormal behaviour or appearance. Detailed clinical examinations were performed weekly and included evaluation of coat condition, skin, eyes, mucous membranes, occurrence of secretions and excretions, autonomic nervous system activity, changes in gait, posture and response to handling.

Individual body weights and food consumption were determined daily for the first week and weekly thereafter. Food efficiency was calculated from the food consumption and body weight data.

An ophthalmological examination was performed prior to grouping and on day 91. A comprehensive neurobehavioural evaluation was conducted prior to grouping and during the final week of the study. Clinical pathology at 13 weeks included haematology, coagulation, serum chemistry and urinalysis.

[12] The workshop report is available at http://www.foodstandards.gov.au/foodmatters/gmfoods/roleofanimalfeedings3717.cfm

At completion of the study, a complete gross pathology examination was conducted on all animals. The following organs were weighed (paired organs weighted together): liver, kidneys, adrenal glands, thymus, brain, spleen, heart, ovaries and uterus (females) and testes and epididymides (males). A large number of tissues and organs from the test and control groups were also examined microscopically.

The statistical analyses used depended on the type of data being analysed. Response variable values from animals in the control group were compared separately to values from animals in the two test groups. Quantitative and categorical data from male and female rats were analysed within gender. For all comparisons, differences between values were considered statistically significant at a p-value < 0.05. Data from animals in the three reference groups were used to construct a within-study range of natural variation for each response variable, but were not included in comparative statistical calculations. Data were analysed using one of the following statistical tests, as appropriate: one-way analysis of variance (ANOVA), Dunn's Type 1 or Fischer's Exact test, as detailed in (Appenzeller *et al*., 2008).

Results

One male rat in the reference group 93B15 died on day 20 from kidney stones. All other animals survived to the scheduled necropsy.

No differences in body weights, body weight gains, food consumption or food efficiency between rats in the control group and those in either test group were observed. There were no clinical signs of toxicity or ophthalmological lesions attributable to dietary exposure. Nor were there any statistically significant differences in the results of the neurobehavioural evaluation for rats in either test group compared with rats in the control group.

No significant differences in mean haematology and coagulation response variables were observed between male and female rats in the control group and the two test groups, except for mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) values for female rats in the herbicide treated soybean 356043 test group. Although these values were statistically significantly higher ($p<0.05$) than the mean values for the control group, the differences are not considered to be diet related or biologically significant. No significant differences in mean MCV and MCH were observed for female rats in the unsprayed soybean 356043 test group, nor in males in either test group, compared to the control group. Also, the study authors state that all individual MCV and MCH values for female rats in the herbicide treated soybean 356043 test group were contained within the range of individual MCV and MCH values obtained for female rats in the reference groups, and so fall within the range of natural variation observed for these variables.

No significant differences in mean serum chemistry response variables were observed between male and female rats in the control group and the two test groups, except for mean blood urea nitrogen (BUN) values for male rats in the herbicide treated soybean 356043 test group. Although these values were statistically significantly higher ($p<0.05$) than the mean values for the control group, the differences are not considered to be diet related or biologically significant. No significant differences in mean BUN were observed for male rats in the unsprayed soybean 356043 test group, nor in females in either test group, compared to the control group. Also, the study authors state that the mean BUN values for male rats in the herbicide treated soybean 356043 test group were within the performing laboratory's historical reference range (at least 12 unrelated rat subchronic dietary toxicity studies conducted under similar conditions with non-transgenic seeds) for control male rats of this age and strain, and so fall within the observed range of natural variation.

No statistically significant difference in urinalysis response variables or in mean relative organ weights were observed for male or female rats in either test group compared to the rats in the control group. Examination of the organs and tissues of male and female rats in the two test groups showed no evidence of altered incidence or severity of pathologic changes or lesions, in comparison with rats in the control group. All observations reported were considered incidental and were consistent with normal background lesions occurring spontaneously in Sprague-Dawley rats.

Analysis of NAAsp and NAGlu

To estimate the dietary intake of N-acetylaspartate and N-acetylglutamate in rats receiving soybean 356043 and the control diets, the levels of these substances were measured in feed samples taken one day before the start of treatment, and again on days 6 and 27 of the study. The results, obtained from HPLC/mass spectrometry analysis, are presented in Table 18.

Table 18: Concentration of NAAsp and NAGlu in rodent diets (mean of duplicate samples)

Soybean diet	Study day	Storage conditions (weeks)	NAAsp µg/g	NAGlu µg/g
Control	-1	Ambient (0)	ND	<LOQ
356043	-1	Ambient (0)	137.8	6.93
Control	6	Ambient (1)	<LOQ	<LOQ
356043	6	Ambient (1)	127.1	7.57
Control	27	Refrigerated (4)	<LOQ	<LOQ
356043	27	Refrigerated (4)	128.6	7.15

ND: not detected
LOQ limit of quantitation (4 µg/g for both NAAsp and NAGlu)

Based on these measurements, the estimated dietary exposure to NAAsp and NAGlu in the rats was calculated to be 9.47 and 0.52 mg/kg bw/day respectively.

Conclusion

There were no test substance related effects on body weights, body weight gain, food consumption or food efficiency. There were no test substance related clinical observations, ophthalmology or neurobehavioural effects. There were no diet-related differences in haematology, serum chemistry or urinalysis parameters or effects on organ weights, gross pathology or microscopic findings.

The results support the conclusion that administration of soybean 356043 at concentrations of 20% meal and 1.5% hulls in the diet for at least 90 days had no adverse effects on the growth or health of Sprague-Dawley rats.

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Attachment 3

HAZARD ASSESSMENT OF GLYPHOSATE RESIDUES

SUMMARY AND CONCLUSIONS

Two novel residues are generated on soybean 356043 plants following glyphosate application, namely N-acetyl glyphosate (NAG) and N-acetyl aminomethylphosphonic acid (N-acetyl AMPA). The current assessment was undertaken to establish the safety of these compounds and to consider whether the existing residue definition for glyphosate remains appropriate for safety assessment purposes.

Using a weight-of-evidence approach, NAG and N-acetyl AMPA were concluded to be less toxic than glyphosate, which itself has low toxicity potential. On this basis, the establishment of a new acceptable daily intake (ADI) for glyphosate and its residues, or a separate ADI for NAG and N-acetyl AMPA, is unnecessary. Therefore the current Australian ADI for glyphosate of 0.3 mg/kg bw/day remains appropriate for dietary risk assessment purposes.

While NAG is the predominant residue detected on commodities derived from soybean 356043 plants that have been sprayed with glyphosate, parent glyphosate, N-acetyl AMPA and aminomethylphosphonic acid (AMPA) are also detectable. Given that glyphosate is the only toxicologically-significant residue present on/in seed derived from soybean 356043 plants, its measurement on material derived from soybean 356043 plants is considered adequate for safety assessment purposes.

ABBREVIATIONS

Weight

bw	Body weight
kg	Kilogram
µg	Microgram
mg	Milligram

Dosing

po	Oral
mg/kg bw/day	mg/kg bodyweight/day

Volume

L	Litre
mL	Millilitre
µL	Microlitre

Concentration

ppm	Parts per million
mCi/mg	Millicuries/mg
µCi/mg	Microcuries/mg

Chemistry

a.e.	Acid equivalents
ALS	Acetolactate synthase
AMPA	Aminomethylphosphonic acid
eq	equivalents
HPLC	High performance liquid chromatography
HPLC/MS/MS	High performance liquid chromatography with tandem mass spectrometry detection
LSC	Liquid scintillation counting
MS	Mass spectrometry
NAG	*N*-acetyl glyphosate
***N*-acetyl AMPA**	*N*-acetyl aminomethylphosphonic acid

Terminology

ADI	Acceptable Daily Intake
AGF	Aspirated grain fraction
AUC	Area under the curve
CHO	Chinese Hamster Ovary
Cmax	Maximum concentration
CV	Co-efficient of variation
GAP	Good Agricultural Practice
GLP	Good Laboratory Practice
GM	Genetically modified
LOEL	Low observed effect level
MRL	Maximum Residue Limit or Level
NOEL	No Observed Effect Level
SD	Sprague Dawley
sd	Standard deviation
STMR	Supervised trial median residues
Tmax	Time taken to reach Cmax

Organisations & publications

FAO	Food and Agriculture Organisation of the United Nations
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
OECD	Organisation for Economic Co-operation and Development
US EPA	United States Environmental Protection Agency
WHO	World Health Organisation

BACKGROUND

As part of its pre-market safety assessment of foods, which are derived from crops that are genetically modified (GM) for pesticide tolerance, FSANZ has regard to the generation of new residues or increased concentrations of known residues on the crop, following application of *the* pesticide. If new residues are generated that have not previously been assessed for safety then their toxicity must be considered as it may have implications for the determination of dietary risk or the residue definition of the maximum residue limit (MRL)[13]. The purpose of the MRL is to ensure the legitimate and safe use of pesticides on commodities grown in, or imported into, Australia or New Zealand (NZ).

The toxicology of glyphosate has been evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) on a number of occasions, most recently in 2004 (WHO 2004). In addition, the toxicology of AMPA was evaluated by the JMPR in 1997, when it was concluded to be of no greater toxicological concern than glyphosate (WHO 1997).

The ADI for humans is the level of intake of a chemical that can be ingested daily over an entire lifetime without appreciable risk to health. In Australia, ADIs for pesticides and veterinary medicines are established by the Office of Chemical Safety within the Department of Health and Ageing. The current ADI for glyphosate of 0.3 mg/kg bw/day was set in 1985[14] based on the no observed effect level (NOEL) of 30 mg/kg bw/day, the highest dose tested in a 2-year rat study, and using a 100-fold safety factor (10-fold intra and interspecies safety factors). There is currently no ADI for NAG or *N*-acetyl AMPA.

In Australia, the APVMA establishes MRLs for pesticides. The APVMA has published a number of principles and options that may assist in the establishment of MRLs[15]. In particular, the inclusion of specific metabolites or degradation products in the residue definition depends on their toxicity. The current Australian MRL for soybean (dry), which appears in Standard 1.4.2 of the Code, is 10 mg/kg; the residue definition is the *sum of glyphosate and aminomethylphosphonic acid (AMPA) metabolite, expressed as glyphosate*. In New Zealand, MRLs are established by the Agricultural Compounds and Veterinary Medicines Group (ACVMG) within the NZ Food Safety Authority (NZFSA). There is no MRL for glyphosate on soybean currently listed in the NZ MRL Standard, however, there is a provision for residues of up to 0.1 mg/kg for agricultural compound/food combinations not specifically listed. In addition, the MRL Standard recognises Codex standards for imported food. The Codex MRL for glyphosate in soybean seed is 20 mg/kg (the residue definition only includes parent glyphosate).

As there is no application under consideration to grow soybean 356043 plants in Australia, food derived from soybean 356043 would be imported most likely from the US. The US Environmental Protection Agency has only recently amended the tolerance (MRL) for herbicide residues on soybean 356043 treated with glyphosate to also include the novel metabolite *N*-acetyl glyphosate.

The Applicant stated that NAG is the predominant residue generated on soybean 356043 plants containing the *gat* gene following the application of glyphosate, with AMPA and *N*-acetyl AMPA also produced.

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[13] The MRL is the maximum concentration of a residue, resulting from the registered use of an agricultural or veterinary chemical legally permitted or recognized as acceptable in or on a food, agricultural commodity, or animal feed.

[14] http://health.gov.au/internet/main/publishing.nsf/Content/E8F4D2F95D616584CA2573D700770C2A/$File/Amended%20ADI%20List%20-%20April%202008.pdf

[15] http://www.apvma.gov.au/guidelines/rgl6.shtml

NAG and *N*-acetyl AMPA are novel residues that have not previously been detected on conventional crops or currently approved GM glyphosate-tolerant soybean (or other) plants containing the *cp4 epsps* gene. Therefore, it is important that FSANZ evaluates the toxicity of these new compounds to determine whether they might impact on the current residue definition for glyphosate.

The Applicant considers that the current Australian MRL for glyphosate of 10 mg/kg for soybean is adequate, based on the existing residue definition for glyphosate in Standard 1.4.2 of the Code.

Soybean 356043 plants also carry a second genetic modification conferring tolerance to acetolactate synthase (ALS)-inhibiting herbicides. FSANZ has not previously assessed any GM lines that are tolerant to ALS-inhibiting herbicides. Therefore soybean 356043 plants would need to comply with existing Australian and New Zealand MRLs for imported material.

AIMS OF THE CURRENT ASSESSMENT

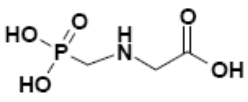
- Conduct a toxicological evaluation of NAG and *N*-acetyl AMPA to determine their toxicity relative to parent glyphosate;
- Determine whether an ADI needs to be established for NAG and/or *N*-acetyl AMPA; and
- Consider whether the existing residue definition for glyphosate in soybean needs to be amended, for safety assessment purposes, to include NAG and/or *N*-acetyl AMPA.

TOXICOLOGICAL ASSESSMENT

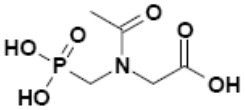
Chemistry

The chemical structures of glyphosate, NAG, AMPA and *N*-acetyl AMPA are given below.

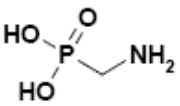
Glyphosate
CAS No. 1071-83-6
Molecular Weight: 169.0
Formula: $C_3H_8NO_5P$
Structure:



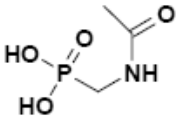
NAG
CAS No. 129660-96-4
Molecular Weight: 211.1
Formula: $C_5H_{10}NO_6P$
Structure:



AMPA
CAS No. 1066-51-9
Molecular Weight: 111.0
Formula: CH_6NO_3P
Structure:



***N*-acetyl AMPA**
CAS No. 57637-97-5
Molecular Weight: 153.0
Formula: $C_3H_8NO_4P$
Structure:



Toxicity studies

The Applicant submitted a number of unpublished toxicity studies on NAG and/or *N*-acetyl AMPA; the full evaluation of these is at Appendix A.

A search of the published scientific literature found no supplementary toxicity data on either compound. All studies were quality assured and conducted according to principles of Good Laboratory Practice (GLP). In addition, all studies complied with national and/or international test guidelines. The studies were considered suitable for determining the toxicity of NAG and/or *N*-acetyl AMPA relative to glyphosate.

Absorption, Distribution, Metabolism and Elimination (ADME)

In a combined pharmacokinetic and metabolism study, Cheng and Howard (2004) administered a single oral gavage dose of [^{14}C]NAG to male rats at a nominal dose of 15 mg free acid equivalents[16] (a.e.)/kg bw. There were no mortalities or clinical signs. The highest radioactivity was detected in urine (66.1%) and faeces (26.4%), with low levels detected in the cage wash/wipe (2.79%) and carcass (0.23%). The majority (>90%) of radioactivity was eliminated by 48 hours post-dose. In the absence of the monitoring of radioactivity in bile, the minimum gastrointestinal absorption of NAG was 66%. The T_{max} and C_{max} in plasma was 2 h and 5.3 μg eq/g, respectively, while the half-life was 15.6 h. In urine and plasma, only unchanged [^{14}C]NAG was detected. In faeces, unchanged [^{14}C]NAG was the main analyte, with a trace (<0.25%) of glyphosate also detected.

Repeated dietary exposure of rats to NAG at levels of 0, 180, 900, 4500 and 18,000 ppm (equal to doses of 0/0, 11/14, 56/68, 283/360 and 1157/1461 mg a.e./kg bw/day in males/females, respectively) for 82/83 days (males/females) generated low levels of glyphosate and *N*-acetyl AMPA in urine at and above 900 ppm. However, NAG was still the main analyte (>97%) in urine. The level of glyphosate generated in urine was approximately an order of magnitude higher in males than females at 4500 and 18,000 ppm. In plasma, predominantly unchanged NAG was detected, while some *N*-acetyl AMPA (<4%) was detected at 4500 and 18,000 ppm. (Shen 2007)

Acute oral toxicity

Following a single oral gavage dose of NAG (5000 mg a.e./kg bw), three of ten rats died and clinical signs occurred in all rats (resolving by four days); macroscopic abnormalities of the lungs and digestive tract were noted in decedents (Vegarra 2004). Following the same oral dose of *N*-acetyl AMPA, clinical signs were observed in all rats up to two days after dosing (Carpenter 2007). The LD_{50} values for NAG and *N*-acetyl AMPA were >5000 mg/kg bw (Vegarra 2004; Carpenter 2007).

Subchronic toxicity

In the study of MacKenzie (2007), NAG was admixed in the diet and fed to groups of rats at dietary concentrations of 0, 180, 900, 4500 or 18000 ppm for approximately 90 days (equal to doses of 0/0, 11/14, 56/68, 283/360 and 1157/1461 a.e mg/kg bw/day in males/females, respectively). The NOEL was 1157 mg/kg bw/day in males and 1461 mg/kg bw/day in females, the highest doses tested, based on the absence of any toxicologically significant effects at these doses.

[16] The dose or amount of glyphosate is typically expressed as *free acid equivalents* to take into account the proportion of herbicidally-active glyphosate acid present in different formulation comprising the monopotassium, isopropylamine, monoammonium or diammonium salts of glyphosate.

Genotoxicity

NAG and *N*-acetyl AMPA were negative for a number of genotoxic endpoints including reverse mutation in bacteria (Mecchi 2004; Wagner & Klug 2007), forward mutation in Chinese Hamster Ovary (CHO) cells (Glatt 2006 & 2007), chromosomal aberrations in CHO cells (Murli 2004; Gudi & Rao 2007) and micronuclei formation in mouse bone marrow (Donner 2006 & 2007).

Residue studies

The Applicant submitted a number of residue chemistry studies that analysed the types and concentrations of residues generated on soybean 356043 plants following glyphosate application. These were evaluated to confirm the identity and concentrations of residues purported by the Applicant and as part of the consideration of the residue definition for glyphosate (Appendix B). All studies were conducted according to principles of GLP or Good Agricultural Practice (GAP) and complied with national and/or international test guidelines. Comparable studies on Optimum® GAT® corn were also submitted but not considered relevant to the current application. Feeding and metabolism studies relevant to the consideration of glyphosate MRLs were submitted but not evaluated as this was outside the scope of the safety assessment.

Two residue trials were conducted at a number of sites in the USA and Canada to determine the levels of glyphosate and its degradates on soybean 356043 plants after a total seasonal glyphosate application of 6.8 kg a.e./ha (one pre-emergent and three foliar applications). In the first trial where glyphosate was applied as the monopotassium salt, total residues (i.e. glyphosate, NAG, AMPA, *N*-acetyl AMPA) in seed ranged from 0.90-6.59 mg/kg, with the level of parent glyphosate (expressed as free acid) at 0.063-0.45 mg/kg. In the second trial, total residues following application of the monopotassium salt were 0.36-8.06 mg/kg and the level of parent glyphosate was 0.015-1.10 mg/kg; application of glyphosate as the free acid resulted in total residues of 0.25-8.83 mg/kg and 0.019-1.8 mg/kg for parent glyphosate.

NAG was the predominant residue in soybean seed (up to 70%) followed by parent glyphosate (up to 16%), *N*-acetyl AMPA and AMPA (maximum residues of 7.9, 1.8, 1.3 and 0.16 mg/kg, respectively; mean residues of 2.3, 0.22, 0.32 and 0.039 mg/kg, respectively). Residue levels detected in aspirated grain fractions (AGF) were 23-43 mg/kg for glyphosate, 2.2-3.6 mg/kg for NAG, 2.5-2.9 mg/kg for AMPA and 0.21-0.78 mg/kg *N*-acetyl AMPA. In processed fractions, no residues were detected in oil; NAG was the predominant residue (1.6/12 mg/kg in meal/hulls) followed by *N*-acetyl AMPA (0.52/3.2 mg/kg in meals/hulls), glyphosate (0.22/2.0 mg/kg in meal/hulls) and AMPA (0.038/0.21 mg/kg in meal/hulls). (Buffington 2006; Schwartz 2007; Shepard 2007)

DISCUSSION

Toxicity and ADI considerations

The toxicological database for NAG and *N*-acetyl AMPA was considered adequate for assessing their respective toxicities relative to glyphosate. A comparison of a number of toxicological parameters for NAG and *N*-acetyl AMPA relative to glyphosate and AMPA is given in Table 1. The gastrointestinal (GI) absorption of NAG was approximately twice that of glyphosate and AMPA, with an equivalent relative increase in the level of urinary excretion; this is consistent with the presence of the *N*-acetyl group increasing the solubility of the compound. The higher level of GI absorption would also mean greater systemic exposure over glyphosate. The limited metabolism, very low acute toxicity and absence of genotoxicity were consistent between NAG and glyphosate/AMPA.

The oral subchronic NOEL for NAG (and AMPA) in rats was approximately 3-fold higher than for glyphosate, with no evidence of any toxicity even at the highest doses tested (1000 and 1400 mg/kg bw/day, respectively). On the basis of these findings, NAG is considered to be less toxic than glyphosate.

Table 1: Comparative toxicity of glyphosate and its residues in Optimum® GAT® soybean

Parameter	Glyphosate[1]	AMPA[1,2]	NAG	*N*-acetyl AMPA
GI Absorption	~30-36%; rapid	~13% in 12 h; rapid	~66%; rapid	No data
Distribution	Widely distributed; no evidence of accumulation	Widely distributed; no evidence of accumulation	No data	No data
Metabolism	Limited (<0.7% AMPA)	Limited (<0.01% CO_2)	Limited (glyphosate: <3% in urine and 0.25% in faeces; *N*-acetyl AMPA: <4% in plasma & urine)	No data
Elimination	Complete by 48 h; 30/70% in urine/faeces	Almost complete by 120 h; 20/75% urine/faeces	90% by 48 h; 66/26% in urine/faeces	No data
Rat acute oral LD_{50} (mg/kg bw)	>5000	8300	>5000	>5000
Rat subchronic oral toxicity [NOEL/LOEL (mg/kg bw/day)]	300/800 Endpoint: Cellular changes in the salivary gland	1000 Highest dose tested	1157/1461 (males/females) Highest doses tested	No data
Genotoxicity	Negative	Negative	Negative	Negative

1 = WHO (2006); 2 = WHO (1997)

Like NAG, *N*-acetyl AMPA had very low acute oral toxicity and was not genotoxic. No data were provided on the ADME or repeat-dose toxicity of *N*-acetyl AMPA. However, the presence of the *N*-acetyl group is highly unlikely to increase the already low repeat-dose toxicity of AMPA. It is worth noting that low levels (<4%) of *N*-acetyl AMPA were detectable in plasma and urine following repeated dietary exposure of rats to NAG (MacKenzie 2007) without evidence of toxicity.

Using a weight-of-evidence approach, NAG and *N*-acetyl AMPA are considered less toxic than glyphosate, which itself has low toxicity potential. On this basis, the establishment of a new ADI for glyphosate and its residues, or a separate ADI for NAG and *N*-acetyl AMPA, is considered unnecessary. Therefore the current Australian ADI for glyphosate of 0.3 mg/kg bw/day remains appropriate.

Residue definition

The residue definition for a pesticide (for compliance with MRLs) is *that combination of the pesticide and its metabolites, derivatives and related compounds to which the MRL applies* (FAO 2002). With regard to GM crops, the principles for determining the residue definition are no different to those used for conventional crops; the residue definition should include toxicologically-significant compounds and those most suitable for monitoring compliance with GAP (FAO 2002; OECD 2006). The residue definition is established on a case-by-case basis and takes into consideration a number of factors including the:

- toxicity of the metabolites, derivatives and related compounds compared to the parent compound;
- results of supervised trials;
- residue composition and levels in animal and plant metabolism studies; and
- analytical methods used to measure the residues (OECD 2006).

Residue trials evaluated as part of the current application indicated that the predominant (up to 70%) residue in soybean seed was NAG followed by parent glyphosate (up to 16%) *N*-acetyl AMPA and AMPA. The residue profile in AGFs was somewhat different in that the main residue was parent glyphosate, followed by NAG, AMPA and *N*-acetyl AMPA. In processed fractions, no residues were detected in oil; NAG was the predominant residue in meal/hulls, followed by *N*-acetyl AMPA, glyphosate and AMPA. From a risk assessment perspective, the relatively high levels of NAG generated on edible material derived from soybean 356043 plants following application of glyphosate is not considered to pose any safety concerns because this compound is much less toxic than parent glyphosate. While *N*-acetyl AMPA is also detectable on GM plant material, it is present at much lower concentrations than NAG (or glyphosate) and similarly has limited toxicity potential. As neither NAG nor *N*-acetyl AMPA are toxicologically-significant compounds, it is unnecessary to include them in the residue definition for glyphosate for dietary risk assessment purposes.

There is no approval or any application under consideration to grow soybean 356043 plants in Australia or New Zealand. Therefore, food commodities derived from soybean 356043 plants would be imported into Australia most likely from the USA. From a practical perspective, the MRL for glyphosate in the Code would be applicable to this imported material. Given that glyphosate is the only toxicologically-significant compound of the four residues considered as part of the current assessment, and that it is detectable on commodities derived from treated soybean 356043 plants (albeit at relatively low levels), its measurement on imported material is considered adequate for safety assessment purposes. On this basis, the current residue definition for glyphosate, which appears in Standard 1.4.2 and is the sum of glyphosate and AMPA expressed as glyphosate, remains appropriate for safety assessment purposes.

CONCLUSIONS

- There are no safety concerns with regard to NAG and *N*-acetyl AMPA, which are less toxic than glyphosate.

- The establishment of a new or amended ADI for glyphosate and its residues, or a separate ADI for NAG and *N*-acetyl AMPA, is unnecessary.

- For the purpose of imported GM soy, the current Australian/Codex residue definition for glyphosate remains appropriate for safety assessment purposes.

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MacKenzie SA (2007) IN-MCX20: Subchronic toxicity 90-day feeding study in rats. Lab: HaskellSM Laboratory for Health and Environmental Sciences, Newark, Delaware, USA. Project ID: DuPont-19008. Sponsor: EI du Pont de Nemours & Company, Wilmington, Delaware, USA. Unpublished.

Mecchi MS (2004) Salmonella-Escherichia coli/mammalian-microsome reverse mutation assay with a confirmatory assay with N-acetyl-glyphosate. Lab: Covance Laboratories Inc, Vienna, VA, USA. Covance Study No. 7535-101. Study No. VER04-COV-03. Sponsor: Verdia Inc, Redwood City, CA, USA. Unpublished.

Murli H (2004) Chromosomal aberrations in Chinese hamster ovary (CHO) cells. Lab: Covance Laboratories Inc, Vienna, VA, USA. Covance Study No. 7535-102. Sponsor: Verdia Inc, Redwood City, CA, USA. Sponsor Study No. VER04-COV-02. Unpublished.

OECD (2006) Guidance document on the definition of residue. ENV/JM/MONO(2006)31. Series on Testing and Assessment Number 63, Series on Pesticides Number 31.

Schwartz NL (2007) Magnitude of residue of glyphosate and its degradates in/on soybean forage, hay, and seed of a soybean line containing event DP-356Ø43-5 containing the gat and gm-hra genes following applications of glyphosate herbicides at maximum label rates - United States and Canadian locations, 2005. Lab: ABC Laboratories, Inc. (Missouri), Columbia, Missouri, USA. Report No. 49989-1. Sponsor: EI du Pont de Nemours & Company, Wilmington, Delaware, USA. DuPont Report No. PHI-2007-10. Unpublished.

Shepard E (2007) Magnitude and decline of residues of glyphosate and its degradates in/on forage, hay and seed of a soybean line containing event DP-356Ø43-5 containing the gat and gm-hra genes following a variety of tank mix applications of glyphosate herbicides and sulfonylurea herbicides (rimosulfuron, tribenuron methyl, chlorimuron ethyl, and metsulfuron methyl) at maximum label rates - United States and Canadian locations, season 2006 [interim report]. Lab: ABC Laboratories, Inc. (Missouri), Columbia, Missouri, USA. Report No. 50283. Sponsor: EI du Pont de Nemours & Company, Wilmington, Delaware, USA. DuPont Report No. DuPont-20123. Unpublished.

Shen ZA (2007) IN-MCX20: Subchronic toxicity 90-day feeding study in rats – Supplement 1. Lab: HaskellSM Laboratory for Health and Environmental Sciences, Newark, Delaware, USA. Project ID: DuPont-19008. Sponsor: EI du Pont de Nemours & Company, Wilmington, Delaware, USA. Unpublished.

Shen ZA (2007) IN-MCX20: Subchronic toxicity 90-day feeding study in rats – Supplement 1. Lab: Haskell^{SM} Laboratory for Health and Environmental Sciences, Newark, Delaware, USA. Project ID: DuPont-19008. Sponsor: EI du Pont de Nemours & Company, Wilmington, Delaware, USA. Unpublished.

Vegarra MM (2004) Acute oral toxicity study in rats with N-acetyl-glyphosate sodium salt (acute toxic class method). Lab: Covance Laboratories Inc, Vienna, VA, USA. Covance Study No. 7535-103. Study No. 25930-0-804. Sponsor: Verdia Inc, Redwood City, CA, USA. Unpublished.

Wagner VO & Klug ML (2007) IN-EY252: Bacterial reverse mutation assay. Lab: BioReliance, Rockville, MD, USA. BioReliance Study No. AB47BT.503.BTL. Sponsor: EI du Pont de Nemours & Company, DuPont Haskell Laboratory. Unpublished.

WHO (2006) Pesticide residues in food – 2004 Joint FAO/WHO Meeting on Pesticide Residues. Part II—Toxicological. WHO/PCS/06.1, 2006

WHO (1997) Toxicological and environmental evaluations 1994. Joint meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group.

APPENDIX A

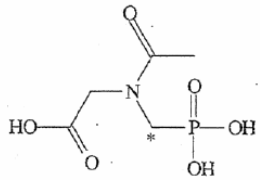
EVALUATION OF TOXICITY STUDIES

ADME

Cheng T & Howard S (2004) Mass balance, metabolism and pharmacokinetics of [^{14}C]N-acetyl-glyphosate following administration of a single oral dose to rats. Lab: Covance Laboratories, Madison, WI, USA. Report No. Covance 7535-100. Sponsor: Pioneer Hi-Bred International Inc, Johnston, IA, USA. Report Date: 6 December 2004. Unpublished.

Statement of compliance with US EPA standards of Good Laboratory Practice (40 CFR 160); Quality Assurance (QA) Statement.

Experimental: Forty-five fasted male Sprague Dawley rats [Crl:CD(SD)IGS BR] (sourced from Charles River Laboratories; 10-weeks of age; 266-292 g bodyweight) were assigned to one of two groups and administered a single oral gavage dose of [^{14}C]NAG (sourced from Sigma; Lot No. 123K9416; 99.181% purity; 8.0 mCi/mmol specific activity) mixed with unlabelled NAG (sourced from Sigma; Lot No. 123K5012; 84.3% purity as the sodium salt and 67.4% as the free acid) in sterile water at a nominal dose of 15 mg a.e./kg bw. The dose volume was approximately 5 mL/kg bw. The position of the radiolabel (*) is given in the following diagram:



Rats were housed individually, with food and water available *ad libitum* from 4 h post-dose. Rats were observed twice daily for mortality and clinical signs. Bodyweights were recorded at assignation and on the day of dosing. For Group 1 (5 rats), urine and faeces were collected at various intervals over 168 hours post-dose (urine: 0-6, 6-12, 12-24, 24-48 and 48-72 hours; faeces: 6-12, 12-24 and 24-48 hours) then rats were sacrificed for analysis of residual radioactivity in the carcass. Cages were washed and wiped following each collection. For Group 2 (40 rats), four rats were sacrificed and blood collected pre-dose and at 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 hours post-dose. Based on the structural similarity of NAG and glyphosate, which does not produce volatile metabolites in rats, expired air was not collected. Radioactivity was measured in carcasses and in plasma, urine and faecal samples by liquid scintillation counting (LSC), with pooled plasma, urine and faecal samples analysed for unchanged parent glyphosate and metabolites by high performance liquid chromatography (HPLC).

Findings: There were no mortalities or clinical signs. In Group 1, the mean total recovery of radioactivity was 98.0%. The highest radioactivity was detected in urine (57.2%) and faeces (37.6%), with low levels detected in the cage wash/wipe (2.91%) and carcass (0.26%). The majority (>90%) of radioactivity was eliminated by 48 hours post-dose. When the results were adjusted to take into account the likely contamination of the faeces of one rat with urine, the mean total recovery was 95.5%, with urine, faeces, the cage wash/wipe and carcass containing 66.1, 26.4, 2.79 and 0.23% of the total administered radioactivity, respectively. Based on the amount of radioactivity excreted in urine and remaining in the carcass, the estimated gastrointestinal absorption of NAG was approximately 67%.

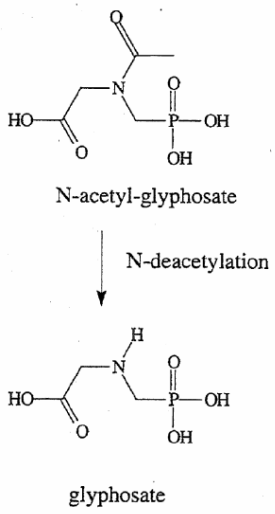
Key pharmacokinetic parameters for Group 2 are given in Table 2.

Table 2: Pharmacokinetic parameters in rats following a single oral dose of [^{14}C]NAG

Matrix	T_{max} (h)	C_{max} (μg eq/g)[1]	$t_{1/2}$ (h)	$AUC_{(0-1)}$ (h μg eq/g)	$AUC_{(0-\infty)}$ (h μg eq/g)
Plasma	2	5.31±1.60	15.6	20.7	20.8

1 = Results expressed as the mean ± 1 standard deviation (sd)

The total recovery of radioactivity in pooled urine was 'nearly 100%', while recoveries in pooled faeces were 92.9-101% for extractable radioactivity and 0.15-5.75% remaining in the post-extraction solid. In pooled plasma collected at 0.5-0.8 h (low levels of radioactivity were detected at 12 and 24 h), recoveries were 88.0-103% for extractable radioactivity and 1.35-9.05% remaining in the post-extraction solid. In urine and plasma, only [^{14}C]NAG was detected. Unchanged [^{14}C]NAG was the main analyte in faeces, with a trace (<0.25%) of [^{14}C]glyphosate also detected. The authors' proposed metabolic pathway of [^{14}C]NAG in rats was as follows:



Shen ZA (2007) IN-MCX20: Subchronic toxicity 90-day feeding study in rats – Supplement 1. Lab: Haskell^{SM} Laboratory for Health and Environmental Sciences, Newark, Delaware, USA. Project ID: DuPont-19008. Sponsor: El du Pont de Nemours & Company, Wilmington, Delaware, USA. Unpublished.

This supplement to MacKenzie (2007) (see evaluation below) analysed the concentrations of NAG, glyphosate and *N*-acetyl AMPA in pooled urine and plasma samples from male and female Crl:CD(SD) rats that were fed NAG in their diet at concentrations of 0, 180, 900, 4500 or 18,000 ppm (equal to doses of 0/0, 11/14, 56/68, 283/360 and 1157/1461 mg a.e./kg bw/day in males/females, respectively). Samples were collected from males/females on day 82/83 of treatment and analysed by HPLC and tandem mass spectrometry (HPLC/MS/MS).

There was a dose-related increase in the concentration of NAG in plasma and urine. Low levels of glyphosate and *N*-acetyl AMPA were detected in urine at and above dietary concentrations of 900 ppm, with the levels of glyphosate markedly higher in males than females at 4500 and 18,000 ppm (Table 3). In plasma, predominantly unchanged NAG was detected; mean (±1 sd) concentrations were 0.33±0.11, 1.85±0.35, 4.77±0.90 and 14.83±2.59 μg/mL in males and 0.44±0.12, 2.32±0.95, 8.35±1.43 and 13.46±2.56 μg/mL in females at 180, 900, 4500 and 18,000 ppm, respectively. No glyphosate was detected in plasma, while some *N*-acetyl AMPA was detected at 4500 and 18,000 ppm. The concentration of *N*-acetyl AMPA detected at 4500 ppm (0.32±0.26 μg/mL) was higher than the concentration detected at 18,000 ppm (trace). The author speculated that this result may have been due to the variation in the instrument response on the different days that samples were analysed resulting in better quantitation of the 4500 ppm samples.

Table 3: Concentrations of NAG, glyphosate and *N*-acetyl AMPA in pooled urine collected at days 82 (males) or 83 (females)

Dose of NAG (mg/kg bw/day)	NAG (μg/mL)		Glyphosate (μg/mL)		N-acetyl AMPA (μg/mL)	
	Males	*Females*	*Males*	*Females*	*Males*	*Females*
0	ND	ND	ND	ND	ND	ND
11/14 (males/females)	53.8	71.5	<0.05	<0.05	<0.05	<0.05
56/68	361	360	0.165	0.360	0.127	0.179
283/360	1150	1110	27.1	2.92	1.50	<0.5
1157/1461	2220	2020	64.4	4.02	5.38	2.89

ND = not detected

These results indicated that in rats, only small levels of glyphosate and *N*-acetyl AMPA are present in urine following dietary exposure to NAG.

Acute toxicity

Vegarra MM (2004) Acute oral toxicity study in rats with N-acetyl-glyphosate sodium salt (acute toxic class method). Lab: Covance Laboratories Inc, Vienna, VA, USA. Covance Study No. 7535-103. Study No. 25930-0-804. Sponsor: Verdia Inc, Redwood City, CA, USA. Unpublished.

Statement of compliance with US EPA standards of Good Laboratory Practice (40 CFR 160) and US EPA and OECD test guidelines [OPPTS 870.1100: Acute Oral Toxicity (adopted August 1998); OECD Test Guideline No. 423: Acute oral Toxicity – Acute Toxic Class Method (adopted 17 December 2001]. QA Statement.

Experimental: Five fasted Crl:CD®(SD)IGS BR rats/sex (sourced from Charles River Laboratories, Raleigh, North Carolina, USA; 8 or 12 weeks of age; 223-247 g bodyweight) were administered a single oral gavage dose of NAG (Sigma-Aldrich Chemical Co.; Lot No. 123K5012; 84.3% purity as the sodium salt, 67.4% as the free acid) in sterile water at 5000 mg a.e./kg bw. The dose volume was 10 mL/kg bw. Rats were housed individually under standard conditions, with food and water available *ad libitum* following dosing. Rats were observed twice daily for mortalities and clinical signs. Bodyweights were recorded pre-dose and at days 1, 8, 15 and 16 post-dose. At day 15 post-dose, surviving rats were fasted overnight prior to sacrifice and necropsy.

Findings: One female died 4 hours after dosing, while a male and female were found dead on day 2. Clinical signs were observed in all rats and included: slight hypoactivity (all); irregular respiration (2 males, 1 female); diarrhoea (2 males, 3 females); soft faeces (4 males, 2 females), perineal staining (all males, 4 females); squinted eyes (3 males) and a brown nasal crust (2 males). In survivors, clinical signs had resolved by day 4. All survivors gained bodyweight over the 14-day observation period. No macroscopic abnormalities were observed in survivors. The female that died on the day of dosing had fluid or a gel-like clear liquid in the digestive tract, with the stomach wall appearing red and a red liquid present in the abdominal cavity. In the male and female that were found dead on day 2, the following macroscopic abnormalities were detected: mottled or discoloured bright red lungs; discoloured black liver; soft stomach with or without yellow fluid; fluid or a reddish liquid in the abdominal cavity and fluid in the small intestine. The LD_{50} was >5000 mg/kg bw.

Carpenter C (2007) IN-EY252: Acute oral toxicity study in rats – up-and-down procedure. Lab: DuPont Haskell Global Centers for Health and Environmental Sciences, Newark, Delaware, USA. Project ID: DuPont-22229. Sponsor: EI du Pont de Nemours & Company, Wilmington, Delaware, USA. Unpublished.

Statement of compliance with US EPA standards of Good Laboratory Practice (40 CFR 160) and US EPA and OECD test guidelines [OPPTS 870.1100: Acute Oral Toxicity, Health Effects Test Guidelines (2002); Section 4 (Part 425): Acute oral Toxicity – Up-and-Down Procedure, Guideline for the Testing of Chemicals (2001)]. QA Statement.

Experimental: Three fasted female Crl:CD (SD) rats (sourced from Charles River Laboratories Inc, Raleigh, North Carolina, USA; 10-11 weeks of age; ~230 g bodyweight) were administered a single oral gavage dose of *N*-acetyl AMPA (supplied by EI du Pont de Nemours and Company, Deleware, USA; Batch No. IN-EY252-003; 79% purity) at 5000 mg/kg bw in deionised water. The dose volume was 20 mL/kg bw. Rats were housed individually under standard conditions, with food and water available *ad libitum* from approximately 3-4 h post-dose. Rats were observed daily for mortalities and clinical signs. Bodyweights were recorded on days -1, 0, 7 and 14. Survivors were sacrificed on day 14 and necropsied.

Findings: There were no mortalities and all rats gained bodyweight over the 14-day observation period. Clinical signs were observed up to two days post-dose and included diarrhoea in all rats and dark eyes, lethargy, high posture, stained and wet fur, ataxia or hyperactivity in two of the three rats. There were no macroscopic abnormalities indicative of toxicity, although dilation of the uterus was observed in one rat, which was considered by the authors to be non-specific in nature. The LD_{50} was >5000 mg/kg bw.

Subchronic toxicity

MacKenzie SA (2007) IN-MCX20: Subchronic toxicity 90-day feeding study in rats. Lab: Haskell^{SM} Laboratory for Health and Environmental Sciences, Newark, Delaware, USA. Project ID: DuPont-19008. Sponsor: EI du Pont de Nemours & Company, Wilmington, Delaware, USA. Unpublished.

Statement of compliance with US EPA standards of Good Laboratory Practice (40 CFR 160) and the following test guidelines: US EPA, OPPTS 870.3100: 90-Day Oral Toxicity in Rodents, Health Effects Test Guidelines (1998); OECD, Section 4 (Part 408): Repeated Dose 90-Day Oral Toxicity Study in Rodents, Guideline for the Testing of Chemicals (1998); EEC, Method B.26 Commission Directive 2001/59/EC: Sub-chronic Oral Toxicity Test. Repeated Dose 90-Day Oral Toxicity Study in Rodents, Methods for the Determination of Toxicity (2001); MAFF Japan, 2-1-9 Notification 12 Nousan 8147 and Notification 13 Seisan 1739, Agricultural Chemicals Regulation Laws (2000 and 2001). QA Statement.

Experimental: NAG (sourced from EI du Pont de Nemours & Company, Wilmington, Delaware, USA; Batch No. IN-MCX20; 81.8% purity as the sodium salt and 63% purity as the free acid) was admixed in the diet and fed to groups of 10 Crl:CD(SD) rats/sex (Charles River Laboratories Inc, Raleigh, North Carolina, USA; approximately 7 weeks of age; bodyweight range of 189-261 and 152-193 g in males and females, respectively, at the start of dosing) at dietary concentrations of 0, 180, 900, 4500 or 18000 ppm for 95 (males) or 96 (females) days. The concentration selection was based on the acute oral toxicity of NAG, the expected similar toxicity to glyphosate and the gastrointestinal absorption of NAG and glyphosate. Samples of the diet were analysed to determine the concentration, homogeneity and stability of NAG in the diet. Rats were house individually under standard conditions, with food and water available *ad libitum*.

Observations for mortalities and clinical signs were made at least twice daily, with more detailed clinical assessments made on a weekly basis. Bodyweight and food consumption were recorded weekly and used to calculate food conversion efficiency. Ophthalmoscopic examinations were performed on all rats 15 days prior to dosing and on all surviving rats on day 85. Prior to dosing and during week 13, an abbreviated Functional Observational Battery (FOB) and motor activity evaluation was performed in replicates over two days. Blood and urine samples collected on days 95-96 were analysed for the standard haematology, clinical chemistry and urinalysis parameters. All surviving males and females were sacrificed on day 95 and 96, respectively, and then necropsied. Histopathology was performed on the standard range of tissues and organs in the control and high-dose groups. The standard organs were weighed. Results were analysed by appropriate statistical tests.

Findings: The test substance was homogenously distributed in the four test diets (±13.7% of nominal concentrations) and stable for up to 21 days at room temperature in the two tested diets (180 and 18,000 ppm). For the stability analysis, two outlying results were obtained for each diet (35% lower than nominal concentration at day 0 in the 180 ppm diet and 40% higher than nominal concentration at day 14 in the 18,000 ppm diet). However, neither result was considered by the author to represent instability because there was no reduction in the concentration of NAG in either diet over time and for the 180 ppm diet, the sample was collected from freshly prepared diet.

Based on bodyweight and food consumption data, the mean (±1 sd) daily intake of NAG over 90 days was 0, 11.31±0.26, 55.72±3.16, 283.46±9.43 and 1157.15±42.27 mg/kg bw/day at nominal concentrations of 0, 180, 900, 4500 and 18000 ppm, respectively in males and 0, 13.88±0.89, 67.78±3.12, 359.51±13.73 and 1460.67±86.30 mg/kg bw/day, respectively in females.

There were no treatment-related mortalities or clinical signs (one male from the 4500 ppm group was found dead on day 42, which was reportedly due to trauma). The mean bodyweight of high-dose males was marginally lower (~8%) than the control group throughout most of the treatment period but was not statistically significant at any time. The overall bodyweight gain of high-dose males was significantly lower ($p<0.05$) than the controls (298.6±36.8 *versus* 347.3±51.9 g, respectively). The bodyweight gain of high-dose males was significantly lower ($p<0.05$) than the controls at week 5-6 (24.4±4.0 *versus* 32.9±7.1 g, respectively), however, as the gain was identical to the previous week (which was not significantly different to the control), the result was not considered treatment-related. No other weekly bodyweight gains in high-dose males were significantly different to the control. Given the small magnitude of the differences in bodyweight and bodyweight gain in high-dose males, the absence of similar results in high-dose females (which received a higher dose of NAG), and the absence of an effect on food consumption and food conversion efficiency, these findings were not considered treatment-related or toxicologically-significant.

Ophthalmoscopy, motor activity assessment and abbreviated FOB and were unremarkable. Monocytes were significantly lower ($p<0.05$) in high-dose males than the control (0.17±0.07 *versus* 0.28±0.11 $x10^3/\mu L$, respectively). An examination of individual animal data indicated that four high-dose males had monocyte counts below the concurrent control range of 0.12-0.49 $x\ 10^3/\mu L$ and the historical control range of 0.14-0.53 $x\ 10^3/\mu L$ (mean = 0.27 $x\ 10^3/\mu L$) for 13-22 week old male Crl:CD(SD) rats[17]. Given the equivocal nature of the clinical significance of reduced monocyte counts and the absence of a similar finding in high-dose females, this finding is not considered to be toxicologically significant.

[17] Giknis MLA & Clifford CB (2006) Clinical Laboratory Parameters for Crl:CD(SD) rats. Available online at http://www.criver.com/flex_content_area/documents/rm_rm_r_clinical_parameters_cd_rat_06.pdf

There was no treatment-related effect on any clinical chemistry or urinalysis parameter. There were no treatment-related macroscopic or histopathological abnormalities, or effects on organ weight.

The NOEL was 1157 mg/kg bw/day in males and 1461 mg/kg bw/day in females, the highest doses tested.

Genotoxicity

Evaluations of submitted genotoxicity studies on NAG and *N*-acetyl AMPA are summarised in Table 4, with neither compound found to be genotoxic under the conditions of each study. Certificates of compliance with GLP in addition to a QA statement were provided with each study report. In addition, statements of compliance with relevant international and national test guidelines were provided [OECD Test Guidelines 471, 473, 474 and 476; US EPA OPPTS Guidelines 870.5300, 870.5375, 870.5395 and 870.5100; EC Commission Directive 2000/32/EC 4E-B.17 No. L136, 4C-B.12 No. L136, 4D-B.13/14 No. 471 and 4A-B.10 No. L136; Japanese MAFF 59 NouSan 4200, 12 NouSan 8147 and Notification No. 12-Nousan-8147 Guideline No. 2-1-19-3]. In all studies, positive and negative controls gave expected results.

Table 4: Results of genotoxicity studies conducted on NAG and *N*-acetyl AMPA

Assay	Strain, Cell Type or Species	Concentration or Dose & Vehicle	Batch & Purity of Test Material	Controls	Result	Reference & Guidelines
NAG						
Bacterial reverse mutation	*Salmonella Typhimurium* strains TA98, TA100, TA1535 & TA1537	100, 333, 1000, 3330 or 5000 μg a.e./plate Deionised water	Lot No. 123K5012 84.3% (sodium salt) 67.4% (free acid)	Vehicle Benzo[a]pyrene 2-nitrofluorene 2-aminoanthracene Sodium azide ICR-191	Negative ± S9	Mecchi (2004)
	Escherichia coli strain WP2 *uvr*A			Vehicle 2-aminoanthracene 4-nitroquinoline-N-oxide		
Mammalian forward mutation	CHO cells	250, 500, 1000, 1500 or 2091 μg a.e./mL Water	Batch No. IN-MCX20-002 63% (sodium salt)	Vehicle Ethyl methanesulfonate Benzo(a)pyrene	Negative ± S9	Glatt (2006)
Chromosomal aberration	CHO cells	960, 1370, 1960 or 2800 μg a.e./mL Water	Lot No. 123K5012 84.3% (sodium salt) 67.4% (free acid)	Vehicle Mitomycin C Cyclophosphamide	Negative ± S9	Murli (2004)
Mouse bone marrow micronucleus test	Male & female Crl:CD1(ICR) mice	0, 500, 1000 or 2000 mg a.e./kg bw, po Water	Batch No. IN-MCX20-002 63% (sodium salt)	Vehicle Cyclophosphamide	Negative	Donner (2006)
***N*-acetyl AMPA**						
Bacterial reverse mutation	*S. Typhimurium* strains TA98, TA100, TA1535 & TA1537	1.5, 5.0, 15, 50, 150, 500, 1500 or 5000 μg/plate Water	Batch No. IN-EY252-001 76%	Vehicle 2-nitrofluorene Sodium azide 9-aminoacridine	Negative ± S9	Wagner & Klug (2007)
	E. coli strain WP2 *uvr*A			Vehicle 2-aminoanthracene Methyl methanesulfonate		

Assay	Strain, Cell Type or Species	Concentration or Dose & Vehicle	Batch & Purity of Test Material	Controls	Result	Reference & Guidelines
Mammalian forward mutation	CHO	100, 250, 500, 1000 or 1531 μg/mL Water	Batch No. IN-EY252-002 72%	Vehicle Ethyl methanesulfonate Benzo(a)pyrene	Negative ± S9	Glatt (2007)
Chromosomal aberration	Human peripheral blood lymphocytes	191.25, 382.5, 765 &1530 μg/mL Water	Batch No. IN-EY252-001 76%	Vehicle Mitomycin C Cyclophosphamide	Negative ± S9	Gudi & Rao (2007)
Mouse bone marrow micronucleus test	Male & female Crl:CD1(ICR) mice	0, 500, 1000 or 2000 mg/kg bw, po Water	Batch No. IN-EY252-002 72%	Vehicle Cyclophosphamide	Negative	Donner (2007)

S9 = microsomal enzymes prepared from Aroclor™-induced SD rat liver

APPENDIX B

EVALUATION OF RESIDUE CHEMISTRY STUDIES

Buffington J (2006) Sample generation and magnitude of residue of glyphosate, N-acetylglyphosate and aminomethyl phosphonic acid (AMPA) in/on soybean forage, hay, and seed of a soybean line containing event DP-356Ø43-5 following applications of a commercial glyphosate formulation - United States and Canada field locations, 2005 growing season. Lab: ABC Laboratories Inc (Missouri), Columbia, Missouri, USA. Report No. 49989. Sponsor: EI du Pont de Nemours & Company, Wilmington, Delaware, USA. DuPont Report No. PHI-2005-056/030. Unpublished

Schwartz NL (2007b) Magnitude of residue of glyphosate and its degradates in/on soybean forage, hay, and seed of a soybean line containing event DP-356Ø43-5 containing the gat and gm-hra genes following applications of glyphosate herbicides at maximum label rates - United States and Canadian locations, 2005. Lab: ABC Laboratories, Inc. (Missouri), Columbia, Missouri, USA. Report No. 49989-1. Sponsor: EI du Pont de Nemours & Company, Wilmington, Delaware, USA. DuPont Report No. PHI-2007-10. Unpublished.

Shepard E (2007a) Magnitude and decline of residues of glyphosate and its degradates in/on forage, hay and seed of a soybean line containing event DP-356Ø43-5 containing the gat and gm-hra genes following a variety of tank mix applications of glyphosate herbicides and sulfonylurea herbicides (rimosulfuron, tribenuron methyl, chlorimuron ethyl, and metsulfuron methyl) at maximum label rates - United States and Canadian locations, season 2006 [interim report]. Lab: ABC Laboratories, Inc. (Missouri), Columbia, Missouri, USA. Report No. 50283. Sponsor: EI du Pont de Nemours & Company, Wilmington, Delaware, USA. DuPont Report No. DuPont-20123, Interim. Unpublished.

GAP; Guidelines: OPPTS 860.1000, 860.1500, 860.1520, Canadian PMRA Residue Chemistry Guidelines, Regulatory Directive Dir 98-02 Sections 0, 9, and 10

Two field trials were conducted at a number of sites in the USA and Canada to determine the levels of glyphosate and its degradates on soybean 356043 plants following application of glyphosate. In the first trial conducted in 2005, glyphosate [as its monopotassium salt with a non-ionic surfactant (~0.25% v/v) and ammonium sulphate (3.4 kg/ha)] was applied as one pre-emergent soil application (3.33 kg a.e/ha) and three post-emergent foliar applications (0.77, 1.75 and 0.876 kg a.e./ha, respectively) at six sites. The three foliar applications were at 10-14 days prior to blooming, full blooming and full maturity, respectively. Application was via a broadcast spray, with spray volumes of 135-153 L/ha used. The total seasonal application rate was 6.726 kg a.e./ha. The second trial was conducted at 15 sites in 2006 using the same formulation and application rats as Trial 1. In addition, glyphosate formulated as its free acid was applied to separate plots at each site in the same manner. Soybean seeds were harvested at maturity (13-17 days after the last glyphosate application) and analysed for residues by HPLC/MS/MS.

Recoveries from freshly fortified soybean seed samples were 71-111% for glyphosate, NAG, AMPA and *N*-acetyl AMPA. In the first trial, total residues (i.e. glyphosate, NAG, AMPA, *N*-acetyl AMPA) ranged from 0.90-6.59 mg/kg, with the level of parent glyphosate (expressed as free acid) at 0.063-0.45 mg/kg. In the second trial, total residues following application of the monopotassium salt were 0.36-8.06 mg/kg and the level of parent glyphosate was 0.015-1.10 mg/kg; application of glyphosate as the free acid resulted in total residues of 0.25-8.83 and 0.019-1.8 mg/kg for parent glyphosate.

The overall supervised trial median residues are summarised in the Table 5. As shown, NAG was the predominant residue in soybean seed (up to 70%) followed by parent glyphosate (up to 16%), *N*-acetyl AMPA and AMPA.

Table 5: Residue levels (ppm or mg/kg) from supervised residue trials on Optimum® GAT® soybean

Soybean Seed	Minimum	Maximum	Median (STMR)	Mean	Standard deviation
Glyphosate	0.01	1.8	0.093	0.22	0.34
NAG	0.01	7.9	1.6	2.3	2.0
AMPA	0.01	0.16	0.020	0.039	0.044
N-acetyl AMPA	0.01	1.3	0.23	0.32	0.28
Total	0.01	8.6	2.1	2.9	2.3

STMR = supervised trial median residues; Limit of quantification = 0.05 mg/kg

Attachment 4

Summary of Public Submissions on First Assessment Report

Submitter	Comments
Sugarcane Gene Technology Group	▪ Supports approval of the Application ▪ Advises that research and trialling of new sugarcane farming systems favour crop rotation with other species such as soybean and peanut, which are cash crops. The availability of herbicide tolerant soybean would be an important new development in the economic viability and sustainability of the sugar industry in Australia.
NSW Food Authority	▪ Notes in the First Assessment Report, that FSANZ undertook to consider whether amendments to Standard 1.4.2, concerning glyphosate residues, may be necessary ▪ Suggests that novel pesticide residues and compositional changes arising from the acetylation of glyphosate, glutamate and aspartate require further consideration. In particular the Safety Assessment contains insufficient information on NAAsp (which is exceptionally high in soybean line DP-356043-5) to assess the impact of these changes on human health. The following points should be addressed: - Information on the fate of a significant ingested dose of the acetylated amino acids is required - NAAsp in soybean line DP-356043-5 fits the CODEX definition of a 'contaminant' and should receive appropraite consideration. ▪ Advises that the use of appropriate primers for detection of the event will add to the cost of GM compliance testing. ▪ Questions the suggestion in the Safety Assessment that *B. licheniformis* is not associated with any safety concerns. ▪ Points out a number of typographical errors and inconsistencies in the text
New Zealand Food Safety Authority	▪ Suggests, from a dietary perspective, that the increased levels of NAAsp and NAGlu in soybean line DP-356043-5 may be significant. Data showing any commonly consumed foods that contain these acetylated amino acids at levels comparable to those in soybean DP-356043-5 would be an appropriate inclusion in the Safety Assessment. ▪ Agrees with the conclusion that the current CODEX residue definition for glyphosate remains appropriate for the safety assessment of soybean DP-356043-5 but notes that FSANZ undertook to consider whether amendments to Standard 1.4.2, concerning glyphosate residues, may be necessary. In particular NZFSA agrees that discussions should be held between the APVMA, FSANZ and NZFSA on any MRL issues arising from the application.
The Food Technology Association of Australia	▪ Supports approval of the Application
Queensland Health (whole of Queensland Government response)	▪ Notes that the First Assessment Report does not state explicitly whether soybean line DP-356043-5 will be grown in Australia. ▪ Notes that the only ALS-inhibiting herbicides that currently have permitted MRLs for soybean in the Food Standards Code are tribenuron, imazethapyr and flumetsulam.

Submitter	Comments
	▪ Seeks a guarantee that all products containing soybean DP356043-5 will comply with these MRLs. Asks whether the applicant intends applying to extend the range of permitted MRLs for soybean. ▪ Requests advice on the progress of submissions by the applicant to regulatory agencies in other countries for the release of soybean DP356043-5 and/or for its use in food and feed ▪ Expresses concern about the lack of independent data used to inform the Safety Assessment. ▪ Advises that a decision to approve the application would impact on the monitoring resources of jurisdictions. ▪ Considers that, since the First Assessment Report states the majority of soybean is processed for soybean meal used in animal feed and soybean oil for human uses, there is an obligation for FSANZ to address the safety of animal feed or animals fed with feed derived from GM plants.
Gregory Damato (Private)	▪ Expresses concern about the lack of independent data used to inform the Safety Assessment. ▪ Cites two recently published reports that have concluded glyphosate-based herbicides can cause cellular death and abnormalities in test tissue. Expresses concern that soybean DP-356043-5 sprayed with glyphosate would pose a safety issue for humans consuming products derived from the line. ▪ Suggests that since compositional analysis of soybean DP-356043-5 has shown that a number of analytes are present at significantly different levels from conventional soybean, soybean DP-356043-5 cannot be regarded as nutritionally equivalent to non-GM soybean. ▪ Cites two studies showing that animals fed GM soybean products have altered gene expression and structural/functional changes in liver tissue compared to animals fed non-GM soybean products. ▪ Details a number of concerns by the African Centre for Biosafety regarding the molecular characterisation of soybean DP-356043-5. These concerns can be summarised as follows: i) Data indicate that there is probably one intact insertion of the cassette (sic). But the results are unclear and inconclusive as they rely on Southern blots with insufficient sensitivity and a limited number of frequently-cutting restriction enzymes. The positive control did not produce the expected pattern with Xba1 enzyme probably because it was methylated, meaning that integrity of the cassette could not be accurately determined and fragment sizes could not be accurately compared to the original construct. ii) Copy number was determined by visual reference to known copies of plasmid DNA and no quantification was made. iii) Data are insufficient to support the conclusion that the cassette (sic) is genetically and phenotypically stable – only a few plants were studied using a few (generally 3) restriction enzymes over only two generations. iv) There are no data to address the question of whether there have been any unintended rearrangements at the site of integration.

Submitter	Comments
	v) The Rsyn7-SynII element of the SCP1 promoter is new to nature and has not been tested for biosafety. The SCP1 promoter also contains a CaMV-35S element; CaMV-35S is associated with increased rearrangements/deletions affecting cassette integrity and genome stability. vi) Global gene expression and horizontal gene transfer have not been addressed. An established technique such as repPCR, RAPD or comparative genome hybridization could have been used to establish the genome similarity between soybean 356043-5 and the non-GM parent line. ▪ The evidence to support the conclusion that neither of the novel proteins are likely to be toxic or allergenic in humans, is incomplete (African Centre for Biosafety). i) The activity of the GAT4601 protein leads to acetylation of amino acids. The exposure to NAA and NAG in soy meal is likely to be high; the health effects of consuming large quantities of NAAsp and NAGlu are uncertain. ii) A feeding study should have been used to observe the immune response of both novel proteins since bioinformatics and simulated gastric/intestinal fluid studies cannot show this. iii) There are doubts about whether the bacterially expressed HRA protein used for the feeding study is sufficiently similar to the plant expressed HRA protein. This questions the validity of the feeding study. ▪ Notes that, contrary to the conclusion of no nutritional impact of soybean 356043 provided by the feeding study and 90-day sub-chronic toxicity study, a 2005 study showed a high death rate and decreased growth rate in rats fed a GM-soy flour diet. In other studies with mice fed Roundup Ready soy, pancreatic cells changed significantly and produced less digestive enzymes. ▪ Cites comments by Jeffrey Smith regarding the possibility of protein produced in Roundup Ready soy triggering allergic reactions, which may be further compounded by the transfer of the gene to gut bacteria that may then continuously produce the protein.
Graham Clarke (Private)	▪ Asks that FSANZ not approve GM food. ▪ Claims there is evidence that genes in GM food are incorporated into the host DNA.
Catherine Page (Private)	▪ Disagrees with altering plant genetic material to create herbicide resistance. ▪ States that current agricultural methods based on chemical use, damage the soil, pollute the air and are unsustainable.
Bee Winfield (Private)	▪ Expresses concern about the lack of independent data used to inform the Safety Assessment. ▪ Suggests that published studies in Austria, the UK and Russia have found disturbing ramifications for rats and mice fed GM food. ▪ Claims that Monsanto has a history of hiding the truth, influencing government decisions, and changing trial parameters in order to get the desired results. ▪ Suggests that DNA from GM food has been found to pass to mouth and stomach bacteria where it confers antibiotic resistance.

Submitter	Comments
	▪ Asks that FSANZ support independent feeding trials of GM food in Australia. ▪ Suggests that all GM foods on supermarket shelves be recalled. ▪ Asks that GM ingredients in food should be labelled even if they are highly refined. ▪ Demands that animal products derived from livestock fed GM feeds be labelled as GM.