Application to FSANZ to Vary Food Standard 1.5.2 to Include the Nematode Resistant and Herbicide Tolerant Soybean (*Glycine max*) Event GMB151

Prepared by [redacted]
on behalf of BASF Agricultural Solutions Seed US LLC

Submitted November 2019

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDED ELECTRONIC DOCUMENTS

Appendix 1 Statutory Declaration verifying that that information provided to FSANZ with this submission is true and correct.

Appendix 2 [redacted] (2016) Description of the amino acid sequence of the HPPD-4 protein. Unpublished report. Document no. M-461393-02. (**Contains confidential commercial information)**

Appendix 3 [redacted] (2016) Description of Vector pSZ8832. Unpublished report. Document no. M-455339-04.

Appendix 4 [redacted] (2018) Molecular characterization of GMB151 soybean by means of next-generation sequencing and junction sequencing analysis. Unpublished report. Document no. GEN170607_H. (**Contains confidential commercial information)**

Appendix 5 [redacted] (2017) DNA sequence determination of the transgenic and insertion loci of GMB151 soybean. Unpublished report. Document no. M-607717-01. (**Contains confidential commercial information)**

Appendix 6 [redacted] (2018) Bioinformatics Analysis of the GMB151 soybean insertion locus. Unpublished report. Document no. 18-RSVLS011. (**Contains confidential commercial information**)

Appendix 7 [redacted] (2018) GMB151 soybean – Identification of open reading frames and homology search of sequences ≥30 amino acids to known allergens and toxins. Unpublished report. Document no. TXVLT032. (**Contains confidential commercial information**)

Appendix 8 [redacted] (2018) GMB151 soybean - Inheritance of the insert over generation. Unpublished report. Document no. M-611752-01.

Appendix 9 [redacted] (2018) Amendment - GMB151 Soybean - Expression of Cry14Ab-1 and HPPD-4 proteins in field samples grown in the USA during 2016. Unpublished report. Document no. M-601077-02.

Appendix 10 Porée, F., Heinrichs, V., Lange, G., Laber, B., Peters, C., Schouten, L. (2014) HPPD variants and methods of use. WO 2014/043435. Document no. M-486300-01.

Appendix 11 Fritze, I. M., Linden, L., Freigang, J., Auerbach, G., Huber, R., Steinbach, S. (2004) The crystal structures of *Zea mays* and *Arabidopsis* 4-hydroxyphenylpyruvate dioxygenase. *Plant Physiology* 134: 1388 – 1400. Document no. M-359884-01.

Appendix 12 [redacted] (2018) Characterization of Cry14Ab-1 protein purified from GMB151 soybean and comparability with the bacterially-produced Cry14Ab-1 protein batch 1514_Cry14Ab-1. Unpublished report. Document no. M-621885-01. (**Contains confidential commercial information**)

Appendix 13 [redacted] (2017) Amendment no 1 to characterization of the recombinant Cry14Ab-1 protein batch 1514_Cry14Ab-1. Unpublished report. Document no. M-566932-02. (**Contains confidential commercial information**)

Appendix 14 [redacted] (2018) Characterization of HPPD-4 protein purified from GMB151 soybean and comparability with the bacterially-produced HPPD-4 protein batch 1338_HPPD-4. Unpublished report. Document no. 17-RSVLN028-A. (**Contains confidential commercial information**)

Appendix 15 [redacted] (2018) Functional characterization of HPPD-4 protein extracted from GMB151 soybean leaf and comparability with the bacterially-produced HPPD-4 protein batch 1338_HPPD-4. Unpublished report. Document no. SEL/6495/3.

Appendix 16 [redacted] (2014) Characterization of the recombinant HPPD-4 protein batch 1338_HPPD-4. Unpublished report. Document M-490041-01. (**Contains confidential commercial information**)

Appendix 17 [redacted] (2018) GMB151 soybean: Processing of grain and analysis of resultant fractions, 2016. Unpublished report. Document no. 16-RSBS0011.

Appendix 18 [redacted] (2016) Description of the amino acid sequence of the Cry14Ab-1 protein. Unpublished report. Document no. M-485425-02. (**Contains confidential commercial information**)

Appendix 19 [redacted] (2018) CryAb-1 protein. Amino acid sequence homology search with known allergens and known toxins. Unpublished report. Document no. TXKIS002. (**Contains confidential commercial information**)

Appendix 20 [redacted] (2018) HPPD-4 protein. Amino acid sequence homology search with known allergens and known toxins. Unpublished report. Document no. TXFAS015. (**Contains confidential commercial information**)

Appendix 21 [redacted] (2014) Cry14Ab-1 protein. *In vitro* digestibility study in human simulated gastric fluid at pH 1.2. Unpublished report. Document no. M-478215-01.

Appendix 22 [redacted] (2014) Cry14Ab-1 protein. *In vitro* digestibility study in human simulated intestinal fluid. Unpublished report. Document no. M-478845-01.

Appendix 23 [redacted] (2015) The effect of temperature on Cry14Ab-1 as assessed by SDS-PAGE and Western blot. Unpublished report. Document no. M-515776-01.

Appendix 24 [redacted] (2014) HPPD-4 protein. *In vitro* digestibility study in human simulated gastric fluid at pH 1.2. Unpublished report. Document no. M-476249-01.

Appendix 25 [redacted] (2014) HPPD-4 protein. *In vitro* digestibility study in human simulated intestinal fluid. Unpublished report. Document no. M-476906-01.

Appendix 26 [redacted] (2015) The effect of temperature on HPPD-4 as assessed by SDS-PAGE and Western blot. Unpublished report. Document no. M-515772-01.

Appendix 27 [redacted] (2016) Cry14Ab-1 protein. Acute toxicity study by oral gavage in mice. Unpublished report. Document no. M-538392-02.

Appendix 28 [redacted] (2016) HPPD-4 protein. Acute toxicity study by oral gavage in mice. Unpublished report. Document no. M-496627-03.

Appendix 29 [redacted] (2018) GMB151 soybean – Composition assessment of GMB151 soybean grown in the USA during 2017. Unpublished report. Document no. 17-RSSB0044-C.

Appendix 30 [redacted] (2018) GMB151 Soybean 90-Day Toxicity Study in the Rat by Dietary Administration - Final Report. Unpublished report. Document no. 00021233.

Executive Summary

BASF Australia Ltd on behalf of BASF Agricultural Solutions Seed US LLC seeks to vary FSANZ Standard 1.5.2 to allow the use of genetically modified soybean (*Glycine max* L. Merr.) derived from transformation event GMB151 in the Australian and New Zealand food industries. Five food products are derived from soybean: whole soybeans, oil, meal, hulls and protein. Soybean oil is the primary food product consumed by humans in Australia, with the other products used either as food products or as components of animal feed.

Soybean event GMB151 contains the stably integrated *cry14Ab-1.b* and *hppdPf-4Pa* gene cassettes. The incorporation and expression of the GMB151 transgenic locus in the *G. max* genome has been characterised according to international standards for the safety assessment of biotechnology products. This information is included with this application to support the food safety of the Cry14Ab-1 and HPPD-4 proteins.

The GMB151 nematode resistant and herbicide tolerant GM soybean (*G. max*) will be commercialized in the major soybean producing countries of the world. GMB151 soybean produces the Cry14Ab-1 protein, a crystal protein derived from *Bacillus thuringiensis*, which confers resistance to soybean cyst nematode and *Pratylenchus*. GMB151 also produces a modified 4-hydroxyphenylpyruvate dioxygenase (HPPD-4), derived from *Pseudomonas fluorescens*, which confers tolerance to HPPD inhibitor herbicides such as isoxaflutole.

Planting nematode resistant/herbicide tolerant soybean GMB151 varieties provides growers with new options for nematode control. Plant-parasitic nematodes are widely prevalent in soybean agricultural production systems. The HPPD inhibitor herbicides to which GMB151 soybean is tolerant will also provide growers with an alternative class of herbicidal chemistry with which to control weeds in the crop.

GMB151 soybean was developed through *Agrobacterium*-mediated transformation using the vector pSZ8832 containing the *cry14Ab-1.b* and *hppdPf-4Pa* expression cassettes. The OECD identifier is BCS-GM151-6.

(i) The *cry14Ab-1.b* gene encodes for the Cry14Ab-1 protein. The *cry14Ab-1.b* coding sequence was derived from *Bacillus thuringensis*. The Cry14Ab-1 protein is a member of Cry (crystal)-type protein family with fully conserved three-domain structure. Cry proteins are produced by *Bacillus thuringiensis* strains and demonstrate specific toxicity towards insects or nematodes. FSANZ has previously assessed an extensive range of Cry proteins for food safety. This is the first time that the Cry14Ab-1 protein has been assessed for food safety in Australia.

(ii) The *hppdPf-4Pa* gene encodes for the HPPD-4 protein. The *hppdPf-4Pa* coding sequence was developed by introducing four amino acid mutations to the wild type *hppd* gene derived from *Pseudomonas fluorescens* strain A32. Expression of the HPPD-4 protein confers tolerance to HPPD inhibitor herbicides such as isoxaflutole.

Soybean is cultivated primarily for the production of seed that has many food, feed and industrial uses. In the human diet, soybeans are one of the major sources of edible vegetable oil that is used as a purified oil, or utilized in margarines, shortenings and cooking and salad oils. Soybeans may also be consumed directly without any processing as soybean seeds, and many non-fermented and fermented oriental soybean foods such as soy sprouts, milk, tofu, tempeh, miso, natto, and soy sauce. Edible soy protein products including grits and flours, concentrates and isolates are used as food ingredients. The different soy protein products are added in bakery products, snack foods, noodle products and comminuted meat products (Hui, 1992; CRC, 1983). Meal derived from soybeans is used as a high protein

supplement in feed rations for livestock. Industrial uses of soybeans range from the production of yeasts and antibodies to the manufacture of soaps and disinfectants.

The GMB151 transgenic locus in the *G. max* genome and the safety of the Cry14Ab-1 and HPPD-4 proteins expressed by the introduced genes, *cry14Ab-1.b* and *hppdPf-4Pa* respectively, have been characterized according to international standards for the safety assessment of biotechnology products. This information is included with this application to support the food safety of the Cry14Ab-1 and HPPD-4 proteins. The GMB151 soybean event will be grown commercially in the soybean producing areas of the USA, Canada and Brazil.

Molecular characterization studies using next generation sequencing (NGS) and junction sequence analysis (JSA) determined that GMB151 contains, at a single locus, a single complete copy of the *cry14Ab-1.b* gene cassette and a single *hppdPf-4Pa* gene cassette that lacks the 5' part of the promoter. Generational stability analysis by NGS/JSA demonstrated that the transgenic locus of GMB151 is stably maintained across multiple generations. Segregation data were consistent with Mendelian principles, confirming that GMB151 has a single insert that is stably inherited over generations.

Bioinformatics analysis of the full DNA sequence revealed no evidence supporting cryptic gene expression or unintended effects resulting from the genetic modification.

Food safety evaluation of the Cry14Ab-1 and HPPD-4 proteins was undertaken utilising guidance provided by Codex (2009). No health-related adverse effects have been associated with the proteins.

A thorough mammalian safety assessment was conducted for both the Cry14Ab-1 and HPPD-4 proteins as expressed in GMB151 soybean. No adverse effects were observed for either protein. The source organism of the Cry14Ab-1 protein, *Bacillus thuringiensis (Bt),* is ubiquitous in the environment, is not known for allergenicity, and has a history of safe use as microbial *Bt*-derived biopesticides. Cry proteins have an established history of safe use and have been used for insect control in crops for over 50 years. The Cry14Ab-1 protein has no amino acid sequence similarity to known allergens or toxins, is rapidly degraded in simulated gastric fluid and exhibited no effects in an acute oral mouse toxicity test. The source organism of the HPPD-4 protein, *Pseudomonas fluorescens,* is a non-pathogenic bacterium which is ubiquitous in nature and has a history of safe use. HPPD proteins are ubiquitous in nature across all kingdoms: bacteria, fungi, plants and animals. The HPPD-4 protein has no amino acid sequence similarity to known allergens or toxins, is rapidly degraded in simulated gastric fluid and exhibited no effects in an acute oral mouse toxicity test.

Food safety for both the Cry14Ab-1 and HPPD-4 proteins are established with data and information within this application for GMB151 soybean - studies confirming the lack of amino acid sequence homology with known toxins and allergens, rapid digestion in simulated gastric fluid, along with complimentary protein expression studies for GMB151 soybean. Acute oral mouse toxicity testing for both proteins and a 90 day toxicity study using GMB151 soybean meal are available and are provided within this submission.

Composition analysis and a comparative assessment demonstrated that GMB151 soybean forage and grain is comparable to that of the non-GM counterpart and reference varieties. These results demonstrate that GMB151 soybean supports the food safety assessment.

Part 1 General Information on the Application

1.1 Applicant Details

(a) Applicant (individual organisation's) name

BASF Australia Ltd on behalf of BASF Agricultural Solutions Seed US LLC

(b) Name of contact person

[redacted]

(c) Address (street and postal)

[redacted]

(d) Telephone numbers

Tel: [redacted]

(e) Email address

[redacted]

(f) Nature of applicant's business

Seeds and traits, biotechnology.

(g) Details of other individuals, companies or organisations associated with the application.

Not applicable.

1.2 Purpose of the Application

This application, on behalf of BASF Agricultural Solutions Seed US LLC, seeks to vary FSANZ Standard 1.5.2 to allow the use of genetically modified soybean (*G. max*) derived from transformation event GMB151 in the Australian and New Zealand food industries.

Five food products are derived from soybean: whole soybeans, oil, meal, hulls and protein. Soybean oil is the primary food product consumed by humans in Australia, with the other products used either as food products or as components of animal feed.

GMB151 soybean will be approved as a single event for food approval in the major soybean product countries of the world. It is anticipated that food products derived from soybean containing this event will enter the Australian and New Zealand food supply via local production and imports from major soybean producing countries such as the United States, Canada and Brazil.

1.3 Justification for the Application

The GMB151 transformation event introduced two genes to the *G. max* genome. These genes confer two novel traits; plant-parasitic nematode resistance, such as to soybean cyst nematode (*Heterodera glycines*) and *Pratylenchus*, and tolerance to HPPD inhibitor herbicides, such as isoxaflutole. Soybean varieties containing the GMB151 event will be produced commercially in the major soybean producing countries of the world such as the United States, Canada and Brazil.

<u>Advantages of GMB151 soybean</u>
The novel traits expressed by GMB151 soybean provide several potential benefits over conventional soybean varieties and other transgenic soybean currently in cultivation. These include:

- The presence of an *Bacillus thuringiensis* derived *cry14Ab-1.b* gene, permits expression of the Cry14Ab-1 protein which shows nematicidal activity against plant-parasitic nematodes, such as the soybean cyst nematode (*Heterodera glycines*) and *Pratylenchus.*
- HPPD inhibitor herbicides are also a broad spectrum weed control system that provides an alternative herbicidal mode of action which also allows for herbicide rotation within soybean farming systems.
  - Broad spectrum weed control reduces cultivation needs, reducing on-farm fuel consumption, decreasing CO_2 emissions and also importantly improving soil health (Brookes, G. and Barfoot, P.; 2016; http://www.tandfonline.com/doi/full/10.1080/21645698.2016.1192754).

Note:

(a) Any public health and safety issues related to the proposed change including details of target groups and population groups that may be adversely affected

(b) Any consumer choice issues related to the proposed change

(c) Any evidence that the food industry generally or other specific companies have an interest in, or support, the proposed change.

In relation to points (a), (b) and (c) above, the data contained within this submission indicates the general safety of GMB151 soybean-derived foods and their close similarity to non-genetically modified (GM) comparators that have been used in studies. From the work conducted there is no indication that there are public health or safety issues related to the proposed change to Standard 1.5.2 of the Food Standards Code. The section below discussing food safety of GMB151 soybean goes into further detail in this respect.

Consumer choice with respect to the proposed change is anticipated to be dealt with by FSANZ via their assessment of the data included in this package. It should be noted that GMB151 soybean when used in breeding systems to deliver soybean-derived food products will result in the primary food product – soybean oil – which contains novel proteins which are below the limit of quantification. This food item therefore does not result in the need for labelling to differentiate it from soybean oil derived from non-GM soybean varieties.

As GMB151 *G. max* is still in the developmental stage with BASF, there is no specific information available to indicate that the food industry have interest in, or support, the proposed change to the Standard 1.5.2. However, due to reasonably rapid uptake by the farming community of GM soybean in past years and the impact that this has had on the price per tonne of seed for crushing to oil and meal it may be anticipated that the food industry generally support technology that leads to lower commodity prices for the soybean that they wish to purchase, use in food production and on-sell. Equally use of lower-priced, soybean meal as a feed for animals would be welcomed by animal producers and in turn would have an impact on the price of food derived from livestock for consumers.

Food safety

Cry proteins expressed by the common soil microorganism, *B. thuringiensis*, have a long history of safe use in agriculture. These proteins have been successfully used for insect control for more than 50 years, and they are expressed by a number of transgenic crops that have been in commercial production over the past decade. Of these proteins, Cry1Ab proteins are the most extensively used and well characterised. FSANZ has assessed several of these proteins previously (see Table 3, Section A.2 (a) (i)), and have not identified public health or safety concerns. Cry14Ab-1 data are presented within this submission to establish the food safety of this member of the Cry protein family.

Tolerance to HPPD inhibitors, such as isoxaflutole, is achieved through expression of the HPPD-4 protein encoded by the *hppdPf-4Pa* gene derived from *Pseudomonas fluorescens*. The HPPD-4 protein has not been assessed previously, however a complete data package as outlined by the FSANZ Application Handbook (FSANZ, 2019) is presented with this submission to support independent evaluation of the food safety of this protein and the Cry14Ab-1 protein expressed by GMB151 soybean.

Information is provided in this application to support claims that the Cry14Ab-1 and HPPD-4 proteins expressed by the GMB151 event share no characteristics consistent with toxins or allergens. The potential for mammalian toxicity is addressed for these proteins. Compositional and nutritional analyses demonstrate that food derived from soybean containing event GMB151 is as safe and nutritious as food derived from conventional soybean varieties.

Should this application for inclusion of GMB151 soybean be included in Food Standard 1.5.2 this may occur late-2020.

The status of submissions for food and feed approval in other cultivation and importing countries for GMB151 is provided in that table below. Further information on the global submission status can be provided to update FSANZ during the application process.

Table 1 Current global submission status for GMB151 soybean

Country	Agency	Submission(S)/Approval(A) date
USA	EPA	Experimental Use Permit (A) 2017
USA	EPA	Section 3 Seed Increase Registration (S) 2018
USA	FDA	Food approval (S) 2019
Canada	Health Canada	Food approval (S) 2019
Canada	CFIA	Feed/cultivation approval (S) 2019
Uruguay	CGR	Food/feed approval (S) 2019

1.4 Regulatory impact information

Costs and benefits, and impacts on trade

Varying FSANZ Standard 1.5.2 to include commercial soybean varieties containing event GMB151 is unlikely to have a detrimental impact on the Australian soybean industry. Despite being a small soybean producer, Australian soybean is sourced for food and feed products on the domestic market and also, culinary quality soybeans produced out of season are exported to the main northern hemisphere producers. Soybean food and feed ingredients are also obtained from imported soybean products, with the US a major source of imports. Once soybean varieties containing the GMB151 event are launched for commercial production in the US as well as other parts of the world, food and feed products derived from soybean containing this event are likely to enter the domestic food and feed supply.

If the soybean event GMB151 is not incorporated into the FSANZ Standards, this could have wide ranging impacts on the price of food and feed products containing the ingredients derived from soybean. These would arise from the need to source varieties that do not contain the GMB151 event. These products may attract a premium price that must be met by the manufacturer, with those costs eventually passed on to the consumer. This would be compounded by the costs of segregating GMB151 soybean products, where trading partners are willing to comply with this requirement. Other factors to consider include disruptions to the food supply, and the significant costs of recalling food products if the GMB151 event were to be distributed in the local food and feed supply.

Varying the FSANZ Standards to include GMB151 will contribute to maintaining stable food prices, consumer choice in the marketplace, and decreased production costs for transgenic soybean varieties in the longer term. The potential trade implications of not including soybean event GMB151 in the FSANZ Standards are significant. Segregating GMB151 soybean products from other soybean products has compliance and identification requirements that are difficult and costly to meet. The US is the major trading partner of Australia, and approved transgenic crops are considered to be substantially equivalent to conventional crops. Therefore, in the US, there are no intentions of segregating or labelling transgenic crops or their products. Products containing event GMB151 imported into Australia from the US, or other trading partners with similar treatments of transgenic crops, may need to be removed from sale. This could expose Australia to disputes with trading partners at the World Trade Organisation.

1.5 Information to support the application

All of the relevant information to support the application is supplied within this summary and the associated electronic dossier that has been supplied to FSANZ. The relevant studies are listed in the “List of Appended Electronic Documents” above, and suitable literature references are provided in a reference list at the end of this document. To navigate the electronic dossier a direction to which Appendix in either the general Appendices or Confidential Commercial Information Appendices within the dossier is supplied in the text of the discussion.

1.6 Assessment Procedure

We consider that the appropriate assessment for this application is the General Procedure since proteins similar to the Cry14Ab-1 and HPPD-4 proteins have been evaluated by FSANZ previously.

1.7 Confidential Commercial Information and Company Names

Information in the BASF and Bayer CropScience (note the accompanying letter of support for utilisation of transferred Bayer study reports now owned by BASF) reports provided in, Appendix 2 (Document M-461393-02), Appendix 4 (Document GEN170607_H), Appendix 5 (Document M-607717-01), Appendix 6 (Document 18-RSVLS011), Appendix 7 (Document TXVLT032), Appendix 9 (Document M-601077-02), Appendix 12 (Document M-621885-01), Appendix 13 (Document M-566932-02), Appendix 14 (Document 17-RSVLN028-A), Appendix 15 (Document SEL-6495-3), Appendix 16 (Document M-490041-01), Appendix 17 (Document 16-RSB0011), Appendix 18 (Document M-485425-02), Appendix 19 (Document TXKIS002), Appendix 20 (Document TXFAS015), Appendix 29 (Document 17-RSSB0044-C), Appendix 30 (Document 00021233), and Appendix 31 (Document 17-RSSB0044-P) contain confidential commercial information. A formal request for this information to be treated as such has been submitted to FSANZ.

1.8 Other Confidential Information

BASF requests that versions of supporting documents submitted with this application that have privacy information removed only are provided to any interested members of the public upon completion of the FSANZ review. The documents included in this request include: Statutory Declaration (Appendix 1), M-455339-04, M-611752-01, SEL/6495/3, M-478215-01, M-478845-01, M-515776-01, M-476249-01, M-476906-01, M-515772-01, M-538392-02, M-496627-03.

1.9 Exclusive Capturable Commercial Benefit (ECCB)

The application is expected to confer an ECCB upon BASF since it will contribute to facilitating commercial activities with GMB151 soybean, once commercially preferred varieties are available, firstly in the USA and Brazil, followed possibly by other soybean producing countries.

1.10 International and Other Standards

All data in relation to GMB151 and the Cry1Ab-1 and HPPD-4 proteins and studies included in the information supporting this application have been conducted according to international standards. In the safety assessment of biotechnology products, BASF refers primarily to the *Codex Alimentarius* Commission weight-of-evidence approach (CAC, 2009), and the relevant Codex Standard is:

Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants. CAC/GL 45-2003. Adopted in 2003, Annexes II and III adopted in 2008. (CAC, 2009).

Other guidelines and recommendations are also considered including those of the World Health Organisation (WHO), the United Nations Food and Agriculture Organisation (FAO), the United States Food and Drug Administration (US-FDA), the United States Environment Protection Agency (US-EPA), and the European Food Safety Agency (EFSA) (see CAC, 2009 above; EFSA, 2011; FAO/WHO, 2001; US-FDA, 2012).

1.11 Statutory Declaration

Included in the application to FSANZ, which is appended as an electronic document at Appendix 1 within the DVD which contains the submission.

1.12 Checklist for the Application

General requirements (3.1.1)		
Check	**Page No.**	**Mandatory requirements**
⊗	13	A Form of application ⊗ Application in English ⊗ Executive Summary (separated from main application electronically) ⊗ Relevant sections of Part 3 clearly identified ⊗ Pages sequentially numbered ⊗ Electronic copy (searchable) ⊗ All references provided
⊗	15	B Applicant details
⊗	16	C Purpose of the application
⊗	16 – 18	D Justification for the application ⊗ Regulatory impact information ⊗ Impact on international trade
⊗	18	E Information to support the application ⊗ Data requirements
⊗	19	F Assessment procedure ⊗ General ☐ Major ☐Minor ☐ High level health claim variation
⊗	19	G Confidential commercial information ⊗ *CCI material separated from other application material* ⊗ Formal request including reasons (included in cover letter) ⊗ Non-confidential summary provided
⊗	19	H Other confidential information ⊗ Confidential material separated from other application material ⊗ Formal request including reasons (included in cover letter)
⊗	20	I Exclusive Capturable Commercial Benefit ⊗ Justification provided
⊗	20	J International and other national standards ⊗ International standards ☐ Other national standards
⊗	20	K Statutory Declaration (included as Appendix 1)
⊗	21	L Checklist/s provided with application ⊗ 3.1.1 Checklist ⊗ All page number references from application included ⊗ Any other relevant checklists for Chapters 3.2–3.7 (see below for relevant section of Chapter 3.5)

Foods produced using gene technology (3.5.1)		
Check	**Page No.**	**Mandatory requirements**
⊗	22	A.1 Nature and identity
⊗	24	A.2 History of use of host and donor organisms
⊗	41	A.3 Nature of genetic modification
⊗	61	B.1 Characterisation and safety assessment
⊗	88	B.2 New proteins
⊗	111	B.3 Other (non-protein) new substances
⊗	111	B.4 Novel herbicide metabolites in GM herbicide-tolerant plants
⊗	113	B.5 Compositional analyses
⊗	128	C Nutritional impact of GM food
⊗	128	D Other information

Part A Technical Information on the Food Produced Using Gene Technology

A.1 Nature and Identity of the Genetically Modified Food

(a) A description of the GM organism from which the new GM food is derived. The description must include the nature and purpose of the genetic modification.

The GM organism is cultivated soybean (*Glycine max* L.). GMB151 soybean was developed through disarmed Agrobacterium-mediated transformation using the vector pSZ8832 containing the *cry14Ab-1.b* and *hppdPf-4Pa* gene cassettes. GMB151 soybean produces the Cry14Ab-1 protein, a crystal protein derived from *Bacillus thuringiensis*, which confers resistance to the plant-parasitic nematode, soybean cyst nematode (*Heterodera glycines*) and *Pratylenchus*. GMB151 also produces a modified 4-hydroxyphenylpyruvate dioxygenase (HPPD-4), derived from *Pseudomonas fluorescens*, which confers tolerance to HPPD inhibitor herbicides, such as isoxaflutole. The OECD identifier of GMB151 soybean is BCS-GM151-6.

The GMB151 event introduced two genes to the *G. max* genome:

(i) (i) The *cry14Ab-1.b* gene encodes for the Cry14Ab-1 protein. The *cry14Ab-1.b* coding sequence was derived from *Bacillus thuringensis*. The Cry14Ab-1 protein is a member of Cry (crystal)-type protein family with fully conserved three-domain structure. Cry proteins are produced by *Bacillus thuringiensis* strains and demonstrate specific toxicity towards insects or nematodes. FSANZ has previously assessed an extensive range of Cry proteins for food safety. This is the first time that the Cry14Ab-1 protein has been assessed for food safety in Australia.

(ii) The *hppdPf-4Pa* gene encodes for the HPPD-4 protein. The *hppdPf-4Pa* coding sequence was developed by introducing four amino acid mutations to the wild type *hppd* gene derived from *Pseudomonas fluorescens* strain A32. Expression of the HPPD-4 protein confers tolerance to HPPD inhibitor herbicides such as isoxaflutole.

The *cry14Ab-1.b* gene is the sequence coding for Cry14Ab-1 protein in GMB151 soybean. The Cry14Ab-1 protein is a crystal protein derived from *Bacillus thuringiensis* as described above under (i), and is expressed by GMB151 soybean for protection against plant-parasitic nematodes.

The coding sequence of the 4-hydroxyphenylpyruvate dioxygenase (HPPD) protein was isolated from the *Pseudomonas fluorescens* strain A32. Four amino acids were substituted

(the coding sequence was modified by the replacement of the amino acid residues [redacted]. The four introduced mutations lead to reduced HPPD inhibitor herbicide binding efficacy. The modified protein is designated as HPPD-4 ([redacted], 2016, M-461393-02, Appendix 2).

(b) The name, line number and OECD Unique identifier of each of the new lines or strains of GM organism from which the food is derived.

The transformation event is named “GMB151”, and soybean transformed with this event will be referred to as GMB151 soybean. The OECD Unique identifier of GMB151 soybean is BCS-GM151-6.

(c) The name the food will be marketed under (if known).

This is unknown as this application is related to a commodity crop rather than a specific food or additive.

A.2 History and Use of the Host and Donor Organisms

The common and scientific names of the host and donor organisms must be stated.

The taxonomic classifications of the organisms from which the genetic elements of GMB151 soybean are derived are presented below in Table 2.

Soybean *Glycine max*
The host species, *G. max* belongs to the subgenus *Soja*, which also contains *G. soja* and *G. gracilis*. *Glycine soja* is the wild species of soybean and is endemic in many Asian countries. Cytological, morphological and molecular evidence suggest that *G. soja* is the ancestor of *G. max*. *Glycine gracilis* is considered to be a weedy or semi-wild form of *G. max*, with some phenotypic characteristics intermediate to those of *G. max* and *G. soja*. *Glycine gracilis* may be an intermediate in the speciation of *G. max* from *G. soja* or a hybrid between *G. soja* and *G. max* (OECD, 2012).

cry14Ab-1.b gene
Soil isolates of *B. thuringiensis* are considered ubiquitous in natural environments and are distributed throughout the world. The genus *Bacillus* consists of more than 20 different spore-forming, gram-positive, rod-shaped bacteria that produce insecticidal crystal proteins (known as “Bt” or “Cry” proteins) during sporulation. Bt proteins have been studied for almost a century and their structure and function are well understood. These proteins are classified as CryI, CryII, CryIII or CryIV depending on their amino acid homology and specificity to insect larvae of the orders Lepidoptera, Diptera and Coleoptera (Kuo and Chak, 1996). Since cloning the first *cry* gene in 1981 (Schnepf and Whitely, 1981), numerous different *cry* genes have been identified (by 2005; OECD, 2007), and those used in agriculture are not toxic to humans or mammals (Betz *et al*., 2000) and are generally classified as non-pathogenic.

Bt proteins are considered one of the most successful biological agents for the control of agricultural insect pests (Kuo and Chak, 1996). Microbial formulations of *B. thuringiensis* were first registered as insecticides in the USA in 1961, and today are registered in many countries worldwide for use in agriculture, forestry and mosquito control (OECD, 2007). More than 100 microbial *B. thuringiensis* products have been registered so far (USEPA, 2007) and account for 1-2% of the global insecticide market (Schnepf *et al*., 1998). Some of the commercially available *B. thuringiensis* formulations include the subspecies *tenebrionis* (e.g. for control of Colorado potato beetle larvae), *kurstaki* (e.g. for control of caterpillar pests), *israelensis* (e.g. for control of mosquito larvae), and *aizawai*. The HD1 isolate of subspecies *kurstaki* produces several Bt proteins and is used as the active ingredient of commercial products such as DiPel® and Foray 48B®. Bt formulations can be sprayed on crops up to just prior to harvest, including on vegetables that are eaten raw with minimal washing such as broccoli, cucumber and lettuce (Rosenquist *et al*., 2005). Therefore, it is expected that humans would frequently directly

consume Bt spores and insecticidal proteins. Numerous acute oral, acute pulmonary, acute intravenous, acute dermal, primary eye irritation, hypersensitivity, subchronic, and chronic toxicity studies in mice, rats, rabbits, and guinea pigs have been conducted using concentration up to 1000-fold higher than field application rates and have demonstrated no significant long-term risks to mammals (Fisher and Rosner, 1959; McClintock *et al*., 1995; Siegel, 2001).

Bacillus thuringiensis microorganisms have been found throughout the natural environment in soils, grain, on leaf surfaces, and in water, as well as in food (e.g. pasta, bread and processed foods that contain flour), animals (e.g. deer, rodents, insectivore mammals), and probably humans through the consumption of foods sprayed with Bt insecticides (OECD, 2007). Strains of *B. thuringiensis* are generally classified as non-pathogenic bacteria; if *B. thuringiensis* were pathogenic to humans, serious illness would be a common occurrence given the long history of frequent and repeated exposure. Extremely rare clinical cases of human infection by *B. thuringiensis* resulting from exposure to commercial products suggest that under highly unusual circumstances they may be an opportunistic pathogen (Siegel, 2001). However, a causal relationship between *B. thuringiensis* subspecies *kurstaki* and the reported gastrointestinal illnesses similar to food poisoning have not been definitively established (Siegel, 2001; Damgaard *et al*., 1997; Hernandez *et al*., 1998; Samples and Buettner, 1983). Studies of environmental and occupational exposure to *B. thuringiensis* formulations have also failed to reveal a causal relationship between the bacterium and any adverse effects on human health. Farm workers have on occasion reported occupational sensitisation to *B. thuringiensis* formulations (Bernstein *et al*., 1999; Doekes *et al*., 2004). However, epidemiological studies of human populations exposed to frequent and repeated aerial applications of *B. thuringiensis* formulations in residential areas revealed little, if any, effect on human health (Levin, 2005; Green *et al*., 1990; Valadares De Amorim *et al.*, 2001; Pearce *et al.*, 2002).

hppdPf-4Pa gene
The coding sequence of the of the 4-hydroxyphenylpyruvate dioxygenase (HPPD) protein was isolated from the *Pseudomonas fluorescens* strain A32 (Genebank A69533; McKellar, 1982) The coding sequence was modified to produce the HPPD-4 protein which confers tolerance to HPPD inhibitors such as isoxaflutole.

Pseudomonas fluorescens Migula 1895 (type strain ATCC 13525; taxonomy ID: 136843; Skerman, 1980)*, Pseudomonas putida* and *Pseudomonas chlororaphis* are closely related to each other and are seen as forming a complex within the fluorescent subgroup of the *Pseudomonas* genus. In addition, *P. fluorescens* is a heterogeneous species comprising several biovars, each of which may deserve species rank, but which are so interconnected that adequate methods have not been devised to clearly separate them (OECD, 1997). *Pseudomonas fluorescens* are Gram-negative, rod-shaped, motile, asporogenous, aerobic bacteria that produce fluorescent pigments and are catalase and oxidase-positive. *Pseudomonas fluorescens* strains are generally not able to grow above 42°C, but grow at 5°C (OECD, 1997; Palleroni, 1981). This organism is a nonpathogenic saprophyte which inhabits soil, water and plant surface environments. It is able to produce a soluble, greenish fluorescent pigment, which relates to its name.

Regulatory Sequences
In the *cry14Ab-1.b* gene expression cassette, the *cry14Ab-1.b* gene coding sequence is under the control of the ubi10 promoter of *Arapidopsis thaliana* (Pubi10At; Grefen *et al*., 2010), followed and terminated by the 3′ untranslated region of the 35S transcript of the Cauliflower Mosaic Virus (CaMV, Sanfaçon *et al.*, 1991).

The *hppdPf-4Pa* gene coding sequence is under the control of the sequence including the double enhanced P2x35S promoter region of the Cauliflower Mosaic Virus (CaMV; Kay *et al.,*

1987), the Ltev sequence including the leader sequence of the Tobacco Etch Virus genomic RNA (TEV, Allison *et al*., 1985), and by the optimized transit peptide as described by Lebrun *et al.* (1996) and terminated by the 3′ untranslated region of the 35S transcript of the Cauliflower Mosaic Virus (CaMV, Sanfaçon *et al.*, 1991).

The plant species *Arabidopsis thaliana,* common name mouse-ear cress, is member of the family Brassicaceae. *Zea mays*, more commonly know as maize or corn, is a member of the Poaceae family. *Helianthus annuus*, or sunflower, is a member of the family Asteraceae. These plant species are not considered to cause disease in humans, plants or animals.

Cauliflower mosaic virus (CaMV) is a pararetrovirus of the family Caulimoviridae. Caulimoviruses are spherical or bacilliform plant viruses containing a circular, double-stranded (ds) DNA genome of 7.1 to 8.2 kb. CaMV can infect a wide range of crucifers (Schoelz and Bourque, 1999) and is commonly found in cabbages, cauliflowers, oilseed rape, mustard and other brassicas in temperate countries (Tomlinson, 1987). This promoter has been widely used in plant biotechnology and is known to have a history of safe use.

Agrobacterium tumefaciens (Depicker et al., 1982) is a soil born, gram-negative bacterium that has been extensively studied since it was identified as the causative agent of crown gall disease in plants. *Agrobacterium tumefaciens* and *A. rhizogenes* are two well known prokaryotic organisms capable of transferring DNA to the eukaryotic cell (De Groot *et al*., 1998). This gene transfer ability may have evolved from bacterial conjugal transfer systems which mobilise plasmids for transfer between bacterial cells (Stachel and Zambryski, 1986) and is exploited in biotechnology. Consequently, *A. tumefaciens* is a widely used transformation system in plant biotechnology.

Table 2 Taxonomy of the donor organisms from which the genetic elements of GMB151 soybean are derived

GENETIC ELEMENT	DONOR ORGANISM TAXONOMY							
	Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Scientific Name**	**Common Name**
Plant Genome								
Genomic DNA	Viridiplantae	Streptophyta	Magnoliophyta	Fabales	Fabaceae	*Glycine*	*Glycine max* (L.) Merr. (2n=40)	Soybean
Gene Construct								
T35S	Viruses	Retro-transcribing viruses	-	-	Caulimoviridae	Caulimovirus	Cauliflower mosaic virus	CaMV
cry14Ab-1.b	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	*Bacillus thuringiensis* (Genbank accession number	
Pubi10At	Plantae	Magnoliophyta	Magnoliopsida	Brassicales	Brassicaceae	Arabidopsis	*Arabidopsis thaliana*	mouse-ear cress
T35S	Viruses	Retro-transcribing viruses	-	-	Caulimoviridae	Caulimovirus	Cauliflower mosaic virus	CaMV
hppdPf-4Pa	Bacteria	Proteobacteria	*Gammaproteobacteria*	Pseudomonadales	Pseudomonadaceae	Pseudomonas	*Pseudomonas fluorescens*	
TPotpY-1Pf	Plantae	Anthophyta	Liliopsida	Poales	Poaceae	Zea	*Zea mays*	corn
	Plantae	Tracheophyta	Magnoliopsida	Asterales	Asteraceae	Helianthus	*Helianthus annuus*	sunflower
Ltev	Viruses	-	-	-	Potyviridae	Potyvirus	Tobacco Etch Virus	TEV
P2x35S	Viruses	Retro-transcribing viruses	-	-	Caulimoviridae	Caulimovirus	Cauliflower mosaic virus	CaMV

https://www.ncbi.nlm.nih.gov/taxonomy (Accessed 1 February 2019)

Where information relating to an organism has been included in previous safety assessments prepared by FSANZ, it is not necessary to provide any further information. Where an organism has not been considered previously by FSANZ, the following information must be provided. A package of data has been provided to FSANZ for GMB151 soybean as the proteins Cry14Ab-1 and HPPD-4 are closely related to numerous proteins that have been previously approved by FSANZ in the case of Cry proteins, and in the case of HPPD-4, the protein is closely related to the HPPD W336 protein that has been previously assessed by FSANZ. Data requirements as per the July 2019 Application Handbook are addressed here.

(a) For the donor organism(s) from which the genetic elements are derived:

(i) Any known pathogenicity, toxicity or allergenicity of relevance to the food;

Soybean (*Glycine max)*

Anti-nutrients in Soybean
There are a several compounds in legumes, and therefore also in soybeans, which are not favourable for human or animal nutrition. These anti-nutritional factors include phytic acid, raffinose and stachyose, protease inhibitors, and hemagglutinins (lectins).

Phytic acid
In most plant tissues, large portions of phosphorus are present in form of phytic acid (1,2,3,4,5,6-hexakis (dihydrogen phosphate) myo-inositol). Phytic acid is regarded as the primary storage form of phosphorus and inositol in almost all seeds. During seed germination, phytin, the calcium-magnesium salt of phytic acid, is hydrolysed by the enzyme phytase and serves as a source of inorganic phosphorus and cations for the emerging seedling. The term phytate is used for the mono to dodeca anion of phytic acid (Ravindran *et al.* 1994; Maga, 1982).

Two-thirds of the phosphorus in soybeans is bound as phytate and unless freed is mostly unavailable to animals (Liener, 1994). Ruminants are able to utilise considerably more phosphorus, since rumen microbes produce phytase that breaks down phytate and releases phosphorus. Phytic acid also chelates mineral nutrients including calcium, magnesium, potassium, iron, and zinc, rendering them unavailable to monogastric animals consuming the beans. In fact, phytic acid chelation of zinc present in corn-soybean meal diets used for growing swine requires supplements of zinc to avoid a parakeratosis (OECD, 2001c). It is becoming common for feed formulators to add a phytic acid degrading enzyme, phytase, to swine and poultry diets to release phytin-bound phosphorus, so that the amount of this mineral added to the diet can be decreased, potentially reducing excess phosphorus in the environment. Phytic acid also impacts on protein bioavailability and enzyme activity since it is a strong anion and it can interfere with the polar side groups of proteins leading to complexion of nutritional proteins or changes in the molecular conformation of enzymes (Fretzdorff and Brümmer, 1992).
Phytic acid contents reported for soybean seeds are 1.0 - 1.5% (Liener, 1994). However, higher values, up to 2.74% have also been reported (Douglas, 1996).

Raffinose and stachyose
The low molecular weight carbohydrates, stachyose and raffinose, are present in defatted toasted soybean meal, as well as in raw soybeans. Raffinose is a trisaccharide containing galactose, glucose and fructose. Stachyose is a tetrasaccharide built of two galactose, one glucose and one fructose molecule. They are considered anti-nutrients, because they remain unhydrolysed in the small intestine of monogastric animals and humans due to a lack of galactosidase and hence are not absorbed. They then pass into the large intestine where microbial fermentation converts them to CO_2, the main components of flatus (Vaidehi and

Kadam, 1989). The raffinose content of soybean seeds ranges from 0.1- 0.9 g per 100 g on a fresh weight basis, while stachyose content is from 1.4 - 4.1 g per 100 g. Further processing of soybean meal into concentrate or isolate, reduces or removes, these oligosaccharides.

Protease inhibitors

Protease inhibitors are typical anti-nutritional compounds present in soybeans, cereals and potatoes. Two types of protease inhibitors are present in soybeans: the Kunitz inhibitor and the Bowman-Birk inhibitor. Trypsin inhibitors are proteins with molecular weights between 6 - 46 kDalton, which form inactive complexes with the proteinase trypsin. The Kunitz inhibitor and the Bowman-Birk inhibitors are active against trypsin, while the latter is also active against chymotrypsin (Liener, 1994). These protease inhibitors interfere with the digestion of proteins resulting in decreased animal growth. The activity of these inhibitors is destroyed when the bean or meal is toasted or heated during processing.

Lectins

Lectins are proteins that bind to carbohydrate-containing molecules. Lectins in raw soybeans can inhibit growth and cause death in animals and it is expected that similar effects would occur in humans (Liener, 1994). The ability of lectins to act as hemagglutinins that cause blood clotting is the basis for most quantitative analytical methods. Soybean lectin is sometimes referred to as soybean hemagglutinin. Lectins are rapidly degraded upon heating but are quite resistant to dry heat.

Isoflavones

Soybeans naturally contain a number of isoflavone compounds reported to possess biochemical activity, including estrogenic, anti-estrogenic, and hypocholesterolemic effects, in mammalian species. These compounds have been implicated in adversely affecting reproduction in animals fed diets containing large amounts of soybean meal (Shutt, 1976). However, it is not universally accepted that isoflavones are antinutrients as they have also been reported to have beneficial anti-carcinogenic effects (Messina and Messina, 1991).

The isoflavones in soybeans and soy products have three basic types: daidzein, genistein, and glycitein. Each of these three isomers, known as aglucones or free forms, can also exist in three conjugate forms: glucoside, acetylglucoside, or malonylglucoside. Therefore, in total there are twelve isomers of isoflavones in soybeans. The isoflavone content of soybeans is greatly influenced by many factors, including variety, growing locations, planting year, planting date and harvesting date. In literature reports on isoflavone contents of soybeans, the specific substances investigated, the analytical methods and the reporting conventions have differed widely from report to report (Douglas, 1996). Isoflavones are heat stable and not destroyed by toasting of soybean meal (Liener, 1994).

Allergies to Soybeans

Several soybean food allergies have been recorded in most countries of the world (Ballmer-Weber and Vieths, 2008). Clinical reactions are similar to those observed with other major food allergens (Besler *et al*., 2000). In the absence of epidemiological data, the estimated prevalence of soybean allergies could be 0.5% in the general population (Sicherer and Sampson, 2006; Ballmer-Weber and Vieths, 2008). Due to its widespread use in the food and beverage industry, soybean allergens are often hidden ingredients. Therefore, labelling regulations (e.g. from Codex, US Food and Drug Administration, European Union) incorporate soybean as part of the major allergenic food lists that should be labelled (Codex 1985, rev. 2018; EU, 2003; US-FDA, 2004).

Saline extracts of soybeans have been reported to contain several antigenic proteins that stimulate the rabbit systemic immune system after injection and/or orally sensitise guinea pigs, calves, pigs, and humans. The presence of these allergenic proteins in the diet of hypersensitive individuals can cause severe adverse reactions in the gastrointestinal tract. The allergenic effect is attributed to the globulin fraction of soybean proteins that comprise

about 85% of total protein. When compared to soybean seeds, sprouts exhibit a similar ability to bind IgE from soy-allergic individuals. A number of immunological or immunochemical tests have been developed to examine allergenic proteins usually based on sera from sensitive subjects (OECD, 2001c).

Many soybean allergenic proteins have been identified, characterized and recorded in multiple allergen databases. AllergenOnline (www.allergenonline.org; update in February 2019), from the Food Allergy Research and Resource Program (FARPP) program, contains the highest number of allergens (see Table 4) and provides a robust resource for searching potential similarities with other proteins. These allergens belong to five major protein families: beta-conglycinin, glycinin, Kunitz trypsin inhibitor, Bd 28K, and Bd 30K. These families have conserved structural features in relation with their biological activity, which explains the wide immunochemical cross-recognition observed among members of the legume family (Ballmer-Weber and Vieths, 2008).

cry14Ab-1.b gene and Cry14Ab-1 protein

Assessments of the *Bacillus thuringiensis* source organism, the *cry14Ab-1.b* gene, and the Cry14Ab-1 protein indicate that they are not pathogenic, allergenic, or toxic to mammals.
The *B. thuringiensis* source organism is a safe microorganism widely used for in food and feed agronomy with little to no pathogenic, toxic, or allergenic effects on humans and animals. The *cry14Ab-1.b* gene is composed of the same essential nucleic acids found in any food or feed DNA, which is commonly consumed as part of human or animal diets. Decades of research have indicated that dietary DNA poses no direct toxicity to human health. The Cry proteins are ubiquitous in nature, and widely consumed from food and feed crops that have been treated with foliar *B. thuringensis* products, or from food and feed crops that express these proteins (e.g. soybean and maize). No health-related adverse effects have been associated with these proteins. Since the Cry14Ab-1 protein is derived from a soil microorganism, the safety profile of the novel protein is expected to remain unchanged relative to its wild-type counterpart. The Cry14Ab-1 protein is highly homologous to and shares similar molecular weight and functionalities with other Cry proteins which have been demonstrated to be non-toxic and non-allergenic over the years through consumption.

hppdPf-4Pa gene

Pathogenicity to humans

The gene *hppdPf-4Pa* in event GMB151 is derived from *P. fluorescens. P. fluorescens* can be an opportunistic pathogen in immunocompromised patients (McKellar, 1982). Some cases of septicemia have been reported due to *P. fluorescens* contamination of transfused blood and blood products, given its ability to grow at 5°C (Gibb *et al*., 1995, Puckett *et al*., 1992). Some *P. fluorescens* strains were also reported to create biofilms on compounded sterile products like catheters and have led to rare infections in immunocompromised populations (Gershman *et al.,* 2008). However, the general virulence of *P. fluorescens* is low due to its inability to multiply rapidly at body temperature and having to compete with defense mechanisms of the host (Liu, 1964).

Pathogenicity to animals

P. fluorescens can infect a wide range of animals including horses, chickens, marine turtles, and many fish and invertebrate species. However, since it is unable to grow at elevated temperatures, it is probably only an opportunistic pathogen for warm-blooded animals (OECD, 1997a).

Pathogenicity to plants

Generally *P. fluorescens* is considered saprophytic but it may be an opportunistic pathogen causing soft rot in plants (OECD, 1997a).

Allergenicity
In general fluorescent pseudomonads have not been described as allergens. However, they do possess an endotoxin (lipopolysaccharide) which may induce an allergic response in some individuals (OECD, 1997a).

Table 3 Gazetted FSANZ Standards for events encoding for the expression of Cry family of proteins and HPPD family of proteins

CROP	EVENTS/LINES EVALUATED	FSANZ APPLICATION NUMBER
Canola	None expressing the Cry family of proteins or HPPD family of proteins	N/A
Cotton	531, 757, 1076, 15985, MXB-13, COT67B, T304-40, GHB119, GHB811 (HPPD W336 protein), MON88702	A341, A436, A518, A615, A1028, A1040, A1147, A1154
Lucerne	None expressing the Cry family of proteins or HPPD family of proteins	N/A
Maize	MON810, DBT418, Bt-176, Bt-11, 1507, MON863, 59122-7, MON88017, MIR604, MON89034, 5307, MON87411, 4114, MZIR098	A346, A380, A385, A386, A446, A484, A543, A548, A564, A595, A1060, A1097, A1106, A1116
Safflower	None expressing the Cry family of proteins or HPPD family of proteins	N/A
Soybean	MON 87701, FG72 (HPPD W336 protein)	A1035, A1051
Sugarbeet	None expressing the Cry family of proteins or HPPD family of proteins	N/A
Wheat	None expressing the Cry family of proteins or HPPD family of proteins	N/A

Regulatory sequences
The promoter and terminator sequences used in GMB151 are derived from common plants, soil bacteria or plant pathogens. These genetic elements constitute a minute component of their respective genomes, no genes that may be implicated in human disease, allergies or toxic effects have been transferred. Many of these organisms from which these elements are derived are model species in plant science with a history of safe use. These elements are described in Table 2, Section A.2.

(ii) history of use of the organism in the food supply or history of human exposure to the organism through other than intended food use (e.g. as a normal contaminant).

Soybean (Glycine max)
Cultivated soybean, *Glycine max* (L.) Merr, is grown as a commercial crop in over 35 countries. Today the major producers of soybeans are the United States, China, Democratic People's Republic of Korea and Republic of Korea, Argentina and Brazil. Soybean is one of the oldest cultivated crops, native to North and Central China. The first recording of soybeans was in a series of books known as Pen Ts'ao Kong Mu written by the emperor Sheng Nung in the year 2838 B.C., in which the various plants of China are described. Historical and geographical evidence suggests that soybeans were first domesticated in the eastern half of China between the 17th and 11th century B.C. (OECD, 1997b). Domestication occurred over many centuries and was highlighted during the Shang Dynasty about 1700-1100 B.C. During the period of strong emperors, soybean remained only in China. In later centuries, increased trading and emigration brought soybean germplasm to other areas of Southeast and South-central Asia, which became the secondary centre of soybean germplasm. These events occurred during the 1st through the 15 - 16th century A.D. (Hymowitz *et al*., 1981). Soybeans were first introduced into the United States, now a major producer, in 1765 (OECD, 1997b), and became established as an oilseed crop by the late 1920s. By World War II soybeans attained major commercial importance, and in the present day soybeans belong to the four principal oilseed crops in the US (soybean, cottonseed, peanuts and sunflowers) (Hui, 1992).
Soybean is grown primarily for the production of seed, has a multitude of uses in the food and industrial sectors, and represents one of the major sources of edible vegetable oil and of proteins for livestock feed use. A major food use is purified oil, utilised in margarines, shortenings and cooking and salad oils. Other food products include tofu, soya sauce, simulated milk and meat products. Soybean meal is also used as a high protein supplement in feed rations for livestock. Industrial uses of soybeans range from the production of yeasts and antibodies to the manufacture of soaps and disinfectants (OECD, 1997b).

cry14Ab-1.b gene
The *cry14Ab-1.b* gene encodes the Cry14Ab-1 protein, a crystal protein derived from *Bacillus thuringiensis,* which confers protection against soybean cyst nematode (*Heterodera glycines*) and *Pratylenchus*. Cry proteins produced by *Bacillus. thuringiensis* (*Bt*) strains demonstrate specific toxicity towards either insects or nematodes. Cry14Ab-1 belongs to the "nematicidal branch" of Cry proteins with the closest homology to Cry14Aa1 (87% identity) (Sanahuja et al., 2011).

hppdPf-4Pa gene
The *hppdPf-4Pa* gene was isolated from *Pseudomonas fluorescens* strain A32. *Pseudomonas fluorescens* are ubiquitous bacterium in the natural environment and are frequently present in water, soil and plant rhizosphere (Bossis *et al*., 2000). The bacterium can be isolated from water, animals, human clinical specimens, the hospital environment, and spoiled foodstuffs such as fish and meat. The survival of *P. fluorescens* is affected by number of biotic and abiotic factors such as soil density, temperature, pH and humidity (OECD, 1997a).

The natural properties of *P. fluorescens* are exploited in agriculture for plant growth-promotion (Fliessbach *et al*., 2009; OECD, 1997a) and pest control. As a growth control agent, the bacterium can enhance plant growth through production of siderophores, which efficiently complex environmental iron rendering it unavailable to other organisms of the soil microflora. As a biopesticide, *P. fluorescens* is able to prevent the growth of frost-forming bacteria on leaves and blossoms of crops and fruits (Compant *et al*., 2005; Raaijmakers *et al*., 2006; US-EPA. 2008a), and prevent damping off diseases caused by fungi (Haas and Defago, 2005; Thrane *et al*., 2001; Voisard *et al*., 1989) and nematodes (Hamid *et al*., 2003) when used as a seed treatment. Naturally occurring strains of *P. fluorescens* have been registered commercially for the control of frost injury and fire blight on pear (Wilson and Lindow, 1993). Since 1992, four products containing *P. fluorescens* strains as active ingredients were approved by US-EPA (US-EPA, 2008b). The US-EPA recognized that this bacterial active ingredient is not expected to cause any adverse health effects in humans, based on various studies that found no evidence that these bacteria are harmful to mammals (US-EPA. 2008a). The US-EPA also established a tolerance exemption for residues of *P. fluorescens* in or on the raw agricultural commodity mushrooms (US-EPA, 1994). The pesticidal activity of *P. fluorescens* is attributed to three mechanisms: competition for an ecological niche or a substrate, production of inhibitory chemicals and induction of systemic resistance in host plants to a broad spectrum of pathogens (Compant *et al*., 2005; Haas and Defago, 2005).
In other applications, strains of *P. fluorescens* have been genetically modified to encapsulate crystal δ-endotoxins (Cry proteins) from the bacterium *Bacillus thuringiensis* (Bt) (Downing *et al*., 2000; Peng *et al*., 2003). The Cry proteins encapsulated by *P. fluorescens* showed high insecticidal activity and retained this activity for two to three times longer than Bt formulations (Peng *et al*., 2003). In pharmaceutical uses, *P. fluorescens* produces the antibiotic pseudomonic acid (also called mupirocin), which is used to prevent *Staphylococcus aureus* infections (Hothersall *et al*., 2007; Tacconelli *et al*., 2003). Further, in addition to the metabolic diversity of *P. fluorescens*, it may be used in bioremediation applications. The bacterium is able to degrade a wide variety of compounds, including 3-chlorobenzoic acid, naphthalene, phenathrene, fluorene and fluoranthene, chlorinated aliphatic hydrocarbons, styrene, pure hydrocarbons and crude oil (OECD, 1997a).

In summary, the source of the *hppdPf-4Pa* gene is ubiquitous in the environment, including soil, water and food. It has many beneficial uses in agriculture, human health and bioremediation. Despite this widespread presence, it is not described as allergenic, toxic or pathogenic to healthy humans and animals.

Regulatory sequences
The promoter and terminator sequences used in GMB151 are derived from common plants or plant pathogens. These genetic elements constitute a minute component of their respective genomes, no genes that may be implicated in human disease, allergies or toxic effects have been transferred. Many of the organisms from which these elements are derived are model species in plant science with a history of safe use.

(b) For the host organism into which the genes were transferred:

(i) Its history of safe use for food

Glycine max (L.) Merr., is a member of the *Leguminosae* plant family, sometimes referred to as the pea family. Its common names are soybean or soya bean. It is cultivated worldwide as a vegetable or oilseed crop. The earliest record showed that soybean has been cultivated since eleventh century BC in China. The *Glycine soja* is the wild counterpart of *Glycine max (G. max)*, which has significant phenotypic and compositional differences from the *G. max*. There are two main types of *G. max* soybeans, vegetable-type and oil-producing type.

Soybean is one of the oldest cultivated crops that were native to China, which was probably first domesticated in the eastern half of North China during the Zhou Dynasty around the eleventh century BC (Hymowitz, 2008; Qiu and Chang, 2010), when the soybean was primarily used as a food and component of medicine. The soybean varieties domesticated in China were mainly *G. max* and *G. soja*. From the first centry AD to the fifteen-seventeenth century AD, soybeans were quickly introduced from China into many surrounding countries, *e.g.*, Japan, Indonesia, Nepal, North India *etc*. The soybean disseminated into European countries at a very slow pace. In 1737, soybean appeared the first time in accountancy books in the Netherlands. In 1740, soybean seeds were planted in France. In those countries, soybeans were grown for taxonomic or display purposes (Hymowitz, 2008). Soybeans were not introduced into the North America until around 1765 by Samuel Bowen, a British seaman who started to grow the soybean crop in Georgia. In the US, the soybean was primarily for forage, as a source of oil, meal (for feed) and industrial products and had little use as grain prior to 1920. The soybean was first introduced to South America by the end of the nineteenth century, *e.g.*, Argentina in 1880 and Brazil in 1882. Soybean seeds were taken to Paraguay in the 1920s from the USA, Brazil, Argentina and Japan, and later arrived to Bolivia, Colombia, Uruguay, and Venezuela (Hungria *et al.*, 2015).

China was the largest soybean producer and exporter during the first half of the twentieth century (Qiu and Chang, 2010). Since the 1950s, soybean production developed rapidly in the USA, Argentina and Brazil. In 2016, these three countries became the largest soybean producers worldwide, representing around 81% of the entire soybean production.
The *G. soja* is the wild counterpart of the *G. max*. However, they have significant phenotypic and compositional differences. The *G. soja* has mainly tiny, black seeds while *G. max* has large yellow seeds. In addition, there are differences in the seed oil and protein concentrations. The seed oil concentration is around 8% in *G. soja* versus 25% in *G. max* (Joshi and Nguyen *et al.*, 2013). However, the seed oil produced by *G. soja* generally has twice the alpha-linolenic acid (ALA) content than that from the *G. max* (Chae and Lee *et al.*, 2015). The ALA is an essential fatty acid for humans and must be obtained in foods or from dietary supplements. The *G. soja*, also contains higher protein than cultivated soybean, as high as 55.7% (Li, 1990).

A small percentage of soybean varieties is cultivated as fresh vegetable, *e.g.*, edamame (Hartman *et al.*, 2011). The current report will focus on the soybean varieties primarily used for producing oil and serving as food and feed for humans and animals (see Section 6).

Soybean is extensively used as livestock feed, dietary protein, and oil for human consumption, and is a component of thousands of processed products. Figure 1 provides an overview of the food, feed, and industrial uses of the different soybean products.

Livestock feed is the major use of soybeans, primarily in the form of soybean meal. Globally, around 98% of soybean meal is used for poultry, pork, cattle and other farm animals and pet feed. In the US, around 50% of soybean feed is for poultry, around 30% for swine, 20% for beef cattle and dairy cattle, and a couple of percentage is for fish and pet (Cromwell, 2012). Soybean meal provides an ideal nutritional balance to amino acid profiles found in corn, sorghum, barley, and wheat.

Soybeans are widely used in human food. Soybean oil for human consumption accounts for 68% of its use in the US. Soybean oil is used in cooking, baking, and frying foods. In liquid or partially hydrogenated form, it is widely used as vegetable oil, in margarine, ingredients to produce salad dressings, baked goods, crackers, barbecue sauce, shortening, non-dairy creamers, potato chips, mayonnaise, breads and whipped topping, and as imitation diary and meat products (Soyconnection, 2018). Soybean meal typically is used as a protein alternative source and as a basis for soymilk which constitutes 2% of its use. In Eastern Asian countries, like China, Japan and Korea, tofu, soysauce, soybean paste, miso, natto, and soybean sprouts are foods consumed by local people widely and made of soybean and soybean products (US Soy, 2018).

Soybean has many industrial uses. In the US, soybean oil comprises the main part of biodiesel, which contributes to 80% of domestic biodiesel production (National Biodiesel Board, 2008). The

burning of biodiesel by soybean oil is cleaner with reduced emission of particulates than that produced by petroleum (NC soybean producers association, 2018). Soybean oil is also used in printing inks, oil paints, and as a drying oil in manufacturing paints and coating applications (Johnson and Myers, 1995; Soyconnection, 2018). Refined soybean oil is transformed into a variety of industrial products, including resins, esters, plastics, fractionated oils *etc*. Soybean protein has many industrial uses, for example, in the manufacture of material for sausages and other processed meat, meat extenders and substitutes for specialized meat applications (E. Nwokolo, J. Smartt, 1996).

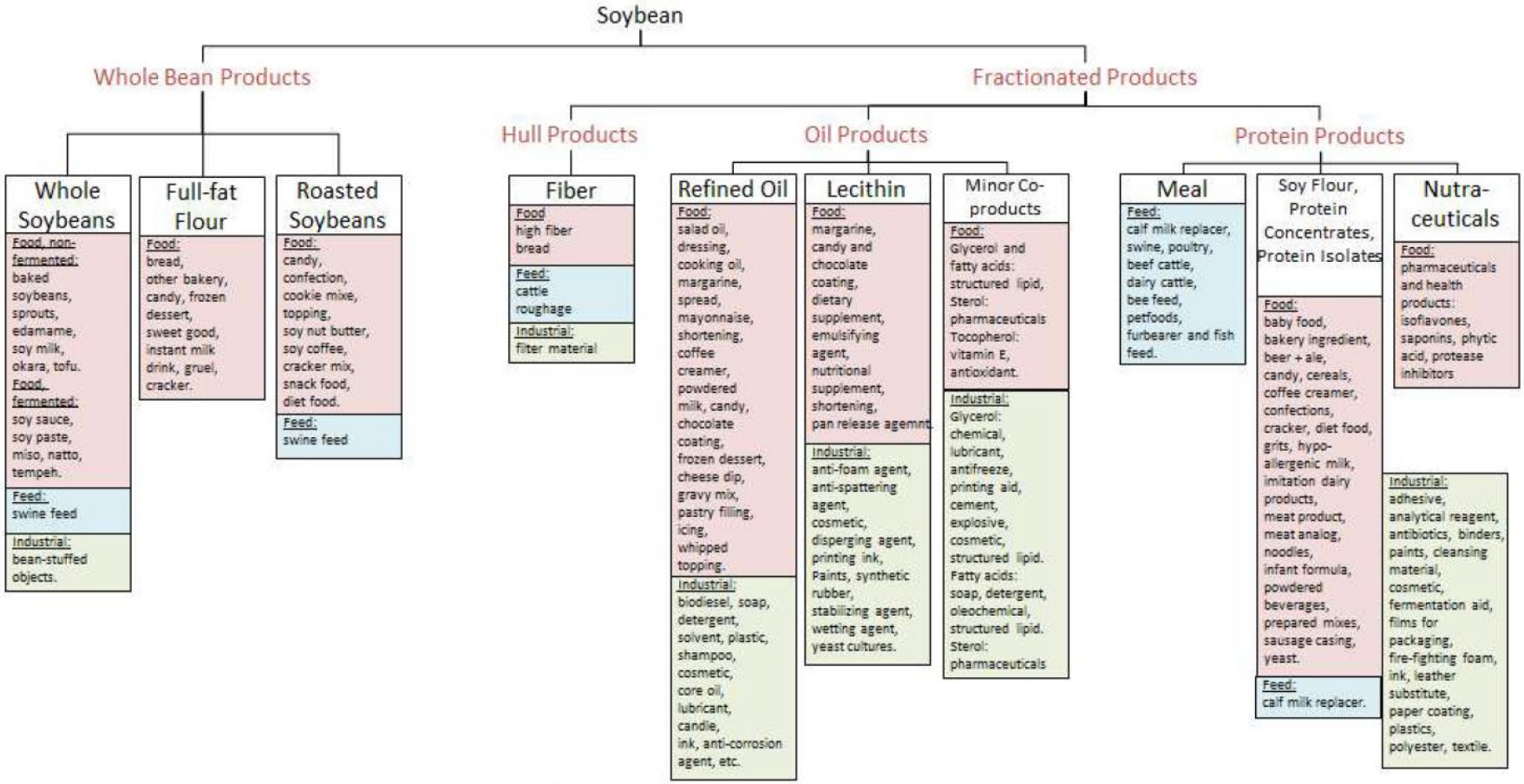


Figure 1. Food and processed products from soybeans.

The oligosaccharides, phytic acid, trypsin inhibitors, and lectins are the main anti-nutrients and toxins in the soybean seed and its derived meal.

Oligosaccharides

Oligosaccharides, including stachyose and raffinose, are low molecular weight carbohydrates in raw soybean seed and its processed products, *i.e.*, defatted and toasted soybean meal. These are similar oligosaccharides as in other foods like sunflower seeds, onions, cabbage and other beans. As there is lack of the enzyme in the intestine for hydrolyzing stachyose and raffinose, they may not be digested but fermented by microorganisms producing gas and flatulence. Therefore, stachyose and raffinose are considered as anti-nutrients in soybean, as they reduce nutrient digestibility and energy availability.

Phytic acid

Phytic acid (1,2,3,4,5,6-hexakis (dihydrogen phosphate) myo-inositol, or the salt form phytate) serves as the primary storage form of phosphorus and inositol in many plant tissues. During germination, it is hydrolysed by the enzyme phytase present in seeds and serves as a source of inorganic phosphorus and cation for the emerging seedling (Ravindran *et al.,*1994; Maga, 1982; OECD 2012).

The concerns with the anti-nutritional properties of phytic acid stem from its ability to chelate with bivalent ions like calcium, zinc, iron, phosphorus, copper, and magnesium (Kaufman, 1971; Vohra, 1966; Thompson, 1979; O'Dell, 1969; Erdman, 1979). It often renders these essential dietary minerals metabolically unavailable, or only partially available, for absorption in monogastric animals and humans and thus induces deficiencies (Maga, 1982; Ravindran *et al.,*1994; OECD 2012). Phytic acid also has a negative effect on protein bio-availability and enzyme activity. It is a strong anion and can interfere with the polar side groups of proteins, leading to complexes of nutritional proteins or changes in the molecular conformation of enzymes (Fretzdorff, 1992).

Trypsin inhibitors

Trypsin inhibitors are protein-based molecules which inhibit the activitiy of proteolytic enzymes and thus impair the protein digestibility efficiency, inadequacy in dietary sulfur amino acids, and pancreatic hypertrophy (Reddy and Pierson, 1994). There are two main groups of trypsin inhibitors: 1) the Kunitz inhibitors, which are heat-labile and active against mainly trypsin; and 2) the Bowman-Birk inhibitors which are also active on chymotrypsin (OECD 2012). The trypsin inhibitors are also present in other legume seeds (*e.g.*, peas, lentils and mung bean *etc*), in potatoes, and in cereals (Liener, 1994; Serna Saldivar *et al.*, 2018).

The inhibition capability is destroyed when the soybean seed or meal is subject to toasting or heat processing. The activity of trypsin inhibitors is destroyed the first time when soybean seeds are flaked before oil separation and again later when the meal is toasted for further processing. Padgette *et al.* (1996) have shown that the toasting process caused a reduction of trypsin inhibitor from 43 Trypsin Inhibition Units (TIU)/mg dry weight in soybean seed to 3 TIU/mg dry weight in soybean toasted meal. Most commercially available soybean products intended for human consumption, such as tofu, soy milk, soy-based infant formula, soy protein isolates and concentrates, and textured meal analogs, have received sufficient heat treatment to cause inactivation of at least 80% of the trypsin inhibitor activity present in raw soybeans (Liener, 1994).

Lectins

Lectins are proteins that bind carbohydrates and are highly selective for specific sugar moieties. They are found in many foods like beans, peas, corn, rice, peanuts and some berries (Vasconcelos and Oliveira, 2004). Some lectins can cause agglutination of cells when binding to carbohydrate

moieties on the cell's surface. The binding or agglutinating of lectins on epithelium cells disrupts the brush border membrane, enzymes and thus impair the nutrient digestibility and even growth in animals (Fasina and Clare *et al.*, 2003), hence they are sometimes considered to be antinutrients. Soybean lectin can be destroyed by moist heat treatment. Unlike trypsin inhibitors soybean lectin is quite resistant to inactivation by dry heat treatment (Liener, 1994).

Isoflavones are a class of phytoestrogens predominately in legumes and beans, *e.g.*, soybean. There are three types of isoflavones in soybeans: diadzein, genistein and glycitein. They exist in a the aglycone or free state or as conjugates such as, glucosides, acetylgucosides or malonylglucosides (OECD 2012). The isoflavones from soy are benefitial to human cardiovascular and bone health. However, due to the structural similarity of isoflavones to human estrogen, 17β-estradiol, it may interrupt endocrine function (Munro *et al.*, 2003) and affect reproduction in animals estrogenically at high exposure levels (OECD 2012). The relationship between isoflavones and its estrogenic effects still need further investigation as recent studies did not reveal its definitive adverse effect on endocrine function (Munro *et al.*, 2003).
Isoflavones are heat stable and not destroyed by toasting of soybean meal (Liener, 1994); hence, toasted soybean meal appears to have the same levels of phytoestrogens as raw soybean (Padgette *et al.*, 1996).

Soybean is among the eight most common allergenic foods for pediatric and adult food allergy patients, which account for 90% of immunoglobulin E (IgE)-mediated food allergies. Soy allergic reactions are typically mild but are unpredictable. The most severe allergic reactions, anaphylaxis, needs immediate medical treatment but this is rare.

Soy and soy products that may contain soybean allergens include: edamame, miso, natto, shoyu, soy flour, soy milk, soya, soybean curd, soy sauce, tofu *etc*. Highly refined soy oil is not considered as soy allergen because it doesn't contain soy proteins. In addition, processed food may also contain soy proteins due to the incorporation of soy flour and other soybean products, *e.g.*, baked goods, cereals, infant formulas, processed meats.

The most common allergic reaction is IgE-mediated, which occurs typically a few minutes to a few hours after the ingestion of the allergic food. Exposure to allergens through the gut, skin or respiratory system stimulates the production of allergen-specific IgE antibodies in plasma cells, and the allergen specific IgE attaches itself to the surface of mast cells in various tissues and basophils in the blood. The process is known as sensitization (Food Allergy Research and Resource Program). Subsequently, an onset of allergic reaction may be triggered post sensitization by releasing active mediators from the affected cells, *e.g.*, histamine.

Multiple allergens are listed for soybean in the COMPARE (comprehensive protein allergen resource) database (http://comparedatabase.org/). Some known and potential soybean allergens are shown in the following table. They are responsible for storage, enzymatic and protective protein functions, and associated with inhalation induced allergy (*e.g.*, Gly m 1, Gly m 2, and Kunitz trypsin inhibitor) or food allergy (P34, β-Conglycinin and glycinin).

The scientific guidance for selecting key allergens for measurement is based on clinically relevant data among publicly available allergen databases and peer-reviewed scientific publications, the abundance of the allergens in foods to be important for public health, and the ability to measure the identified allergen (Bjorksten et al., 2008; EFSA, 2010; Ladics et al, 2014; Selb et al., 2017).

Table 4 Known and potential soybean allergens:

No.	Allergen Nomenclature	Protein Family	Soybean Protein
1	Gly m 1	Lipid transfer protein	Hydrophobic seed protein
2	Gly m 2	Storage protein	Defensin
3	Gly m 3	Profilin	Soy profilin
4	Gly m 4	Bet v 1 family	SAM22, PR-10
5	Gly m 5	Cupin, vicilin-like proteins	β-Conglycinin, 7S globulin fraction, Gly m Bd 60 K
6	Gly m 6	Cupin	Glycinin, 11S globulin fraction
7	Gly m 7	n/a	Seed biotinylated protein
8	Gly m 8	Prolamin, plant LTP	2S albumin
9	Gly m Bd 30 K	Thiol proteinases of papain family, peptidase C1	Soybean vacuolar protein, P34
10	Gly m Bd 28 K	Vicilin-like glycoprotein, Cupin	Soy cupin, unknown Asn-linked glycoprotein
11	Soybean lectin	Agglutinin, Lectin legB	Soy agglutinin
12	Lipoxygenase	n/a	Lipoxidase
13	Kunitz trypsin inhibitor	Trypsin inhibitors	Soybean trypsin inhibitor, Gly TI
14	Gly m 39KD	n/a	P39, unknown 39K
15	Gly m 50KD	n/a	Unknown 50K
16	P22-25	n/a	n/a
17	Gly m CPI	Squash aspartic acid proteinase inhibitor	Cystatin, cysteine protease inhibitor
18	Gly m EAP	Late embryogenesis abundant protein	Embryonic abundant protein
19	Unknown possible allergen	n/a	Protease inhibitor/seed storage/LTP family

Source: OECD 2012; Selb *et al.* 2017; EFSA http://www.efsa.europa.eu/sites/default/files/consultation/160726-tableC.pdf; WHO/IUIS database http://www.allergen.org/ retrieved in June 2018.

(ii) The part of the organism typically used as food

The two primary products of soybeans used in food and feed, oil and meal respectively, are derived from the bean or seed. The various food (and feed) uses of these products are detailed above in Section A.2(b)(i).

(iii) The types of products likely to include the food or food ingredient

See the information under Section A.2 (b)(i) above.

(iv) Whether special processing is required to render food derived from the organism safe to eat.

Three basic methods are used to process soybeans for use as food as feed: solvent

extraction, hydraulic extraction and expeller extraction. Almost all soybean oil is extracted from the seed using the solvent process. Prior to processing, seeds are cleaned, cracked to loosen the seed coat or hulls, dehulled and then conditioned to 10 - 11% moisture. The conditioned meats are then flaked and extracted with hexane to remove the oil. Hexane and oil in the miscella are separated by evaporation and the hexane is recovered. Residual hexane in the flakes is removed by steam treatment in a desolventiser-toaster. The heat treatment inactivates antinutritional factors, such as trypsin inhibitors and lectins, in the raw flakes and increases protein digestibility. A metric ton of soybeans yields about 180 kg oil and 790 kg meal. (Hui,1992). Figure 2 below shows the solvent extraction process.

Soybean oil
Soybean oil is the most valuable of the soybean products and is consumed almost entirely (more than 95%) as food. Food-grade soybean oil is used as salad and cooking oil, shortenings and margarines. For non-food uses, soybean oil is converted into alkyd resins for protective coatings, plasticisers, dimer acids, surfactants and a number of other products (Hui, 1992). To be suitable for human consumption, the extracted oil must undergo further processing, which is referred to as refining. Figure 2 below shows the oil refining procedure.

Soybean meal
Most soybean meal obtained via processing is used as a protein supplement in animal feeds. Only in the last 30 years have appreciable amounts been converted into products for human consumption, and these have been almost exclusively derived from defatted soybean flakes (Hui, 1992).
Soybean meal normally contains 41 – 50% protein, depending on the amount of hull removed. Because of their high protein content, protein meals are essential ingredients of poultry and livestock feeds. Soybean meal is often blended with corn meal in animal feeds because the two protein sources complement each other; soy supplies the lysine and corn the methionine necessary to provide a balanced ration at relatively low cost (Hui, 1992).

Soybean hulls
The hull is the tough protective covering of the seed which must be removed before the oil can be extracted. The primary use for soybean hulls is animal feed. Hulls are routinely removed during crushing of soybeans but are returned to the processing stream to be added to the meal fraction. Hulls are withheld from the meal only if their inclusion would cause the product to exceed the limit of allowable fibre. Excess hulls may be sold as feedstuffs or discarded as waste.

Soybean protein products
Three classes of protein products are derived from soybeans: defatted flours and grits, protein concentrates and protein isolates. Flours and grits (containing 40 – 50% protein) are made by grinding and sieving flakes. Concentrates (containing about 70% protein) are prepared by extracting and removing the soluble sugars from the defatted flakes by leaching with dilute acid at pH 4.5 or leaching with aqueous ethanol. Isolated soy proteins are obtained by extracting the soluble proteins with water at pH 8-9, precipitating at pH 4.5, centrifuging the resulting protein curd, washing, redispersing in water, and finally spray drying. Flours and concentrates are further processed into textured products that are used as meat extenders and substitutes. Protein isolate is used primarily as adhesives for clays used in coating of paper and paperboard to render surfaces suitable for printing (Hui, 1992).

Soy Lecithin - Phospholipids
Soybean has the highest phospholipid content of the common oilseeds. Crude lecithin is obtained by degumming the crude soybean oil. This process involves mixing the crude oil with about 2% water at a temperature of 60 – 80°C. The mixture is then centrifuged to separate the lecithin emulsion which is vacuum dried in a thin film evaporator to a water content of 0.2 - 0.8%. Crude lecithin consists of 45 – 60% phosphatides and 30 – 35% triglycerides, the

remaining 5 – 10% are free fatty acids, carbohydrates, glycolipids, sterols, and tocopherols (Pardun, 1989). Soy lecithin is used as ingredient in margarine, chocolate, icecream and baked goods. Its non-food applications are in cosmetics, pharmaceuticals and as additives in technical products.

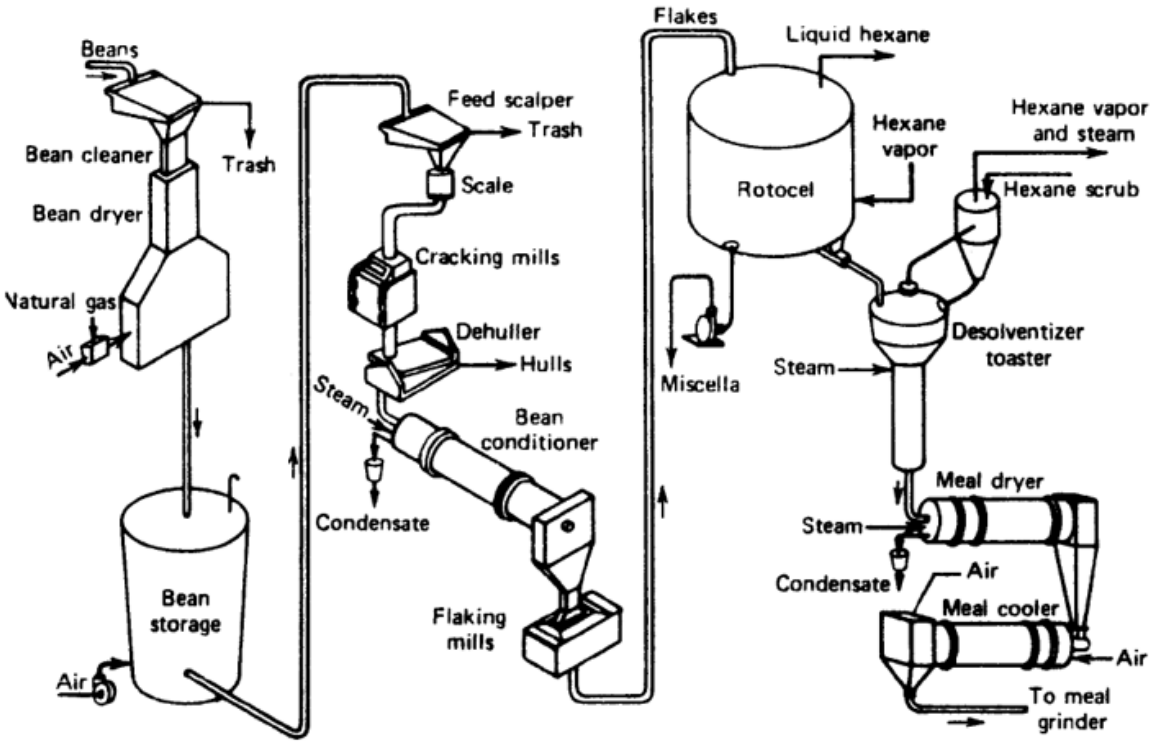


Figure 2 Processing of soybean into oil and meal by solvent extraction, courtesy of Dravo Corp. (Hui, 1992)

A.3 The Nature of the Genetic Modification

(a) A description of the method used to transform the host organism.

Explants from soybean variety Thorne were prepared and transformed with the transformation vector pSZ8832 using an *Agrobacterium tumefaciens* (*A. tumefaciens*) transformation method. Explants were exposed to a culture of a disarmed *A. tumefaciens*, strain LBA4404 (Hoekema *et al.*, 1983), harbouring two plasmids: the helper Ti-plasmid pAL4404 and the T-DNA region-containing transformation vector pSZ8832, derived from plasmid pGSC1700 (Cornelissen and Vandewiele, 1989). After the exposure, the explants were transferred to selection media containing the selection agent tembotrione to select for transformed cells and Ticarcillin to eliminate residual *A. tumefaciens*. Transformed shoots were transferred to fresh selection media for shoot elongation and were subsequently transferred to a rooting medium. Rooted shoots were transferred to the greenhouse where they were allowed to flower and set seed.

See Table 5 below for a description of the vector ([redacted] 2016; M-455339-04; Appendix 3), Sections A.3 (b), (i) and (ii)).

(b) A description of the construct and the transformation vectors used, including:

(i) The size, source and function of all the genetic components including marker genes, regulatory and other elements; and

The vector pSZ8832 is derived from pGSC1700 (Cornelissen and Vandewiele, 1989). The genetic elements are represented on the vector map (see Figure 3 below) and are further described in Table 5. ([redacted]; 2016; M-455339-04, Appendix 3).

Table 5 Genetic elements comprising the pSZ8832 vector used in GMB151 *G. max*

GENETIC ELEMENT	NT POSITION	SIZE (KB)	ORIENTATION	DESCRIPTION AND FUNCTION	REFERENCE
ftiR	1 – 184			Ti- plasmid sequence of pTiAch5 flanking the T-DNA right border region.	Zhu *et al*., 2000
	185 - 189			Polylinker sequences: sequence used in cloning	
RB	190 - 214			Right border region of the T-DNA of *Agrobacterium tumefaciens*.	Zambryski, 1988
	215 - 344			Polylinker sequences: sequence used in cloning	
T35S	345 - 614		Counter clockwise	Sequence including the 3' untranslated region of the 35S transcript of the Cauliflower Mosaic Virus.	Sanfaçon *et al*., 1991
	615 - 625			Polylinker sequences: sequence used in cloning	
cry14Ab-1.b	626 - 4183		Counter clockwise	Coding sequence of the delta-endotoxin gene of *Bacillus thuringiensis* (GenBank accession number:	
Pubi10At	4184 - 5490		Counter clockwise	Sequence including the promoter region of ubiquitin-10 gene of *Arabidopsis thaliana*.	Grefen *et al*., 2010
	5491 - 5595			Polylinker sequences: sequence used in cloning	
T35S	5596 - 5790		Counter clockwise	Sequence including the 3' untranslated region of the 35S transcript of the Cauliflower Mosaic Virus.	Sanfaçon *et al*., 1991
	5791 - 5802			Polylinker sequences: sequence used in cloning	
hppdPf-4Pa	5803 - 6879		Counter clockwise	Coding sequence of a variant of 4-hydroxyphenylpyruvate dioxygenase gene of *Pseudomonas fluorescens*.	Porée *et al*., 2014
TPotpY-1Pf	6880 - 7251		Counter clockwise	Coding sequence of an optimized transit peptide derivative (position 55 changed into Tyr), containing sequence of the RuBisCO small subunit genes of *Zea mays* and *Helianthus annuus*.	Le Brun *et al*., 1996
	7252 - 7272			Polylinker sequences: sequence used in cloning	
Ltev	7273 - 7399		Counter clockwise	Sequence including the leader sequence of the Tobacco Etch Virus genomic RNA	Allison *et al*., 1985
	7400 - 7405			Polylinker sequences: sequence used in cloning	
P2x35S	7406 - 8155		Counter clockwise	Sequence including the double enhanced promoter region of the Cauliflower Mosaic Virus 35S genome transcript.	Kay *et al*., 1987
	8156 - 8282			Polylinker sequences: sequence used in cloning	
LB	8283 - 8307			Left border region of the T-DNA of *Agrobacterium tumefaciens*	Zambryski, 1988
ftiL	8308 - 8612			Ti- plasmid sequence of pTiAch5 flanking the T-DNA left border region	Zhu *et al*., 2000
TaadA	8613 - 8864		Counter clockwise	Sequence including the 3' termination region of the aminoglycoside adenyltransferase gene of transposon Tn7 of *Escherichia coli*	Fling *et al*., 1985
aadA	8865 - 9656		Counter clockwise	The coding sequence of the aminoglycoside adenyltransferase gene (aadA) of the transposon Tn7 of *Echerichia coli*.	Fling *et al*., 1985
PaadA	9657 - 10394		Counter clockwise	Sequence including the promoter region of the aminoglycoside adenyltransferase gene of transposon Tn7 of *Escherichia coli*.	Fling *et al*., 1985

GENETIC ELEMENT	NT POSITION	SIZE (KB)	ORIENTATION	DESCRIPTION AND FUNCTION	REFERENCE
	10395 - 10400			Polylinker sequences: sequence used in cloning	
ORIpVS1	10401 - 13187			Fragment including the origin of replication of the plasmid pVS1 of *Pseudomonas aeruginosa*.	Heeb *et al*., 2000
ORI ColE1	13188 - 14251			Fragment including the origin of replication from the plasmid pBR322 for replication in *Escherichia coli*.	Bolivar *et al*., 1977
	14252 - 14361			Polylinker sequences: sequence used in cloning	

(ii) A detailed map of the location and orientation of all the genetic components contained within the construct and vector, including the location of relevant restriction sites.

The vector pSZ8832 is derived from pGSC1700 (Cornelissen and Vandewiele, 1989). The genetic elements are represented on the vector map (see Figure 3) and are further described in Table 5. The locations of restriction sites are not provided, as restriction enzyme analysis was not employed in the molecular characterization by Next Generation Sequencing/Junction Sequence Analysis study ([redacted] 2018, GEN170607_H, Appendix 4).

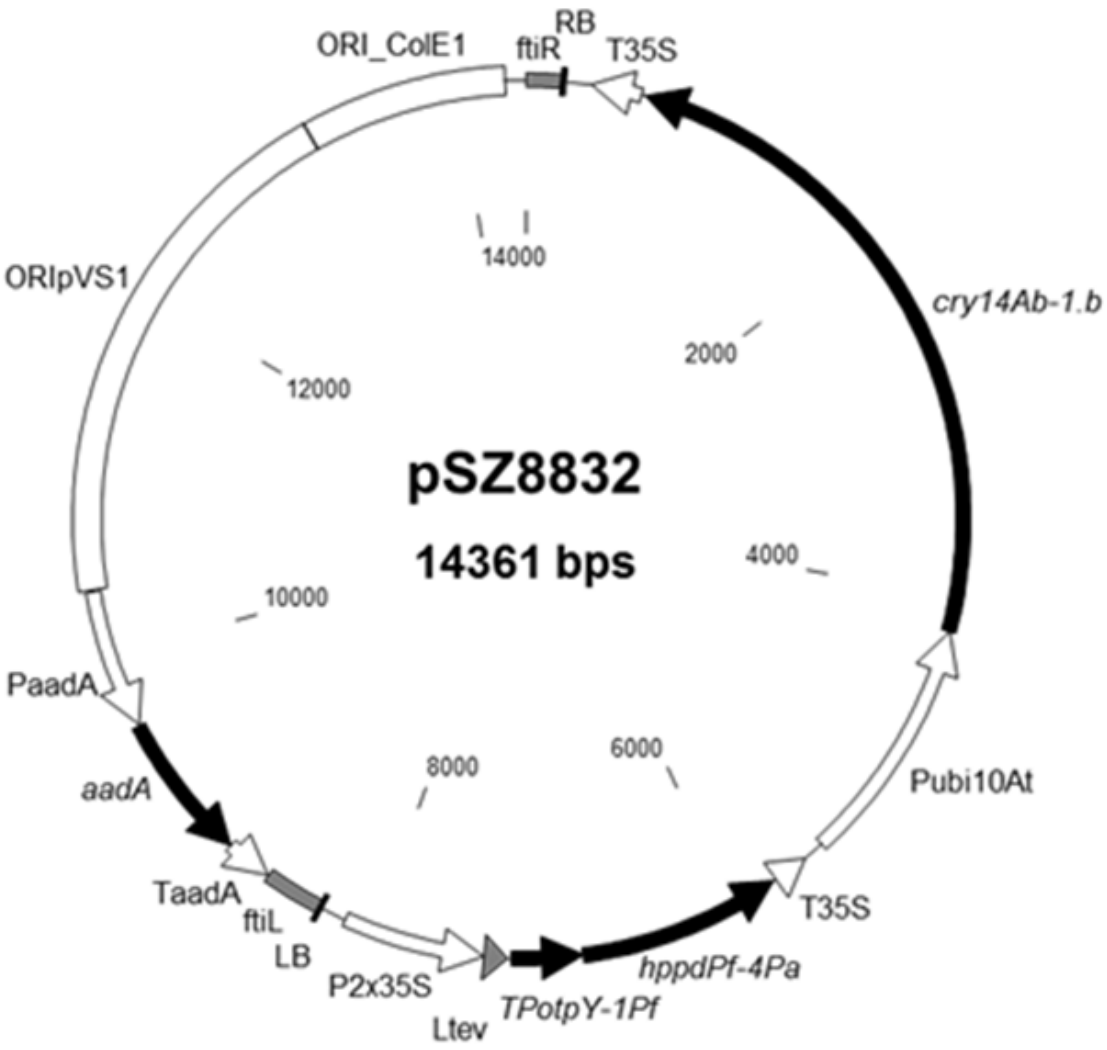


Figure 3 Map of plasmid vector pSZ8832 used in GMB151 *G. max*

(c) A full molecular characterisation of the genetic modification in the new organism, including:

(i) Identification of all transferred genetic material and whether it has undergone any rearrangements;

The inserted sequences of GMB151 soybean were characterized by means of next generation sequencing (NGS) and junction sequencing analysis (JSA) on genomic DNA (gDNA) prepared from seeds ([redacted] 2018, GEN170607_H, Appendix 4 (CCI)).

To characterize the inserted DNA sequences in GMB151 soybean, a JSA was performed on whole-genome sequence data from GMB151 soybean (T2 generation) as well as appropriate control samples. The non-GM counterpart Thorne was used as a negative control sample. As a positive control, the non-GM counterpart Thorne was supplemented with an equimolar amount of the transforming plasmid pSZ8832. The non-GM counterpart Thorne supplemented with 1/10 ^{h} equimolar amount of the plasmid pSZ8832 was used as a sensitivity control sample.

Ready-to-sequence library pools with an average fragment size of 500-600 base pairs (bp) were prepared for each GMB151 soybean sample and for the negative, positive, and sensitivity control samples and used as templates for whole-genome sequencing. Whole-genome sequencing was performed using the Illumina HiSeq 2500 technology. For each fragment, paired-end reads of 125 bp each were obtained.

Low quality sequencing data were trimmed. The remaining sequencing reads were mapped to both the soybean genome and transforming plasmid reference sequences. Duplicate reads, which were the result of a PCR amplification step during library preparation, were removed, and the effective median genome coverage was examined by the alignment of the reads to a known single copy locus of the soybean genome (lectin gene, Glyma.02G012600, Chr.02:1123507-1125658). The median coverage depth of a known single copy locus represented the genome coverage at the insertion locus and was higher than 75-fold for each of the GMB151 soybean and control samples.

Alignment of the sequencing reads to the transforming plasmid reference sequence demonstrated that the median coverage depths of the transforming plasmid in the positive and sensitivity control samples were 96-fold and 7-fold, respectively, and that 100% of the transforming plasmid sequence was covered. This demonstrated that the coverage was adequate to perform a high quality JSA. A 100% nucleotide identity between the obtained transforming plasmid sequences and the transforming plasmid reference sequence was obtained, demonstrating that the entire transforming plasmid sequence was amenable to sequencing with the technology used.

Alignment of sequencing reads of the negative control sample to the transforming plasmid reference sequence demonstrated that the median coverage depth was 0-fold. The few reads that did map to the transformation plasmid sequence were the result of low quality of short sequence similarities between the transformation plasmid and host genome sequences.

An in-depth molecular characterization was performed through the identification of all novel sequence junctions created upon transformation. A novel junction created upon transformation is typically covered by sequence reads containing sequence from both pSZ8832 and the soybean genome (junction reads). Therefore, reads partially mapping to the transforming plasmid were selected and further analyzed using a bioinformatics JSA. Based on the results of the parameter optimization for the bioinformatics JSA pipeline, aiming for the maximal sensitivity and specificity, the minimal length of the mapped subsequence was

set to 30 bp. Hence insertions of plasmid sequences smaller than 30 bp were below the detection limit.

The junction positions in the transforming plasmid which separated the mapped portion from the unmapped portion of the junction reads, were collected. The selected junction reads were grouped based on their junction position(s) within the transforming plasmid.

Due to the presence of a sequence repeat in the P2x35S promoter of the transforming plasmid, the correct position of some junction reads could not be defined, leading to two possible junction positions. This was resolved by broken pair analysis. Broken pairs are sequence reads coming from both ends of a DNA fragment that either map to a different target sequence or that map to the same target sequence but with an unexpected insert length or non-standard orientation. Analysis of the broken pairs defined the junction regions. The junction position of the true junctions are located in these junction regions.

The consensus sequences of all junction reads corresponding to the same junction position within the transforming plasmid were generated. The junctions were further identified as "plasmid/flank" junctions or "plasmid/plasmid" junctions. Junctions containing flanking sequences are unique for each insertion site, and the number of plasmid/flank junctions defines the number of transgenic loci. Junctions containing different parts of the transforming plasmid are the result of rearrangements within the inserted plasmid sequences.

Junction sequence analysis demonstrated that all analyzed samples had one junction (position 8156 in pSZ8832) in common. This junction is background, coming from endogenous soybean sequences mapping to a very small region in the transforming plasmid (pSZ8832: bp 8155 – 8188). The number of reads supporting this junction was very low and similar in all samples. No other junctions were found in the negative control, the positive control, or the sensitivity control samples.

<u>GMB151 contains a single transgenic locus which consists of a single T-DNA copy</u>

Junction sequence analysis of GMB151 soybean identified two "plasmid/flank" junctions, which are not found in the non-GM counterpart, and no "plasmid/plasmid" junctions. This result demonstrated that GMB151 soybean contains one copy of the T-DNA insert without rearrangements at a single insertion site.

Alignment of junction reads and their mates to the transforming plasmid reference sequence demonstrated that the junctions in pSZ8832 are at positions 213 and 7673. Mapping of all reads to the transforming plasmid showed that the region between these two junction positions is sufficiently covered.

These results demonstrated that GMB151 soybeans contain a single transgenic locus which consists of a single T-DNA copy, lacking the 5' part of the P2X35S promoter.

(ii) A determination of the number of insertion sites, and the number of copies at each insertion site;

As detailed above in Section A.3 (c)(i) above and below in Section A.3 (c)(iii), NGS/JSA analysis and full DNA sequencing of the GMB151 soybean transgenic locus revealed that the inserted genetic material contains a single transgenic locus which consists of a complete *cry14Ab-1.b* gene cassette and a *hppdPf-4Pa* gene cassette which lacks the 5' part of the P2x35S promoter. The arrangement of the GMB151 soybean transgenic locus is shown in Figure 4 below. The NGS/JSA analysis is detailed in [redacted] (2018;

GEN170607_H; Appendix 4, (CCI)), and sequencing of the transgenic locus and corresponding insertion locus is detailed in [redacted] (2017, M-607717-01-1, Appendix 5, (CCI)).

(iii) Full DNA sequence of each insertion site, including junction regions with the host DNA;

The DNA sequence of the soybean GMB151 transgenic locus and the corresponding insertion locus was determined ([redacted] 2017, M-607717-01-1, Appendix 5 (CCI)).

Two overlapping PCR fragments were prepared for the determination of the GMB151 transgenic locus, using GMB151 gDNA as a template. To determine the GMB151 insertion locus, one fragment was amplified from gDNA extracted from the non-GM counterpart (Table 6). Sanger sequencing was performed using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Table 6 Overview of the sequencing fragments prepared

	Fragment ID	Template DNA	Primer pair	Target	Length of final consensus sequence (bp)
GMB151 transgenic locus	FR-VLT137-A-01	GMB151 soybean	GLPA210 GLPB167	GMB151 – 5' integration fragment	9498
	FR-VLT137-A-02		GLPB170 GLPA212	GMB151 – 3' integration fragment	
GMB151 insertion locus	FR-VLT137-A-03	Non-GM counterpart	GLPA210 GLPA212	GMB151 – insertion locus	2063

The obtained consensus sequences of the transgenic and insertion loci were annotated by pairwise alignments using the Clone Manager software (Sci-Ed Central). An alignment between the GMB151 transgenic locus and the GMB151 insertion locus sequence was made to identify sequence regions of soybean origin within the GMB151 transgenic locus as well as the target site deletion (TSD) within the GMB151 insertion locus. The consensus sequence of the GMB151 transgenic locus was compared with the pSZ8832 sequence to identify the T-DNA region. Further sequence annotation within the T-DNA was performed by comparing the GMB151 transgenic locus sequence with each feature of the pSZ8832 T-DNA region.

By means of pairwise alignment, two large sections of sequence identity were found between the GMB151 soybean transgenic locus sequence and the GMB151 soybean insertion locus sequence. These regions are identified as the 5' flanking genomic sequence (Region A – Table 7) and the 3' flanking genomic sequence (Region B – Table 7) of the GMB151 soybean transgenic and insertion loci sequences. One base pair difference between the GMB151 soybean transgenic locus (position 8675) and the GMB151 soybean insertion locus (position 1240) was found. Sequence differences between plants are commonly found and are due to natural genetic variation (Zhu *et al*., 2003).

In the GMB151 soybean insertion locus sequence, 63 bp of genomic DNA were observed which are not present in the GMB151 transgenic locus sequence. These base pairs were deleted during the transformation process and are referred to as the target site deletion.

Pairwise alignment between the GMB151 transgenic sequence and the pSZ8832 plasmid sequence identified on large region sharing 100% sequence identity (Table 8). Region D on the GMB151 soybean transgenic locus sequence is identical to the T-DNA region of pSZ8832. Two base pairs at the 5‘ end of the T-DNA region (bp 999 to bp 1000) were identical to both the transforming plasmid pSZ8832 and the GMB151 soybean insertion locus sequence. These base pairs were annotated as 5‘ flanking genomic sequence. Base pairs 1001 to 8459 of the GMB151 soybean transgenic locus (7459 bp) were annotated as T-DNA.

The GMB151 soybean transgenic locus sequence corresponds to one copy of the T-DNA region of the transforming vector pSZ8832, which includes the complete *cry14Ab-1.b* gene cassette and an incomplete *hppdPf-4Pa* gene cassette, lacking 482 bp of the P2x35S promoter.

Between the T-DNA sequences and the 3‘ flanking genomic sequences, 39 bp filler DNA was found. Twenty-one bp of this filler DNA shows sequence identity to a region containing part of the ORIpVS1 from the transforming plasmid pSZ8832 (Region E in Table 8) and 17 bp has sequence identity to the 3‘ flanking genomic sequence (Region C in Table 7). Considering the small size of these separate regions showing sequence identity, the 39 bp sequence was annotated as filler DNA.

A schematic representation of the GMB151 soybean transgenic locus sequence in relation to the transforming plasmid pSZ8832 and the GMB151 soybean insertion locus is provided in Figure 4.

Table 7 Results of the pairwise alignment between the sequence of the GMB151 soybean transgenic and insertion loci

Region showing sequence identity	% matches	Length (bp)	GMB151 soybean insertion locus sequence		GMB151 soybean transgenic locus sequence	
			Start	End	Start	End
Region A: 5' flanking genomic sequence	100	1000	1 bp	1000 bp	1 bp	1000 bp
Region B: 3' flanking genomic sequence	99*	1000	1064 bp	2063 bp	8499 bp	9498 bp
Region C: Part of filler DNA	100	17	1090 bp	1106 bp	8482 bp	8489 bp

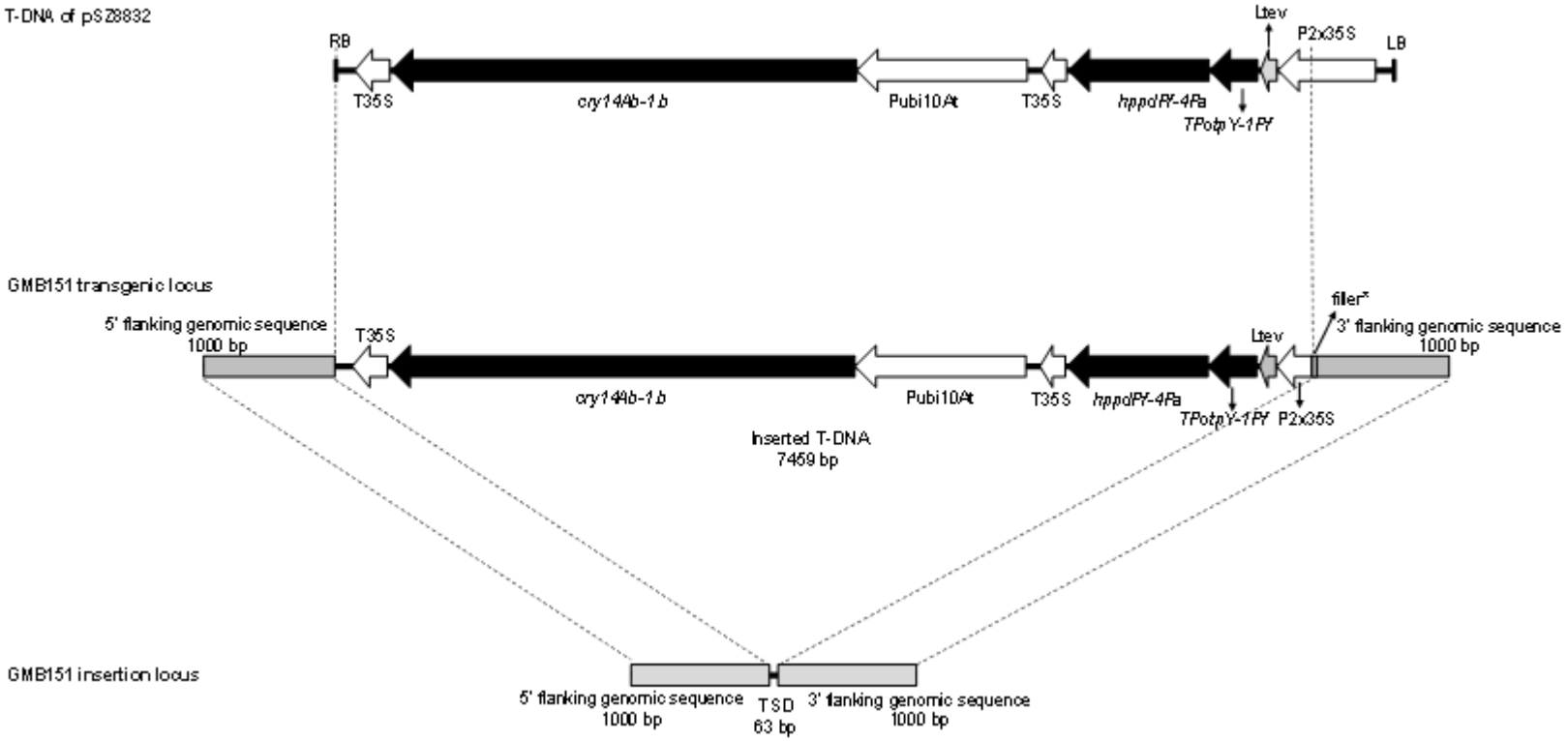
*One base pair difference between the GMB151 soybean transgenic locus (3' flanking genomic sequence position 8675) and the GMB151 soybean insertion locus (position 1240) was found. Sequence differences between plants are commonly found and are due to natural genetic variation (Zhu *et al.*, 2003)

Table 8 Results of the pairwise alignment between the sequence of the GMB151 soybean transgenic locus and the transforming plasmid pSZ8832 sequence

Region showing sequence identity	% matches	Length (bp)	Transforming plasmid pSZ8832		GMB151 soybean transgenic locus sequence	
			Start	End	Start	End
Region D T-DNA	100	7461*	213 bp	7673 bp	999 bp	8459 bp
Region E Part of filler DNA	100	21	11923 bp	11943 bp	8481 bp	8461 bp

*Two base pairs at the 5' end of the T-DNA region (bp 999 to bp 1000) were identical to both the transforming plasmid pSZ8832 and the GMB151 soybean insertion locus sequence. These base pairs were annotated as 5' flanking genomic sequence. Base pairs 1001 to 8459 of the GMB151 soybean transgenic locus (7459 bp) were annotated as T-DNA.

The results demonstrated that upon transformation, 63 bp from the non-genetically modified counterpart were replaced by 7498 bp of inserted sequences, including 7459 bp of T-DNA sequence and 39 bp filler DNA. Part of the filler DNA shows sequence identity to the ORIpVS1 from pSZ8832, another part shows sequence identity to the 3' flanking genomic sequence. The flanking genomic sequences obtained at the transgenic locus were similar to the homologous sequence from the insertion locus, demonstrating the soybean origin. The GMB151 soybean transgenic locus contains one copy of the T-DNA region of transforming vector pSZ8832, comprising the complete *cry14Ab-1.b* gene cassette and the *hppdPf-4Pa* gene cassette lacking the 5' part of the P2x35S promoter.



*39 bp filler DNA contains 21 bp showing sequence identity to a region containing ORIpVS1 from the transforming plasmid pSZ8832 and 17 bp showing sequence identity to the 3' flanking genomic sequence
TSD = target site deletion

Figure 4 Schematic drawing of the GMB151 soybean transgenic locus in relation to the GMB151 insertion locus and the T-DNA of transforming plasmid pSZ8832

(iv) A map depicting the organisation of the inserted genetic material at each insertion site; and

The organisation of the GMB151 transgenic locus within the *G. max* genome, as confirmed by NGS/JSA ([redacted], 2018, GEN170607_H; Appendix 4 (CCI)) and DNA sequencing of the transgenic locus and corresponding insertion locus is detailed in [redacted] (2017, M-607717-01-1, Appendix 5, (CCI)), and described above in Sections A.3(c)(i-iii), is shown in Figure 5 below.

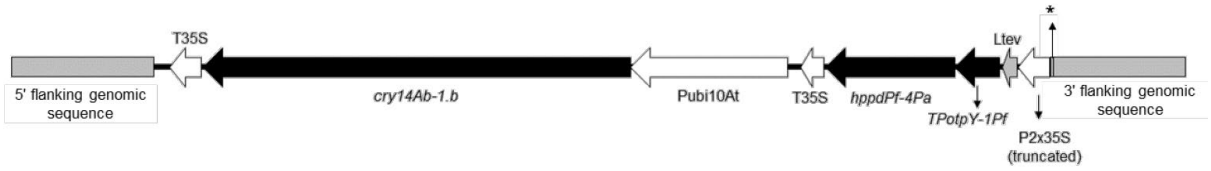


Figure 5 Organisation of the GMB151 transformation event in the *G. max* genome

* GMB151 soybean contains 39 bp filler DNA between the 3' end of the inserted T-DNA region and the 3' flanking genomic region. Filler DNA showed 21 bp sequence identity to the ORIpVS1 of the transforming plasmid pSZ8832 and 17 bp sequence identity to the 3' flanking genomic region.

Vector backbone analysis

The presence of vector backbone sequences in GMB151 soybean was investigated by NGS/JSA on the T_2 generation, as described in Section A.3 (c)(i), above.

JSA of GMB151 identified only two unique plasmid/flank junctions, at positions 213 and 7673 of the transformation plasmid pSZ8832. Since these junction positions are in the T-DNA and not within the vector backbone of the transformation plasmid, the absence of vector backbone in GMB151 soybean was indicated. However, consensus sequences were generated from the junctions, and BLAST searches of these sequences, performed to identify host genomic sequences in the flanking regions, identified 39 bp of filler DNA between the GMB151 T-DNA sequence and the 3' flanking genomic sequences. Twenty-one bp of this filler DNA showed sequence identity to the ORIpVS1 in the vector backbone region of the transformation plasmid. The short size of this fragment was below the limit of detection (30 bp) in the NGS/JSA. In conclusion, the GMB151 transgenic locus has 21 bp containing sequence identity to ORIpVS1 in the pSZ8832 vector backbone region adjacent to the 3' end of the T-DNA insert. These 21 bp do not originate from an antibiotic resistance gene.

(v) Details of an analysis of insert and junction regions for the occurrence of any open reading frames (ORFs).

Bioinformatics analysis of the GMB151 soybean insertion locus

A bioinformatics analysis was performed on the GMB151 insertion locus sequence, to identify the position of the insertion locus in the genome and to determine whether regulatory sequences or endogenous soybean genes were interrupted upon the insertion of T-DNA sequences ([redacted] 2018; 18-RSVLS011, Appendix 6).

The GMB151 insertion locus sequence was used as the query sequence and consists of a 5' and a 3' flanking genomic sequence region and a target site deletion of 63 bp that is removed upon integration of the GMB151 T-DNA.

BLASTn compares a nucleotide query sequence against a nucleic sequence database. This tool was used to identify similarities between the GMB151 insertion locus and sequences within the nucleotide collection and the Expressed Sequence Tag (EST) databases available on the NCBI website. The MEGABLAST algorithm (Zhang *et al*., 2000), which uses parameters that efficiently find long alignments between very similar sequences, and the *Glycine max* dataset of the NCBI Genomic Reference Sequences database were used to locate the GMB151 insertion locus in the soybean genome.

BLASTx compares the six-frame theoretical translation products of the nucleotide query sequence (both strands) against a protein sequence database. A BLASTx search of the GMB151 insertion locus sequence against the NCBI non-redundant protein database was performed.

The bioinformatics analysis demonstrated that the GMB151 soybean insertion locus originates from *Glycine max* chromosome 7. The results indicate that the insertion of T-DNA sequences in the GMB151 soybean is located in the 3' untranslated region of a putative endogenous gene annotated as a BON1-associated protein 1-like protein. Based on literature, the BON1-associated protein 1 has a function in a signal transduction cascade in *Arabidopsis thaliana*. The biological function of BON1-associated protein 1-like protein in *Glycine max* is yet uncharacterized.

Identification of Open Reading Frames and homology search of sequences with known allergens and toxins

A bioinformatics analysis was performed on the transgenic locus sequence of the GMB151 soybean to identify open reading frames (ORF) ([redacted], 2018, TXVLT032, Appendix 7).

The GMB151 transgenic locus, containing the inserted DNA together with the 5' and 3' flanking sequences, was used as query sequence. The GetORF search program was used to identify all ORF crossing a junction or overlapping the inserted DNA, between two translation stop codons, with a minimum size coding for 3 amino acids. The 3' flanking genomic sequence of GMB151 soybean has a one basepair sequence difference when compared to the non-genetically modified counterpart Thorne. Sequence differences between plants are commonly found and are due to natural genetic variation. Nevertheless, the ORF containing this one basepair are included in this search. 601 ORFs were identified.

In the next step, the translated amino acid sequences from the identified ORF with a minimum size of 30 amino acids were used as query sequences in homology searches to known allergens and toxins. After elimination of duplicates, they represented 115 unique sequences.

Two *in silico* approaches were used to evaluate the potential amino acid sequence identity with known allergens contained in the public allergen database COMPARE (www.comparedatabase.org):

- An 8-mer search was carried out to identify any short sequences of 8 amino acids or longer that share 100% identity to an allergenic protein. This search was performed using SeqMatchAll from the EMBOSS suite, which compared each ORF sequence with all known allergens present in the allergen database.
- An overall identity search was carried out by using FASTA algorithm, which compared each complete query sequence with all protein sequences present in the allergen database. The scoring matrix was BLOSUM50. An E-value threshold of 1 was used. The criterion indicating potential

relevant identity to an allergen was ≥35% identity over at least 80 amino acids for sequences of ≥80 amino acids, or ≥35% recalculated over a hypothetical 80 amino acid window for sequences of <80 amino acids.

In addition, each query sequence was evaluated for potential identity with known toxins. An overall identity search was carried out by using FASTA algorithm with all protein sequences present in the NCBI non-redundant database, using the BLOSUM50 scoring matrix. An E-value threshold of 0.1 was used for pre-selecting the most identical proteins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the familiarity and potential toxic properties of the potential polypeptide.

The overall search showed no biologically relevant identity between the query sequences and any known allergenic proteins. One ORF matched with a contiguous 8- amino acid sequence (SSPTTTTS) present in the Cas s 5 allergen. No match with these proteins was found in the overall search for an allergen match, and an 8-mer match in isolation is unlikely to indicate any potential cross-reactivity. Also, translation of this 8-mer is not possible due to the absence of a translation start codon and a RNA splice site.

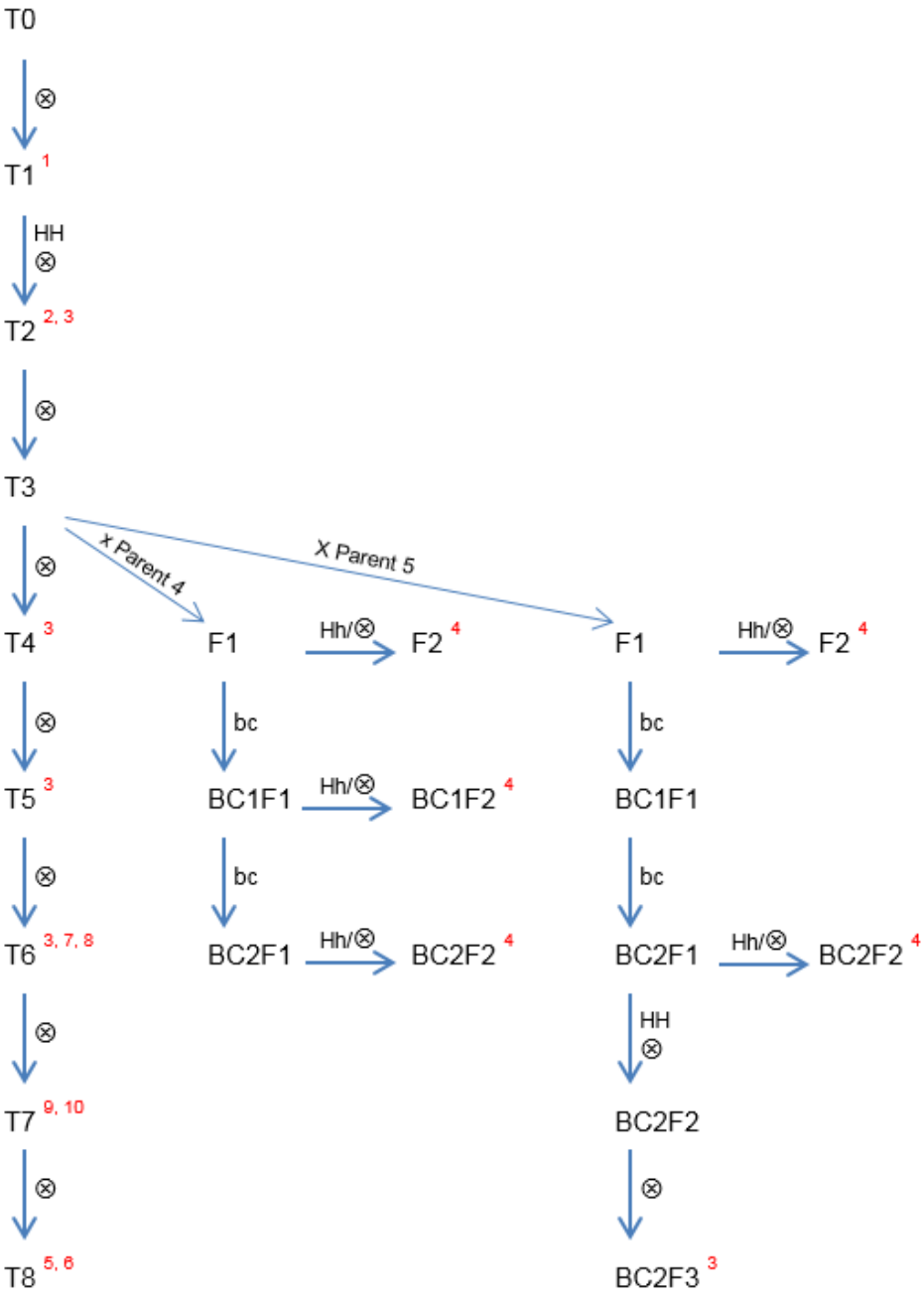
In addition, no biologically relevant identities were found with any toxic protein from the NCBI non-redundant database.

In conclusion, there are neither allergenic nor toxicological *in silico* findings associated with the potential ORF polypeptides.

(d) A description of how the line or strain from which food is derived was obtained from the original transformant (i.e. provide a family tree or describe the breeding process) including which generations have been used for each study.

Following Agrobacterium-mediated transformation of the conventional breeding line Thorne, the GMB151 T_0 plant was self-pollinated to generate T_1 seed. All subsequent T generations were produced through self-pollination. The T_3 generation was used for crossing into conventional soybean lines.

The breeding diagram is shown in Figure 6 below. Table 9 describes the GMB151 generations used for analysis and the associated reports describing these studies.



Legend:

T_0 - original transformant

⊗ - self pollination

bc - backcross

x - cross

HH - selection of homozygous plants

Hh - selection of hemizygous plants

Figure 6 Pedigree of GMB151

Table 9 GM151 soybean generations used for analysis

No. in Tree	Experiment	Generation(s)	Comparator
1	DNA sequencing of insert and flanking region	T1	Thorne
2	Molecular Characterization by NGS / JSA	T2	Thorne
3	Structural Stability by NGS/JSA	T2, T4, T5, T6, BC2F3 (parent 5)	Thorne, parent 5
4	Inheritance of the Insert	F2 (parent 4) BC1F2 (parent 4) BC2F2 (parent 4) F2 (parent 5) BC2F2 (parent 5)	None
5	Agronomic and phenotypic Analysis	T8	Thorne
6	Composition Analysis*	T8	Thorne
7	Protein Expression Analysis*	T6	Thorne
8	Plant protein characterization and equivalency studies – Cry14Ab-1	T6	Thorne
9	Plant protein characterization and equivalency studies – HPPD-4	T7, T6 (functional equivalence)	Thorne
10	90-day rat feeding study	T7	Thorne

* the grain analyzed in the study is the next generation from that planted.

(e) Evidence of the stability of the genetic changes, including:

(i) The pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored; and

The structural stability of GMB151 soybean was investigated by performing NGS/JSA on plants from five different breeding generations (T2, T4, T5, T6 and BC2F3 generations) as described in [redacted] 2018; GEN170607_H; Appendix 4 (CCI).

For each of the breeding generations, two junctions were determined after NGS/JSA. The position of both junctions within the plasmid were the same in all generations. Also, multiple sequence alignment between the obtained junction consensus sequences showed that both novel junctions are conserved between all breeding generations analyzed (Table 10). This demonstrated that the transgenic locus of GMB151 soybean is stably maintained across different breeding generations.

Table 10 The junctions identified in GMB151 soybeans and the non-GM counterparts using junction sequence analysis following background subtraction

Sample	Number of junctions	Junction positions in pSZ8832
GMB151 soybean – T2	2	213 and 7673
GMB151 soybean – T4	2	213 and 7673
GMB151 soybean – T5	2	213 and 7673
GMB151 soybean – T6	2	213 and 7673
GMB151 soybean – BC2F3	2	213 and 7673
Non-GM counterparts	0	-

(ii) The pattern of inheritance and expression of the phenotype over several generations and, where appropriate, across different environments.

The segregation ratios of the GMB151 insert over five segregating generations were determined to confirm inheritance of the insert in a predictable and stable manner, according to Mendelian inheritance principles ([redacted] 2018, M-611752-01, Appendix 8).

Genomic DNA from individual plants of five segregating GMB151 soybean generations was tested for the genotype of GMB151 by polymerase chain reaction (PCR). In addition, the presence or absence of the *cry14Ab-1.b* and *hppdPf-4Pa* genes was confirmed. The results from event-specific PCR analysis were used to calculate the segregation ratios of the GMB151 insert. Chi-square analysis of the segregation data was performed to test the hypothesis that the GMB151 soybean insert is inherited in a manner that is predictable according to Mendelian principles and is consistent with the insertion into a single locus.

To determine the genotype of the GMB151 insert, plant samples were analyzed using event specific PCR. This included the amplification of the GMB151 event-specific sequence and the amplification of the pre-insertion locus sequence. Samples with fluorescent signal corresponding to the GMB151 event-specific sequence only were recorded as homozygous for the GMB151 insert. Samples with fluorescent signal corresponding to the pre-insertion locus sequence only were recorded as null segregant. Samples with fluorescent signal corresponding to the GMB151 event-specific sequence and the pre-insertion locus sequence were recorded as hemizygous for the GMB151 insert.

To confirm the presence or absence of the *cry14Ab-1.b* and *hppdPf-4Pa* genes; plant samples were also analyzed using gene-specific PCR. This PCR analysis included the amplification of the gene-specific sequence and the amplification of an endogenous gene sequence. Samples with signal corresponding to the gene-specific sequence and the endogenous sequence were recorded as positive for the gene tested. Samples with signal corresponding to the endogenous sequence only were recorded as negative. The gene-specific PCR analysis confirmed that the *cry14Ab-1.b* and *hppdPf-4Pa* genes were present for samples positive for GMB151 and were absent for samples negative for GMB151.

Chi-square (χ^2) analysis was performed to confirm the segregation and stability of the GMB151 insert. The Chi-square analysis is based on testing the observed segregation ratio relative to the segregation ratio expected from Mendelian inheritance principles. The expected segregation ratio of homozygous, hemizygous and null segregate was 1:2:1. The critical value to reject the hypothesis of a 1:2:1 ratio at the 5% significance level with two degrees of freedom is 5.99 (Strickberger, 1976). The χ^2 values were calculated using the following equation.

$$\chi^2 = \sum \frac{|(\text{Observed} - \text{Expected})|^2}{\text{Expected}}$$

The results are summarized in Table 11.

Table 11 Observed versus Expected (1:2:1) identity for five generations of GMB151 soybean as determined by PCR analysis

GMB151 Insert	F2 (14MRGM010348)		BC2F2 (16MRGM006629)		F2 (15MRGM011089)		BC1F2 (16MRGM005436)		BC2F2 (16MRGM012834)	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
Homozygous	56	61.5	57	61.5	54	54	62	55.5	56	57.25
Hemizygous	133	123	120	123	102	108	101	111	104	114.5
Wildtype	57	61.5	69	61.5	60	54	59	55.5	69	57.25
Total Number of Plant Samples	246		246		216		222		229	
χ^2 Value *	1.634		1.317		1.000		1.883		3.402	

* The critical value to reject the null hypothesis at the 5% significance level is < 5.99 with two degrees of freedom.

Segregation ratios determined for plants from two F_2, two BC_2F_2, and one BC_1F_2 generations of GMB151 soybean confirmed that the GMB151 insert is inherited as expected for a single insert. These data are consistent with Mendelian principles and support the conclusion that the GMB151 event consists of a single insert integrated at a single locus within the soybean genome that is stably inherited over generations. In addition, the presence or absence of the *cry14Ab-1.b and hppdPf-4Pa* genes was confirmed.

Expression of the phenotype across different environments

Protein expression levels of Cry14Ab-1 and HPPD-4 were determined by Enzyme-Linked immunosorbent assay (ELISA) in field-grown soybean matrices from GMB151 plants treated and not treated with trait-specific herbicide grown at three field trials in the USA in 2016 ([redacted] 2018; M-601077-02, Appendix 9).

Protein expression analysis was conducted on tissue samples harvested from plants grown in the USA in 2016. Four field sites were located in areas representative of the commercial production of soybean in the USA and sampled throughout the growing season for different tissues. The three randomly selected sites for expression analysis (Pennsylvania, Missouri and Kansas) were representative of the locations of commercial soybean production with respect to cultural practices, soil type and climatic conditions.

Each site had two plots of GMB151 soybean. One plot was treated with trait-specific herbicide while the other plot was not treated. The isoxaflutole application to the treated GMB151 entry was made at a rate of 70.1 (69.2 – 71.1) g ai/ha before emergence (BBCH 00). All entries were of the Thorne background.

The matrices analyzed are summarized below:

Table 12 Plant matrices analyzed for Cry14Ab-1 and HPPD-4 expression.

Growth Stage[1]	Matrix	Sample Description[2]
BBCH 13-14	Leaf	All true leaves[3]
	Root	All roots
BBCH 16-17	Leaf	All true leaves
	Root	All roots
BBCH 60-66	Leaf	All true leaves
	Root	All roots
	Flowers	Composite sample of flowers
BBCH 76-79	Leaf	All true leaves
	Root	All roots
	Forage	Entire above-ground portion
BBCH 89-99	Grain	Grain
	Whole plant	Whole plant (including roots)

[1]The BBCH-scale is a system for a uniform coding of phenologically similar growth stages of mono- and dicotyledonous plant species.
[2] A sample represents the indicated martrix type from one plant, with the exception of flowers which was a composite sample collected from plants in a row.
[3]True leaves in this study are defined as all leaves except cotyledons.

[redacted]

Expression of Cry14Ab-1 in Soybean Matrices

The level of Cry14Ab-1 protein in not treated and treated GMB151 soybean leaf, root, flower, forage, grain and whole plant matrices ranged from 0.34 to 290.44 µg/g DW and 0.75 to 279.73 µg/g DW, respectively (Table 13).

Leaf at BBCH 16-17 growth stage demonstrated the highest mean Cry14Ab-1 protein expression levels (Table 13). Mean (±SD) Cry14Ab-1 expression levels in not treated and treated leaf at BBCH 16-17 of GMB151 soybean were 191.99 ± 50.01 µg/g DW and 168.73 ± 64.98 µg/g DW, respectively.

Root at BBCH 60-66 growth stage demonstrated the lowest mean Cry14Ab-1 protein expression levels (Table 13). Mean (± SD) Cry14Ab-1 expression levels in not treated and treated leaf at BBCH 60-66 of GMB151 soybean were 8.78 ± 9.90 µg/g DW and 4.80 ± 4.26 µg/g DW, respectively.

Mean (± SD) Cry14Ab-1 expression levels in not treated and treated grain of GMB151 soybean were 95.91 ± 43.11 µg/g DW and 83.14 ± 37.69 µg/g DW, respectively.

Expression of HPPD-4 in Soybean Matrices

The level of HPPD-4 protein in not treated and treated GMB151 soybean leaf, root, flower, forage, grain and whole plant matrices ranged from <LLOQ to 779.67 µg/g DW and 1.31 to 1028.76 µg/g DW, respectively (Table 14).

Leaf at BBCH 13-14 growth stage demonstrated the highest mean HPPD-4 protein expression levels (Table 14). Mean (± SD) HPPD-4 expression levels in not treated and treated leaf at BBCH 13-14 of GMB151 soybean were 430.04± 207.57 µg/g DW and 429.22 ± 271.72 µg/g DW, respectively.

Whole plant demonstrated the lowest mean HPPD-4 protein expression levels (Table 14). Mean (± SD) HPPD-4 expression levels in not treated and treated whole plant of GMB151 soybean were 2.44 ± 1.02 µg/g DW and 2.89 ± 1.33 µg/g DW, respectively.

Mean (± SD) HPPD-4 expression levels in not treated and treated grain of GMB151 soybean were 4.46 ± 2.90 µg/g DW and 4.45 ± 3.57 µg/g DW, respectively.

Table 13 Expression of Cry14Ab-1 in Soybean Matrices Harvested from Treated and Not treated GMB151 Grown at Three Sites

Matrix	BBCH Growth Stage	Entry	Cry14Ab-1 (µg/g DW)				Cry14Ab-1 (µg/g FW)			
			Mean	SD	Min	Max	Mean	SD	Min	Max
Leaf	13-14	C (PE)	156.36	49.91	98.54	280.32	36.07	17.01	18.92	75.59
		D (PE)	104.34	46.76	31.3	212.07	20.45	7.74	7.14	37.23
Root	13-14	C (PE)	28.28	16.19	9.86	58.56	4.99	2.69	1.49	10.54
		D (PE)	20.61	11.55	6.11	37.24	2.88	1.45	0.95	5.41
Leaf	16-17	C (PE)	191.99	50.01	129.6	290.44	44.48	15.08	27.01	72.95
		D (PE)	168.73	64.98	56.92	279.73	37.77	17.59	9.9	68.31
Root	16-17	C (PE)	21.59	13.48	3.65	48.61	4.01	3.02	0.64	10.6
		D (PE)	12.92	8.6	3.72	33.01	2.37	1.37	0.6	5.28
Leaf	60-66	C (PE)	79.62	39.18	42.87	186.45	19.9	12.09	10.8	53.68
		D (PE)	73.98	28.09	42.22	139.53	18.98	8.91	10.09	40.19
Root	60-66	C (PE)	8.78	9.9	0.61	29.9	1.99	2.09	0.17	6.28
		D (PE)	4.8	4.26	0.75	12.9	1.13	0.87	0.22	2.82
Flower	60-66	C (PE)	49.47	10.63	34.36	66.37	8.94	1.7	6.44	11.3
		D (PE)	48.03	17.08	30.71	72.1	8.52	3.1	4.62	12.71
Leaf	76-79	C (PE)	126.15	37.34	99.65	234.43	35.25	11.07	23.18	63.52
		D (PE)	107.77	42.86	46.41	214.47	29.86	12.49	14	59.99
Root	76-79	C (PE)	13.24	12.95	2.23	38.01	4.05	3.91	0.71	11.79
		D (PE)	13.24	12.65	3.57	40.91	4.09	4.05	1.06	13.3
forage	76-79	C (PE)	51.34	9.25	36.92	64.05	12.19	3.02	7.56	17.01
		D (PE)	48.72	9.38	38.25	66.9	11.37	3.59	7.04	18.52
Grain	89-99	C (PE)	95.91	43.11	43.13	166.22	84.99	38.66	38.45	147.89
		D (PE)	83.14	37.69	15.17	144.81	73.93	33.86	13.5	129.82
Whole plant	89-99	C (PE)	40.84	18.13	0.34	69.19	33.66	15.06	0.28	55.05
		D (PE)	49.03	16.15	25.7	72.64	40.22	13.68	22.15	62.25

Entry C (PE) = GMB151 (not treated); Entry D (PE) = GMB151 (treated).
Mean and standard deviation (SD) for each entry was based on the total sample population (N=12).

Table 14 Expression of HPPD-4 in Soybean Matrices Harvested from Treated and Not treated GMB151 Grown at Three Sites

Matrix	BBCH Growth Stage	Entry	HPPD-4 (µg/g DW)				HPPD-4 (µg/g FW)			
			Mean	SD	Min	Max	Mean	SD	Min	Max
Leaf	13-14	C (PE)	430.04	207.57	191.44	779.67	102.86	66.4	35.66	219.07
		D (PE)	429.22	271.72	191.29	1028.76	89.78	63.5	38.09	205.75
Root	13-14	C (PE)	57.71	29.15	26.43	107.86	10.49	5.46	3.78	20.54
		D (PE)	73.67	41.89	26.42	138.82	10.74	5.7	3.55	20.15
Leaf	16-17	C (PE)	416.62	158.43	175.6	688.68	97.3	44.48	37.33	174.06
		D (PE)	350.86	236.25	59.11	830.47	78.91	59.82	13.27	202.79
Root	16-17	C (PE)	61.9	16.24	40.71	91.93	11.3	4.81	7.04	20.83
		D (PE)	51.05	16.86	25.9	83.86	9.49	2.47	5.81	14.85
Leaf	60-66	C (PE)	201.65	100.9	82.72	372.05	50.53	29.72	20.84	101.8
		D (PE)	178.76	73.51	90.83	350.74	46.18	23.27	21.7	101.04
Root	60-66	C (PE)	24.6	12.98	9.7	49.41	5.94	2.69	2.48	11.33
		D (PE)	20.93	7.83	9.59	40.96	5.4	1.98	2.6	9.8
Flower	60-66	C (PE)	54.29	13.73	40.95	75.72	9.86	2.5	7.39	13.98
		D (PE)	70.87	26.45	39.59	121.6	12.45	4.62	7.53	20.67
Leaf	76-79	C (PE)	287.64	208.74	112.19	715.95	78.85	56.72	36.04	193.98
		D (PE)	225.73	141.37	34.57	449.58	61.38	39.21	11.34	125.74
Root	76-79	C (PE)	34.89	22.58	12.81	74.92	11.12	7.57	3.82	23.88
		D (PE)	24.69	9.71	9.58	40.41	7.45	2.94	2.88	12.76
forage	76-79	C (PE)	120.18	42.47	78.96	203.83	29.09	12.99	15.55	54.92
		D (PE)	129.03	45.32	87.61	196.27	30.48	14.07	15.26	53.51
Grain	89-99	C (PE)	4.46	2.9	1.23	9.83	3.95	2.59	1.11	8.74
		D (PE)	4.45	3.57	1.31	12.71	3.97	3.2	1.18	11.39
Whole plant	89-99	C (PE)	2.44	1.02	<LLOQ	4.12	2.03	0.88	<LLOQ	3.28
		D (PE)	2.89	1.33	1.33	4.59	2.4	1.15	1.01	3.93

Entry C (PE) = GMB151 (not treated); Entry D (PE) = GMB151 (treated).
Mean and standard deviation (SD) for each entry was based on the total sample population (N=12), except for whole plant Entry C (PE). Mean and SD for whole plant Entry C were calculated on a population of N=11, since one of the samples yielded HPPD-4 values below the Lower Limit of Quantification (<LLOQ).

(g) an analysis of the expressed RNA transcripts, where RNA interference has been used.

RNA interference has not been used to develop this food product.

B.1 Characterisation and safety assessment of new substances

(a) A full description of the biochemical function and phenotypic effects of all new substances (e.g. a protein or an untranslated RNA) that are expressed in the new GM organism, including their levels and site of accumulation, particularly in edible portions

HPPD-4 protein

The coding sequence of the 4-hydroxyphenylpyruvate dioxygenase (HPPD) protein was isolated from the *Pseudomonas fluorescens* strain A32. Four amino acids were substituted [redacted] to reduce HPPD inhibitors binding efficacy. The modified protein is designated as HPPD-4 (Porée, F. et al, 2014, M-486300-01-1, Appendix 10).

Background information and History of Safe Use of HPPD-4

The *hppd* gene was isolated from the bacterium *Pseudomonas fluorescens*, strain A32. *P. fluorescens* is a Gram-negative, rod-shaped, motile, asporogenous, aerobic bacterium. *P. fluorescens*, is ubiquitous in the environment, including soil, water and food (OECD; 1997a). It has many beneficial uses in agriculture, human health and bioremediation. It is not described as allergenic, toxic or pathogenic to healthy humans and animals and has an overall history of safe use.

HPPD proteins are ubiquitous in nature across all kingdoms: bacteria, fungi, plants and animals including mammals. HPPD amino acid sequences have been determined in bacteria such as *Streptomyces avermitilis* (Accession number Q53586), in fungi such as *Aspergillus fumigatus* (Accession number Q4WPV8), in plants such as *Arabidopsis thaliana* (Accession number P93836), and in animals such as *Caenorhabditis elegans* (Accession number Q22633), mouse (*Mus musculus*, Accession number P49429), and human (*Homo sapiens*, Accession number P32754).

In particular, HPPD proteins have been characterized in organisms present in human food, such as carrot (*Daucus carota*, Accession number O23920), barley (*Hordeum vulgare* Accession number O48604; Falk *et al*., 2002), pork (*Sus scrofa*, Accession number Q02110)) and beef (*Bos Taurus*, Accession number Q5EA20).

In conclusion, HPPD proteins are present in food from plant, fungal or animal origin, with good safety records. Therefore, HPPD proteins have a history of safe use.

Biochemistry and mode of action of HPPD-4

4-Hydroxyphenylpyruvate dioxygenase (HPPD) is an Fe(II)-dependent, non-heme oxygenase. HPPD is a key enzyme involved in the catabolism of tyrosine which catalyzes the conversion of 4-hydroxyphenylpyruvate to homogentisate. In plants, HPPD enzyme is also involved in several anabolic pathways; its reaction product homogentisate (2,5-dihydroxyohenylacetate) being the aromatic precursor of tocopherol and plastoquinone, which are essential to the photosynthetic transport chain and antioxidative systems (Fritze, I. M.; et al.; 2004; M-359884-01, Appendix 11). Figure 7 shows a diagram of the different metabolic pathways in which HPPD is involved in plants and non-photosynthetic organisms.

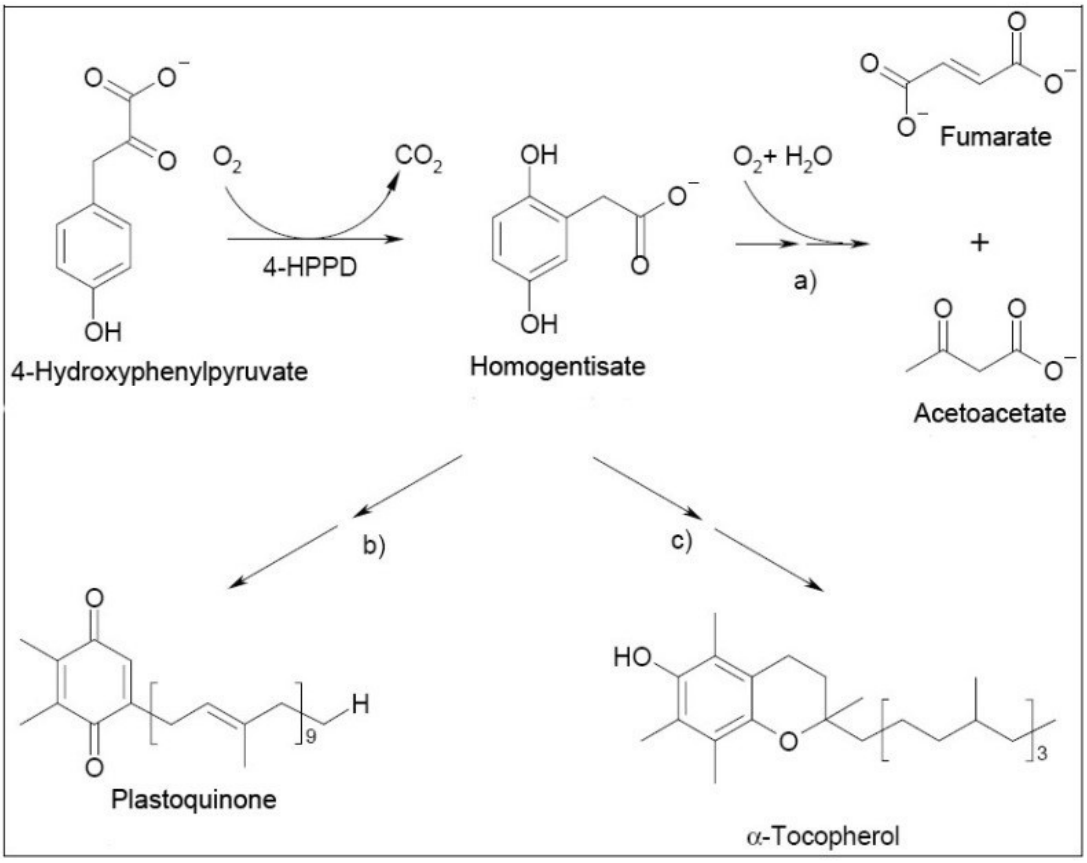


Figure 7. Biochemical pathways of HPPD proteins

a) catabolism of tyrosine, b) biosynthesis of plastoquinone (plants)
c) biosynthesis of tocopherol and tocotrienols (plants)

HPPD enzymes require a α-keto acid and molecular oxygen to oxidize or oxygenate a third molecule. The activity of HPPD is suppressed by HPPD-inhibiting herbicides (Figure 8). HPPD enzyme inhibition results in the disruption of the biosynthesis of carotenoids. Plants lacking carotenoids cannot protect themselves from the radicals generated by the light activation of chlorophyll, causing bleaching, necrosis, and death.

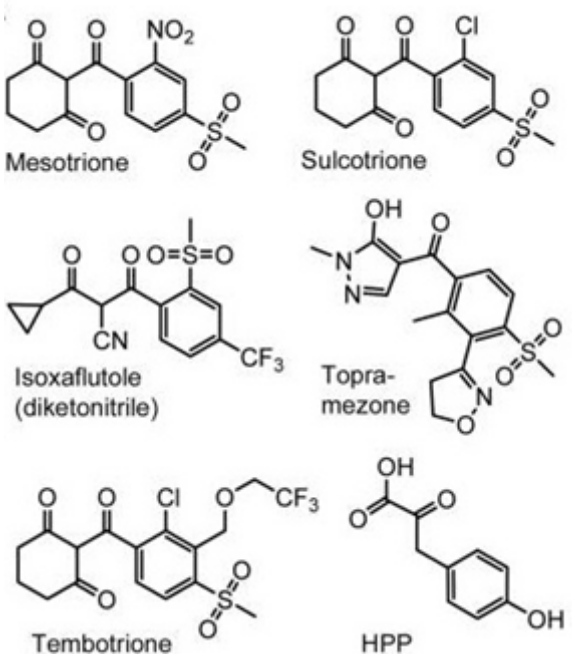


Figure 8. Structures of HPPD-inhibiting herbicides and substrate 4-hydroxyphenylpyruvate**. (Siehl D. L. et al., 2014)**

Cry14Ab-1 protein

The Cry14Ab-1 protein is a member of the Cry-type protein family with a fully conserved three-domain structure. Cry proteins are produced by *Bacillus thuringiensis* (*Bt*) strains and demonstrate specific toxicity towards insects or nematodes. Cry14Ab-1 belongs to the “nematicidal branch” of Cry proteins.

Background information and history of safe use of the Cry14Ab-1 protein

Cry proteins are synthesized by the Gram positive, spore-forming bacterium *Bacillus thuringiensis*. To date, more than 700 *cry* gene sequences that code for Cry proteins have been identified. These sequences were categorized based on their amino acid sequence similarity in at least 70 different cry gene groups, among which Cry5, Cry6, Cry12, Cry13, Cry14, and Cry21 displayed nematicidal activity (Bravo et al., 2013). The Cry proteins require specific conditions (e.g., specific proteases and receptors), resulting in a narrow host range. Several of the receptors for Cry proteins, including nematicidal Cry, have been characterized (Griffitts et al*.,* 2003; Griffitts and Aroian, 2005).

Cry proteins typically have narrow target specificity that limits activity of individual Cry proteins to just a few genera. They have been successfully used as biopesticides against Lepidoptera, Coleoptera, and Diptera (Palma et al., 2014).

Biochemistry and mode of action of the Cry14Ab-1 protein

Many studies and reviews have described the mode of action of Cry proteins (WHO/IPCS, 1999; Betz et al., 2000; Siegel, 2001; Bravo et al., 2007; OECD, 2007; Federici and Siegel, 2008). Cry proteins are active only when ingested.

Cry14Ab-1 activity has been observed only with nematodes. Target organism panel assessments have revealed negligible effects against arthropods, annelids, and vertebrates. With an insecticidal

Cry protein, the crystals are solubilized in the midgut upon ingestion by an insect. The toxins are then proteolytically activated by midgut proteases and bind to specific receptors located in the cell membrane, leading to cell disruption and death (Palma et al., 2014). Nematicidal Cry proteins have been observed to use a similar mode of action. The gastrointestinal tract epithelial surface of non-target animals, including humans, lack specific high-affinity Cry protein receptors (Koch et al., 2015).

Comparison of proteins (equivalence study)

For the safety assessment of GM crops, certain safety tests and studies require large amounts of protein. The expression levels of Cry14Ab-1 and HPPD-4 in GMB151 were too low to allow for purification of sufficient quantities of the two proteins directly from GMB151 for use in the safety assessment studies. Therefore, the Cry14Ab-1 and HPPD-4 proteins were produced in high-expressing recombinant host organisms, *Bacillus thuringiensis* and *E. coli* respectively. The recombinant proteins were engineered to match the amino acid sequences of their counterparts expressed in GMB151. The equivalence of GMB151 soybean-produced and bacterially-produced proteins were examined to ensure that the proteins from the two host sources were equivalent so that the bacterially-produced proteins could be used as surrogates in the studies.

Equivalence of GMB151-purified and microbially-produced Cry14Ab-1 proteins.

Purification of Cry14Ab-1 protein from the GMB151 soybean leaf matrix was performed using affinity chromatography. GMB151 soybean-purified Cry14Ab-1 protein was characterized and evaluated for equivalence with bacterially-produced Cry14Ab-1 protein based on a panel of analytical tests and assays, including analysis of a Coomassie-stained SDS-PAGE; western blot analysis; glycostaining analysis; *mass spectrometry*; N-terminal sequence analysis; and [redacted] bioassay ([redacted]., 2018, M-621885-01; Appendix 12 and [redacted] 2017, M-566932-02-1, Appendix 13).

Assessment and comparison of the apparent molecular mass

The GMB151 soybean-purified Cry14Ab-1 protein and the bacterially-produced Cry14Ab-1 protein were compared side by side by means of an SDS-PAGE analysis (Figure 9). Additionally, the bacterially-produced Cry14Ab-1 protein was spiked into the sample resulting from non-GM soybean subjected to the same affinity purification procedure as the plant-purified Cry14Ab-1 protein sample (*i.e.* non-GM control) to allow comparison in a similar soybean plant matrix.

A specific, predominant band was observed for both the plant-purified and the bacterially-produced Cry14Ab-1, which corresponds to the expected molecular mass of the Cry14Ab-1 protein (131.5 kDa). This demonstrated that the apparent molecular mass of the GMB151 soybean-purified and the bacterially-produced Cry14Ab-1 protein are comparable. No signal was observed for the negative control sample.

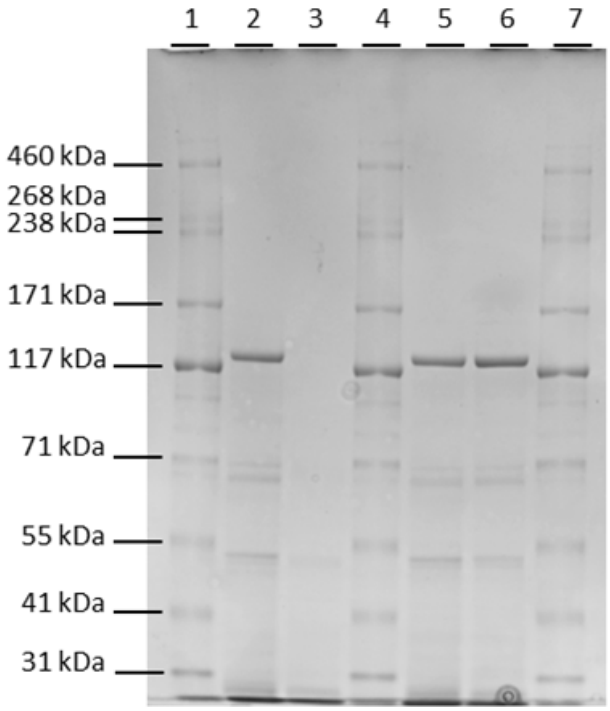


Figure 9: Assessment and comparison of the apparent molecular mass of the GMB151 soybean-purified Cry14Ab-1 protein and the bacterially-produced Cry14Ab-1 protein

Both plant-purified and bacterially-produced Cry14Ab-1 protein samples were loaded on a Criterion XT Tris-acetate 3-8 % gel and SDS-PAGE gel electrophoresis was performed in 1 x Tris-Acetate running buffer, followed by Coomassie staining.

Loading order:
Lane 1: 5 µL of the HiMark™ Pre-Stained High Molecular Mass Protein Standard (Life technologies)
Lane 2: 1 µg of plant-purified Cry14Ab-1 protein from GMB151 soybean
Lane 3: 10 µL (= corresponding volume) of the non-GM control
Lane 4: 5 µL of the HiMark™ Pre-Stained High Molecular Mass Protein Standard (Life technologies)
Lane 5: 1 µg of plant-purified Cry14Ab-1 protein from GMB151 soybean
Lane 6: 1 µg of bacterially-produced Cry14Ab-1 protein spiked in 10 µL non-GM control
Lane 7: 5 µL of the HiMark™ Pre-Stained High Molecular Mass Protein Standard (Life technologies)

Assessment and comparison of the immuno-reactivity

The GMB151-purified Cry14Ab-1 protein and the bacterially-produced Cry14Ab-1 protein were compared side by side by means of western blot analysis (Figure 10).

Using a Cry14Ab-1-specific polyclonal antibody, a signal corresponding to the expected molecular mass of the Cry14Ab-1 protein was detected for all plant-purified and bacterially produced Cry14Ab-1 samples. No signal was observed for the negative control samples.

The obtained results confirmed the immuno-reactivity of the GMB151-purified Cry14Ab-1 protein and the comparability to the bacterially-produced Cry14Ab-1 protein.

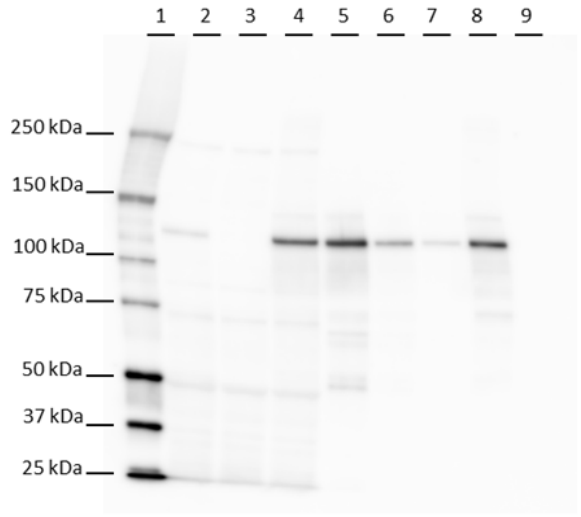


Figure 10: Assessment and comparison of immuno-reactivity of GMB151 soybean-purified Cry14Ab-1 protein and bacterially-produced Cry14Ab-1 protein

Both plant-purified and bacterially-produced Cry14Ab-1 protein samples were loaded on a Criterion XT Tris-Acetate gel 3-8% gel and SDS-PAGE gel electrophoresis was performed in 1x Tris-Acetate running buffer. After semi-dry blotting, the proteins were visualized by Enhanced chemiluminescence detection using the rabbit anti-Cry14Ab-1 antibody as primary antibody, the goat anti-rabbit HRP conjugated as secondary antibody and the Precision Protein™ StrepTactin-HRP Conjugate to detect the ECL Molecular mass marker.

Loading order:
Lane 1: 5 µL of Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)
Lane 2: 10 µL of crude protein extract from GMB151 soybean
Lane 3: 10 µL of crude protein extract from the non-GM counterpart
Lane 4: 40 ng bacterially-produced Cry14Ab-1 protein spiked in 10 µL of the crude protein extract from the non-GM counterpart
Lane 5: 40 ng of plant-purified Cry14Ab-1 protein from GMB151 soybean
Lane 6: 10 ng of plant-purified Cry14Ab-1 protein from GMB151 soybean
Lane 7: 4 ng of plant-purified Cry14Ab-1 protein from GMB151 soybean
Lane 8: 40 ng of bacterially-produced Cry14Ab-1 protein
Lane 9: 4 µl of a 1/10 diluted non-GM control

Assessment and comparison of the glycosylation status

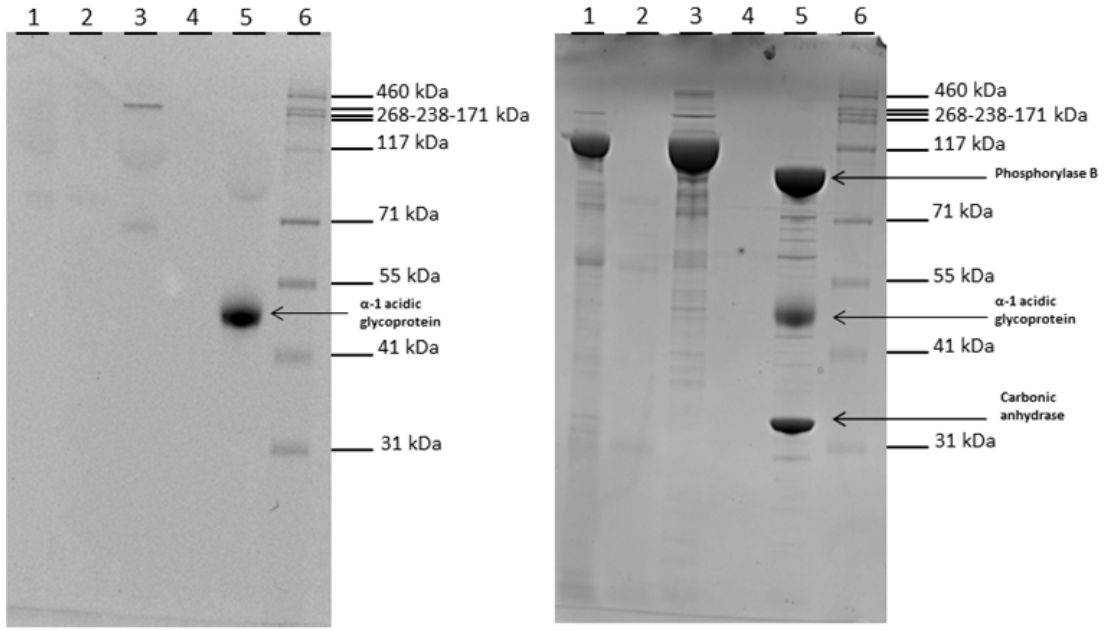
The glycosylation status of plant-purified Cry14Ab-1 protein and the bacterially-produced Cry14Ab-1 protein were assessed side by side. The results of the glycostaining analysis are shown in Figure 11.

The alpha-one acidic glycoprotein of the glycoprotein mix was visualized as a bright band. For the Cry14Ab-1 protein samples, weak background signals, similar to the background signal of the non-glycosylated proteins in the glycoprotein mix, were observed. In the bacterially-produced Cry14Ab-1 sample, a weak band was observed between 171 kDa and 238 kDa which is most probably an impurity

consisting of a potential glycosylated or glycosylation-like structure from the *Bacillus thuringiensis* spores (Figure 11, panel A).

The presence of sufficient Cry14Ab-1 protein on the gel was demonstrated by staining the gels with Coomassie after the glycostaining procedure (Figure 11, panel B).

The absence of glycosylation was demonstrated for both the GMB151-purified Cry14Ab-1 protein and the bacterially-produced Cry14Ab-1 protein. Consequently, both Cry14Ab-1 protein samples have a comparable glycosylation status.



Panel A Panel B

Figure 11: Assessment and comparison of the glycosylation status of the plant-purified Cry14Ab-1 protein and the bacterially-produced Cry14Ab-1 protein

One Criterion XT Bis-Tris 12 % SDS-PAGE gel was prepared and cut into two pieces, each part containing 7 µg Cry14Ab-1 protein of both the plant-purified Cry14Ab-1 protein and bacterially-produced Cry14Ab-1 protein together with the appropriate positive and negative controls to assess the glycosylation status.

Panel A shows the result of the staining using the Glycoprotein Detection Kit (Sigma) to demonstrate the absence of glycosylation of both the Cry14Ab-1 protein samples.
Panel B shows a Coomassie staining of the SDS-PAGE gel to demonstrate the presence of the proteins on the gel.

Loading order of Panel A and B:
Lane 1: 53.21 pmoles of plant-purified Cry14Ab-1 protein from GMB151 soybean
Lane 2: 9.46 (corresponding volume) µL of the non-GM control
Lane 3: 53.21 pmoles µg of bacterially-produced Cry14Ab-1 protein
Lane 4: empty
Lane 5: 45.45 pmoles of Glycoprotein mix
Lane 6: 5 µL of the HiMark™ Pre-Stained High Molecular Mass Protein Standard (Life technologies)

Assessment of the peptide mapping

The peptide mapping was established using UPLC-UV-MS analysis for the GMB151-purified and the bacterially-produced Cry14Ab-1 protein.

Peptides resulting from a trypsin digest of the GMB151-purified Cry14Ab-1 protein were analysed using UPLC-UV-MS. Figure 12 provides an overview of the mapped peptides against the theoretical amino acid sequence of the Cry14Ab-1 protein. A coverage of 70.8 % was determined, which confirms the identity of Cry14Ab-1 protein. The observation of a 42 atomic mass unit (amu) mass increment at the N-terminal peptide indicates acetylation of the N-terminus. Cleavage of the N-terminal methionine and N-terminal acetylation are by far the most common modifications of eukaryotic proteins (Polevoda and Sherman, 2000 and 2003; Bradshaw et al., 1998). These post-translational modifications affect the majority of soluble eukaryotic proteins. Over 70% of the analyzed proteins contain a modification on the N-terminus. Examples of N-terminal acetylation of plant proteins are described for soybean (Peariso et al., 2007).

Peptides resulting from a trypsin digest of bacterially-produced Cry14Ab-1 protein were analysed using UPLC-UV-MS. Figure 13 provides an overview of the mapped peptides against the theoretical amino acid sequence of the Cry14Ab-1 protein. A coverage of 86.8 % was determined, which confirmed the identity of the Cry14Ab-1 protein.

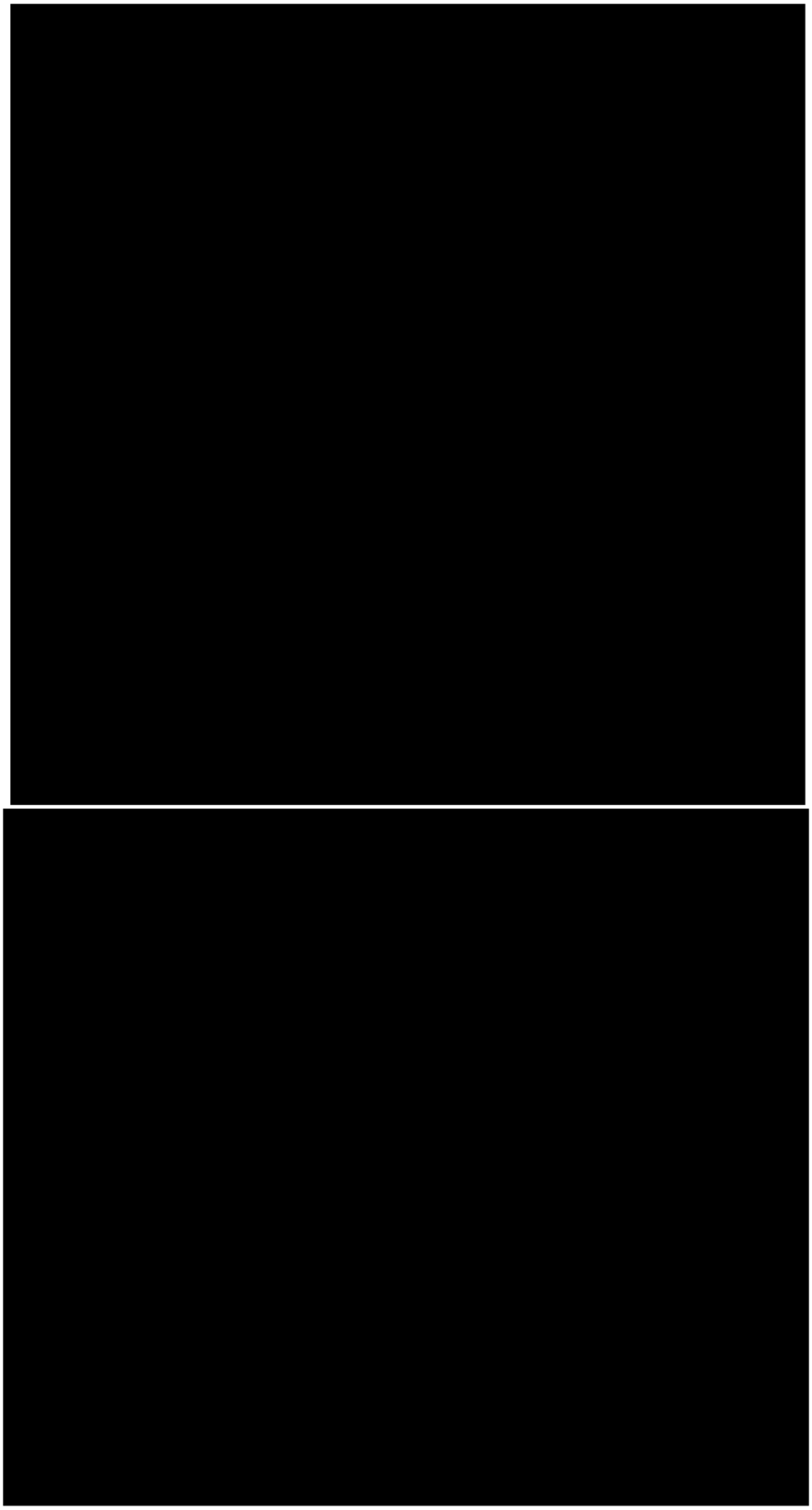


Figure 12: Schematic overview of the coverage of the theoretical Cry14Ab-1 sequence by the tryptic peptides from the soybean GMB151-purified Cry14Ab-1 protein

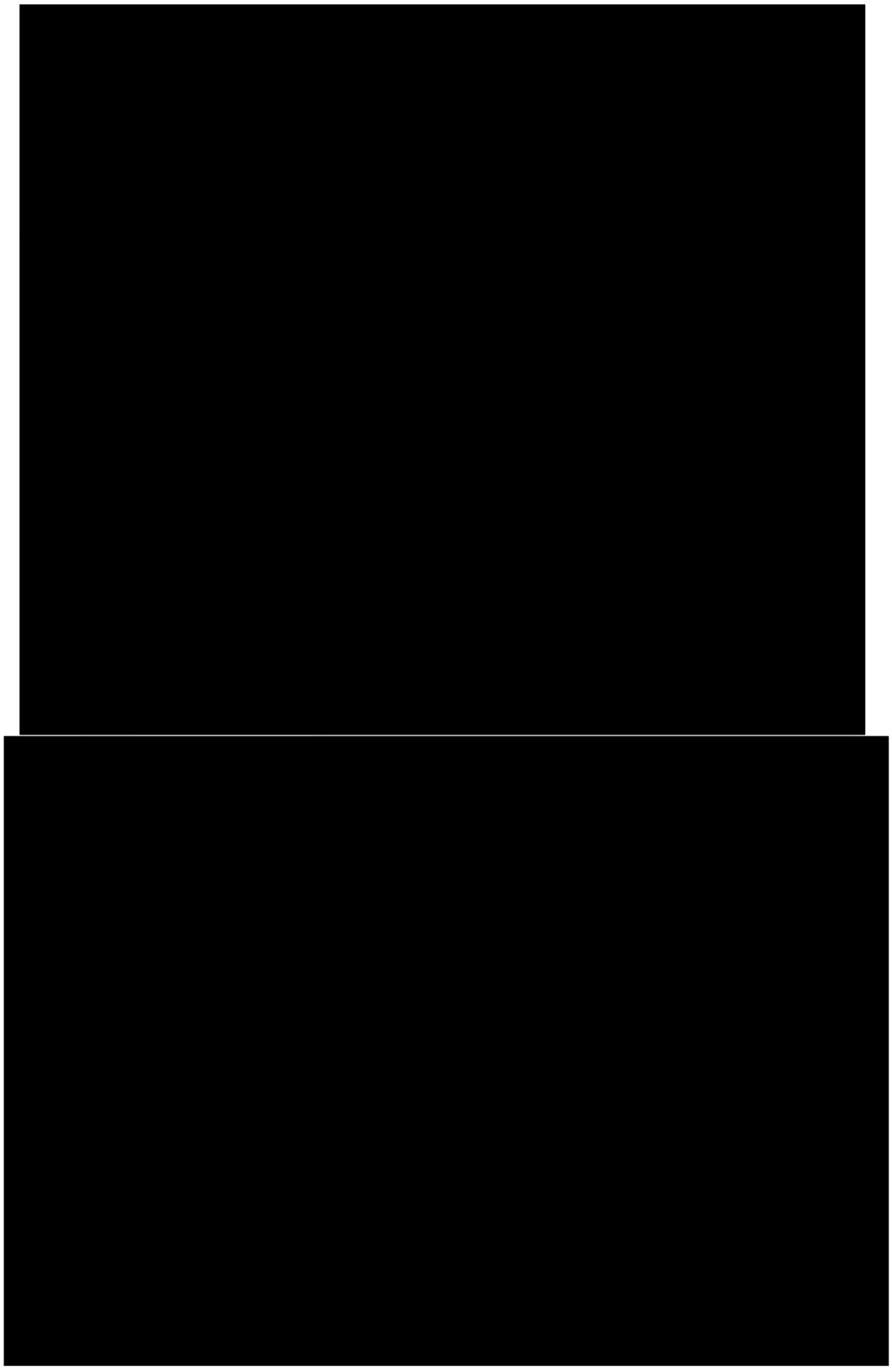


Figure 13: Schematic overview of the detected peptides derived from tryptic digestion of bacterially-produced Cry14Ab-1

Assessment of the N-terminal sequence

The N-terminal sequence of the GMB151 soybean-purified Cry14Ab-1 protein was determined by UPLC-UV-MS^E analysis of a Chymotrypsin digested sample.

The obtained mass of the N-terminal peptide, containing amino acid residues 1 to 13, was in agreement with the theoretical monoisotopic mass of the acetylated N-terminal peptide.

The N-terminal sequence of bacterially-produced Cry14Ab-1 protein was determined by Edman degradation. The obtained N-terminal sequence confirmed the theoretical amino acid sequence ( ) with the exception of . .

Assessment and comparison of the Cry14Ab-1 functional activity

The functional activity of the GMB151 soybean-purified and the bacterially-produced Cry14Ab-1 proteins was assessed by means of a bioassay.

Since the of the plant-purified Cry14Ab-1 protein was within 10-fold of the of the bacterially-produced Cry14Ab-1 protein, plant-purified and bacterially-produced Cry14Ab-1 proteins were considered functionally equivalent.

Conclusion

The equivalence of the GMB151 soybean-purified Cry14Ab-1 protein with bacterially-produced Cry14Ab-1 protein was demonstrated based on a panel of analytical tests and assays, including analysis of a Coomassie-stained SDS-PAGE; western blot analysis; glycostaining analysis; mass spectrometry; N-terminal sequence analysis; and bioassay.

Equivalence of GMB151-purified and microbially-produced HPPD-4 proteins.

Purification of HPPD-4 protein from the GMB151 soybean leaf matrix was performed using affinity chromatography. GMB151 soybean-purified HPPD-4 protein was characterized and the equivalence with bacterially-produced HPPD-4 protein was evaluated based on a panel of analytical tests and assays, including analysis of a Coomassie-stained SDS-PAGE; western blot analysis; glycostaining analysis; mass spectrometry; and N-terminal sequence analysis ([redacted], 2018, 17-RSVLN028-A, Appendix 14; [redacted] 2018, SEL/6495/3, Appendix 15; [redacted] 2014, M-490041-01, Appendix 16).

Assessment and comparison of the apparent molecular mass

The GMB151 soybean-purified HPPD-4 protein and the bacterially-produced HPPD-4 protein were compared side by side by means of an SDS-PAGE analysis (Figure 14). The bacterially-produced HPPD-4 protein was spiked into the sample resulting from non-GM soybean subjected to the same affinity purification procedure as the plant-purified HPPD-4 protein sample (*i.e.* non-GM control) to allow comparison in a similar soybean plant matrix.

A specific, predominant band which corresponds to the expected molecular mass of the HPPD-4 protein (40.3 kDa) was observed for both the plant-purified HPPD-4 protein and the bacterially-produced HPPD-4 protein samples. This demonstrated that the apparent molecular mass of the GMB151 soybean-purified and the bacterially-produced HPPD-4 protein are comparable. The non-GM control showed some non-specific background staining derived from the plant matrix. These background fragments are visible in all soybean samples.

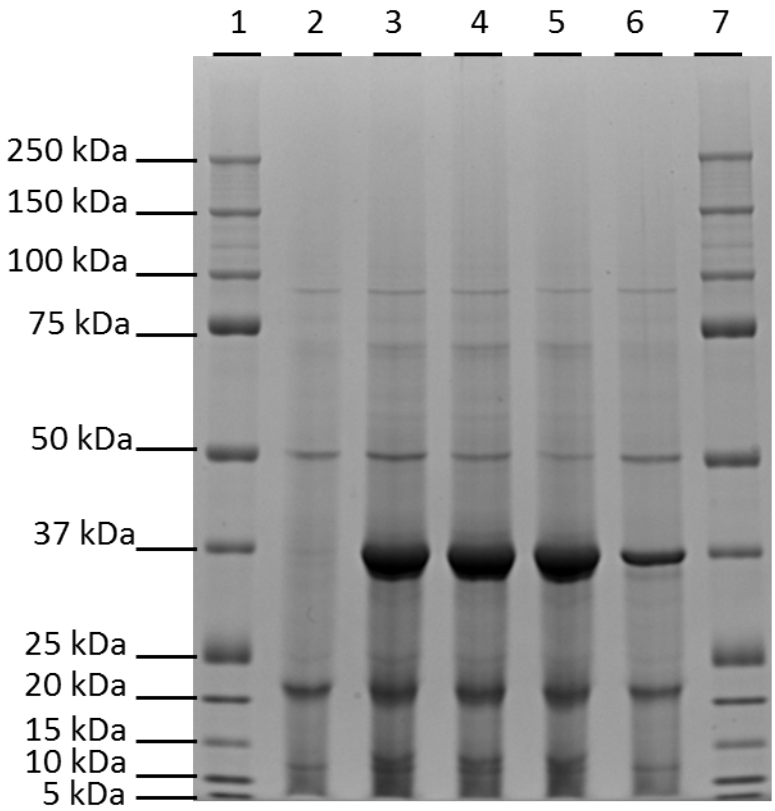


Figure 14: Assessment and comparison of apparent molecular mass GMB151 soybean-purified HPPD-4 protein and the bacterially-produced HPPD-4 protein

Both plant-purified and bacterially-produced HPPD-4 protein samples were loaded on a NuPAGE Bis-Tris 4 - 12 % gel and SDS-PAGE gel electrophoresis was performed in 1 x MOPS running buffer, followed by Coomassie staining.

Loading order:
Lane 1: 2 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)
Lane 2: 10 µL of the non-GM control
Lane 3: 10 µl of plant-purified HPPD-4 protein sample GM/VLN028-A/05 from GMB151 soybean
Lane 4: 10 µl of plant-purified HPPD-4 protein sample GM/VLN028-A/06 from GMB151 soybean
Lane 5: 10 µl of plant-purified HPPD-4 protein sample GM/VLN028-A/07 from GMB151 soybean
Lane 6: 1 µg of bacterially-produced HPPD-4 protein spiked in 10 µL non-GM control
Lane 7: 2 µL of Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)

Assessment and comparison of the immuno-reactivity

The GMB151 soybean-purified HPPD-4 protein and the bacterially-produced HPPD-4 protein were compared side by side by means of a western blot (Figure 15).

Using a HPPD-4-specific polyclonal antibody, a signal corresponding to the expected molecular mass of the HPPD-4 protein was detected for all plant-purified and bacterially produced HPPD-4 samples.

The obtained results confirmed the immuno-reactivity of the GMB151 soybean-purified HPPD-4 protein and the comparability to the bacterially-produced HPPD-4 protein.

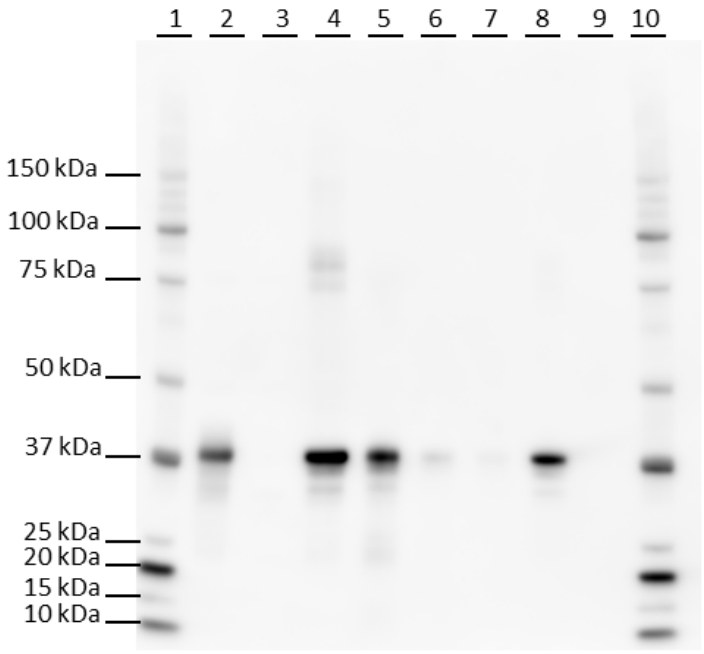


Figure 15: Assessment and comparison of immuno-reactivity of GMB151 soybean-purified HPPD-4 protein and bacterially-produced HPPD-4 protein

Both plant-purified and bacterially-produced HPPD-4 protein samples were loaded on a NuPAGE Bis-Tris 4 - 12 % gel and SDS-PAGE gel electrophoresis was performed in 1x MOPS running buffer. After semi-dry blotting, the proteins were visualized by Enhanced chemiluminescence detection using a 1:5000 dilution of the rabbit anti-HPPD-4 antibody as primary antibody and a 1:10000 dilution of the goat anti-rabbit HRP conjugated as secondary antibody and a 1:10000 dilution of the Precision Protein™ StrepTactin-HRP Conjugate to detect the ECL Molecular mass marker.

Loading order:
Lane 1: 5 µL of Precision Plus Protein™ WesternC Standards (Bio-Rad)
Lane 2: 5 µL of crude protein extract from GMB151 soybean
Lane 3: 5 µL of crude protein extract from the non-GM counterpart
Lane 4: 40 ng bacterially-produced HPPD-4 protein spiked in 5 µL of the crude protein extract from the non-GM counterpart
Lane 5: 40 ng of plant-purified HPPD-4 protein from GMB151 soybean
Lane 6: 10 ng of plant-purified HPPD-4 protein from GMB151 soybean
Lane 7: 4 ng of plant-purified HPPD-4 protein from GMB151 soybean
Lane 8: 40 ng of bacterially-produced HPPD-4 protein
Lane 9: 6.56 µl of a 1/100 diluted non-GM control
Lane 10: 5 µL of Precision Plus Protein™ WesternC Standards (Bio-Rad)

Assessment and comparison of the glycosylation status

The results of the glycostaining analysis are shown in Figure 16.

The glycosylated proteins of the horseradish peroxidase positive control and the alpha-one acidic glycoprotein of the glycoprotein mix were visualized as bright bands on the gel, while for the HPPD-4 protein samples, no signal was observed (Figure 16, panel A).

The presence of sufficient HPPD-4 protein on the gel was demonstrated by staining the gels with Coomassie after the glyco-staining procedure (Figure 16, panel B).

The absence of glycosylation was demonstrated for both the GMB151 soybean-purified HPPD-4 protein and the bacterially-produced HPPD-4 protein. Consequently, both HPPD-4 protein samples have a comparable glycosylation status.

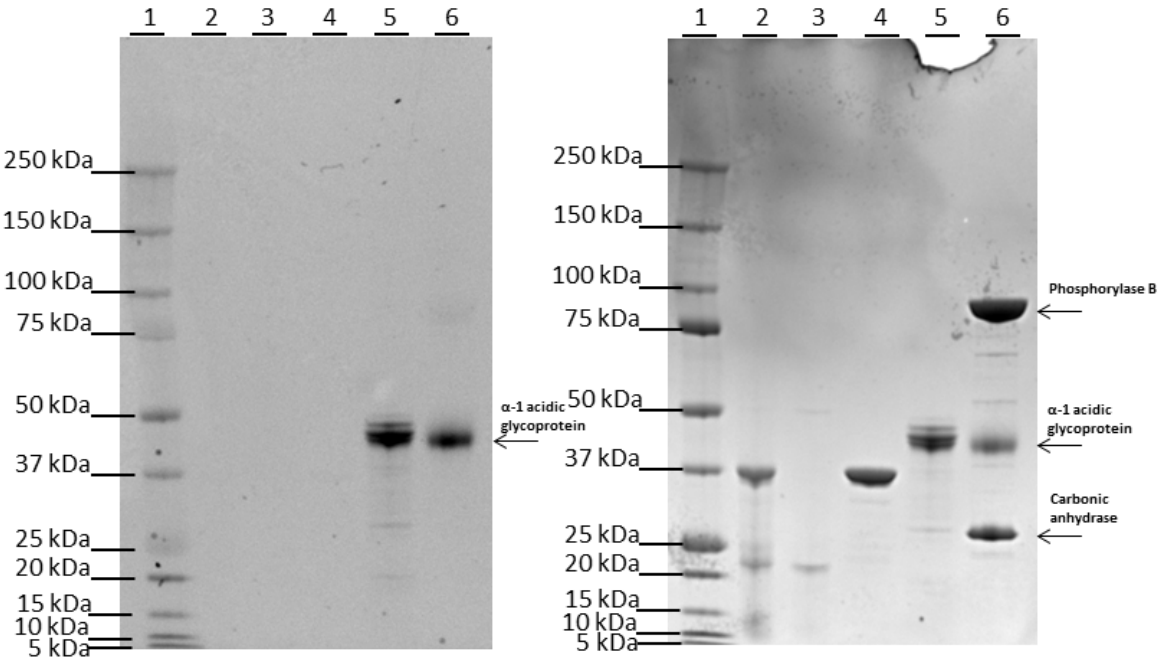


Figure 16: **Assessment and comparison of the of the glycosylation status of the GMB151 soybean-purified HPPD-4 protein and the bacterially-produced HPPD-4 protein**

One Criterion XT Bis-Tris 4 - 12 % SDS-PAGE gel was prepared and cut into two, each part containing 2 µg HPPD-4 protein of both the plant-purified HPPD-4 protein and bacterially-produced HPPD-4 protein together with the appropriate positive and negative controls to assess the glycosylation status.

Panel A shows the result of the staining using the Glycoprotein Detection Kit (Sigma) to demonstrate the absence of glycosylation of both the HPPD-4 protein samples.
Panel B shows a Coomassie staining of the SDS-PAGE gel to demonstrate the presence of the proteins on the gel.

Loading order of Panel A and B:
Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards (Bio-Rad)
Lane 2: 2 µg of plant-purified HPPD-4 protein from GMB151 soybean
Lane 3: 3.28 (corresponding volume) µL of the non-GM control
Lane 4: 2 µg of bacterially-produced HPPD-4 protein
Lane 5: 45.45 pmoles Horseradish Peroxidase (positive control)
Lane 6: 45.45 pmoles of Glycoprotein mix

Assessment of the intact molecular mass

The intact molecular mass of the GMB151-purified HPPD-4 protein was determined using UPLC-UV-MS. The determined intact molecular mass allowed the identification of three major molecular masses. The molecular mass of 39748.3 Da corresponds to the mature form of the HPPD-4 protein, minus the [redacted], which lacks the [redacted]. A second molecular mass (40014.7 Da) corresponds to the mature HPPD-4 protein with [redacted], lacking the [redacted]. The third observed molecular mass (40415.1 Da) corresponds to the mature HPPD-4 protein with [redacted]. An overview of the different forms mapped against the theoretical form is shown in Figure 17.

Degradation of the plant-purified HPPD-4 protein sample was noticed in several experiments. The forms lacking four residues at the C-terminus are considered as partially degraded HPPD-4 protein forms derived from the two major forms present in the plant.

Cleavage of the [redacted] and is a common process in many organisms. The additional [redacted] derived from incomplete cleavage of the transit peptide.

The intact molecular mass of the bacterially produced HPPD-4 protein was determined using LC-UV-MS analysis. The determined intact molecular mass (40148.1 Da) confirms the theoretical molecular mass of the protein corresponding with the mass of the HPPD-4 protein lacking the [redacted] (40149 Da).

Both bacterially-produced and GMB151 plant-purified HPPD-4 proteins consist of the mature HPPD-4 protein lacking [redacted]. The GMB151 plant-purified HPPD-4 also contains a second form: the mature HPPD-4 protein carrying a [redacted]. This [redacted] is derived from incomplete cleavage of the transit peptide.


[redacted]

Figure 17: Schematic overview of the results of the intact molecular mass determination

Mapping of the three major forms of the plant-purified HPPD-4 protein and the bacterial-produced HPPD-4 against the N- and C termini of the theoretical amino acid sequence of the mature HPPD-4 protein. [redacted] which is derived from the transit peptide.

Assessment of the peptide mapping

The peptide mapping for the GMB151-purified HPPD-4 protein was established using UPLC-UV-MS^{E} analysis. Peptides resulting from a trypsin digest of the GMB151 soybean-purified HPPD-4 protein were analysed. Figure 18 provides an overview of the mapped peptides against the theoretical amino acid sequence of the HPPD-4 protein. A coverage of 72.9 % was determined, which confirmed the identity of the HPPD-4 protein. Numerous peptides resulted from protein cleavage at sites which are not trypsin-specific which could possibly have been caused by protein degradation.

The peptide mapping for bacterially-produced HPPD-4 protein was established using LC-UV-MS analysis. Peptides resulting from a trypsin digest of bacterially-produced HPPD-4 protein were analysed. Figure 19 provides an overview of the mapped peptides against the theoretical amino acid sequence of the HPPD-4 protein. A coverage of 96.6 % was determined, which confirmed the identity of HPPD-4 protein.

Figure 18: **Schematic overview of the coverage of the theoretical HPPD-4 sequence by the tryptic peptides from the soybean GMB151-purified HPPD-4 protein detected by UPLC-UV-MS^E**

Mapping of the peptides derived from the trypsin-digested plant-purified HPPD-4 protein sample against the theoretical amino acid sequence of the HPPD-4 protein. The black bar represents the N-terminal peptide corresponding to the HPPD-4 protein with an N-terminal cysteinic sulfinic acid, derived from the transit peptide.

Figure 19: Schematic overview of the coverage of the theoretical HPPD-4 sequence by the tryptic peptides from the bacterially-purified HPPD-4 protein detected by UPLC-UV-MS^{E}.

Mapping of the peptides derived from the trypsin digested bacterially produced HPPD-4 protein sample against the theoretical amino acid sequence of the HPPD-4 protein

Assessment of the N-terminal sequence

The N-terminal sequence of the GMB151 soybean-purified HPPD-4 protein and the bacterially produced HPPD-4 protein were determined by Edman degradation.

The obtained data for the GMB151 soybean-purified sample suggested the presence of [redacted]. The obtained sequence result was resolved as [redacted], corresponding to the [redacted] of the mature HPPD-4 lacking [redacted], corresponding to incomplete cleavage of the transit peptide of the HPPD-4 protein. These results support the data obtained with the intact molecular mass determination.

The N-terminal sequence of bacterially-produced HPPD-4 protein was determined by Edman degradation. The obtained [redacted] corresponds to the [redacted] of the mature HPPD-4 lacking [redacted]. This confirmed the results observed with the intact molecular mass determination.

Assessment and comparison of the HPPD-4 functional activity

The functional activity of the HPPD-4 protein in GMB151 soybean and the bacterially-produced HPPD-4 protein was assessed by means of a quantitative enzyme assay.

A protein extraction was performed from GMB151 soybean leaf samples, and the concentration of HPPD-4 was determined by means of ELISA. The activity of the HPPD-4 was determined in this crude extract by measuring the homogentisic acid (HGA) formed over time, using LC MS/MS.

As the soybean background negatively impacts the enzymatic assay, the measurement of the activity of the bacterially produced HPPD-4 protein had to be performed in a similar matrix. Therefore, the bacterially produced HPPD-4 protein was added during protein extraction from non-GM soybean, and the determination of its activity was performed in the same way.

The GMB151 soybean extracted HPPD-4 protein was shown to have a specific activity of 1.88E-02 nMol/min/µg enzyme. This activity is comparable with the bacterially-produced HPPD-4 protein as measured in the leaf extracts (2.77E-02 nMol/min/µg). As the two values are comparable, the HPPD-4 in GMB151 soybean and the bacterially-produced HPPD-4 protein can be considered as functionally equivalent.

Conclusion

The equivalence of the GMB151 soybean-purified HPPD-4 protein with bacterially-produced HPPD-4 protein was demonstrated based on a panel of analytical tests and assays, including analysis of a Coomassie-stained SDS-PAGE; western blot analysis; glycostaining analysis; *mass spectrometry*; and N-terminal sequence analysis. Both bacterially-produced and GMB151 plant-purified HPPD-4 proteins consist of the mature HPPD-4 protein lacking the [redacted]. In GMB151 a second form is also present: the mature HPPD-4 protein carrying a modified cysteine residue at the N-terminus. This additional residue is derived from incomplete cleavage of the transit peptide. Overall, the comparison of the plant-purified HPPD-4 protein from GMB151 soybean with the bacterially-produced HPPD-4 protein demonstrated that both HPPD-4 proteins are comparable for all assessed structural characteristics.

Expression of Cry14Ab-1 and HPPD-4 in grain and processed fractions

The concentration of Cry14Ab-1 and HPPD-4 proteins in grain and processed fractions of GMB151 soybean treated and untreated with trait-specific herbicide were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA) ([redacted] et al., 2018, 16-RSBS0011, Appendix 17).

The concentration of the Cry14Ab-1 protein in grain and hull samples ranged from 24.24 to 92.84 µg/g DW (Table 17). The highest concentration of Cry14Ab-1 was observed in grain. The Cry14Ab-1 protein concentration was below the lower limit of quantitation (LLOQ) in toasted meal, RBD oil, and protein isolate.

The concentration of the HPPD-4 protein in grain, protein isolate, and hull samples ranged from 0.27 to 5.57 µg/g DW (Table 18). The highest concentration of HPPD-4 was observed in grain. The HPPD-4 protein concentration was below the LLOQ in toasted meal and RBD oil.

Table 15. Concentrations of Cry14Ab-1 in GMB151 Soybean grain and Processed Fractions

Matrix	Trait-Specific Herbicide Treatment	Cry14Ab-1 (µg/g FW)[1]	Cry14Ab-1 (µg/g DW)[1]
Grain	Not Treated	71.79	81.48
	Treated	81.89	92.84
Toasted meal	Not Treated	<LLOQ	<LLOQ
	Treated	<LLOQ	<LLOQ
RBD oil	Not Treated	<LLOQ	NA
	Treated	<LLOQ	NA
Protein isolate	Not Treated	<LLOQ	<LLOQ
	Treated	<LLOQ	<LLOQ
Hulls	Not Treated	20.90	24.24
	Treated	23.50	26.99

[1]Values were calculated to full precision and rounded to 2 decimal places for reporting consistency.
LLOQ = Lower Limit of Quantitation
NA = No Applicable. DW calculations for oil samples were not performed since oil samples were analyzed as received and moisture data for these samples were not obtained.

Table 16. Concentrations of HPPD-4 in GMB151 Soybean Grain and Processed Fractions

Matrix	Trait-Specific Herbicide Treatment	HPPD-4 (µg/g FW)[1]	HPPD-4 (µg/g DW)[1]
Grain	Not Treated	3.34	3.79
	Treated	4.91	5.57
Toasted meal	Not Treated	<LLOQ	<LLOQ
	Treated	<LLOQ	<LLOQ
RBD oil	Not Treated	<LLOQ	<LLOQ
	Treated	<LLOQ	<LLOQ
Protein isolate	Not Treated	0.78	0.80
	Treated	0.47	0.48
Hulls	Not Treated	0.23	0.27
	Treated	0.36	0.42

[1]Values were calculated to full precision and rounded to 2 decimal places for reporting consistency.
LLOQ = Lower Limit of Quantitation
NA = No Applicable. DW calculations for oil samples were not performed since oil samples were analyzed as received and moisture data for these samples were not obtained.

(b) Information about prior history of human consumption of the new substances, if any, or their similarity to substances previously consumed in food.

See the relevant parts of Section B.1(a) above on history of safe use and refer to the relevant studies.

HPPD-4 protein

The *hppd* gene was isolated from the bacterium *Pseudomonas fluorescens*, strain A32. *P. fluorescens* is a Gram-negative, rod-shaped, motile, asporogenous, aerobic bacterium. *P. fluorescens*, is ubiquitous in the environment, including soil, water and food (OECD; 1997a). It has many beneficial uses in agriculture, human health and bioremediation. It is not described as allergenic, toxic or pathogenic to healthy humans and animals and has an overall history of safe use.

HPPD proteins are ubiquitous in nature across all kingdoms: bacteria, fungi, plants and animals including mammals. HPPD amino acid sequences have been determined in bacteria such as *Streptomyces avermitilis* (Accession number Q53586), in fungi such as *Aspergillus fumigatus* (Accession number Q4WPV8), in plants such as *Arabidopsis thaliana* (Accession number P93836), and in animals such as *Caenorhabditis elegans* (Accession number Q22633), mouse (*Mus musculus*, Accession number P49429), and human (*Homo sapiens*, Accession number P32754).

In particular, HPPD proteins have been characterized in organisms present in human food, such as carrot (*Daucus carota*, Accession number O23920), barley (*Hordeum vulgare* Accession number O48604; Falk *et al*., 2002), pork (*Sus scrofa*, Accession number Q02110)) and beef (*Bos Taurus*, Accession number Q5EA20).

In conclusion, HPPD proteins are present in food from plant, fungal or animal origin, with good safety records. Therefore, HPPD proteins have a history of safe use. The specific amino acid sequence of the HPPD-4 protein was determined to permit further elucidation of its potential toxicity by *in silico* assessment methods.

HPPD-4

The coding sequence of the 4-hydroxyphenylpyruvate dioxygenase of *Pseudomonas fluorescens* strain A32 was modified by the replacement of the [redacted] The four introduced mutations lead to reduced HPPD inhibitor herbicide binding efficacy. The modified protein is designated as HPPD-4. ([redacted] 2016, M-461393-02, Appendix 2)

[redacted]

Figure 20. Amino acid sequence of the HPPD-4 protein

Cry14Ab-1 protein

Cry proteins expressed by the common soil microorganism, *B. thuringiensis*, have a long history of safe use in agriculture. These proteins have been successfully used for insect control for more than 60 years, and they are expressed by a number of transgenic crops that have been in commercial production for more than 20 years in many parts of the world. FSANZ has assessed several Cry proteins previously (see Table 3, Section A.2(a)(i)), and have not identified public health or safety concerns.

In conclusion, Cry proteins are present in food and feed from plant and microbial sources with good safety records. Therefore, Cry proteins have a history of safe use.

Cry14Ab-1

The amino acid sequence of the Cry14Ab-1 protein is described by [redacted](2016, M-485425-02, Appendix 18).

Figure 21. Amino acid sequence of the Cry14Ab-1 protein

(c) Information on whether any new protein has undergone any unexpected post-translational modification in the new host

Post-translational modification is determined by glycosylation analysis. For Cry14Ab-1 and HPPD-4, glycosylation testing was performed on both proteins purified from GMB151 soybean and the microbially-produced proteins. No glycosylation was determined in either the GMB151 plant-purified proteins or the microbially-produced proteins. Therefore, it is deduced that there is no unexpected post-translational modification via glycosylation for Cry14Ab-1 or HPPD-4 protein.

(d) Where any ORFs have been identified (in subparagraph A.3 (c)(v) of this Guideline (3.5.1)), bioinformatics analyses to indicate the potential for allergenicity and toxicity of the ORFs.

A bioinformatics analysis was performed on the transgenic locus sequence of the GMB151 soybean to identify open reading frames (ORFs) (2018; TXVLT032; Appendix 7).

The GMB151 soybean transgenic locus, containing the inserted DNA together with the 5' and 3' flanking sequences, was used as query sequence. The GetORF search program was used to identify all ORF crossing a junction or overlapping the inserted DNA, between two translation stop codons, with a minimum size coding for three amino acids. This search identified 601 ORFs.

In the next step, the translated amino acid sequences from the identified ORFs with a minimum size of 30 amino acids were used as query sequences in homology searches to known allergens and toxins. They represented 115 unique sequences.

Two *in silico* approaches were used to evaluate the potential amino acid sequence identity with known allergens contained in the public allergen database COMPARE (www.comparedatabase.org):

- An 8-mer search was carried out to identify any short sequences of eight amino acids or longer that share 100% identity to an allergenic protein. This search was performed using SeqMatchAll

from the EMBOSS suite, which compared each ORF sequence with all known allergens present in the allergen database.

- An overall identity search was carried out by using FASTA algorithm, which compared each complete query sequence with all protein sequences present in the allergen database. The scoring matrix was BLOSUM50. An E-value threshold of 1 was used. The criterion indicating potential relevant identity to an allergen was ≥35% identity over at least 80 amino acids for sequences of ≥80 amino acids, or ≥35% recalculated over a hypothetical 80 amino acid window for sequences of <80 amino acids.

In addition, each query sequence was evaluated for potential identity with known toxins. An overall identity search was carried out by using FASTA algorithm with all protein sequences present in the NCBI non-redundant database, using the BLOSUM50 scoring matrix. An E-value threshold of 0.1 was used for pre-selecting the most identical proteins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the familiarity and potential toxic properties of the potential polypeptide.
The overall search showed no biologically relevant identity between the query sequences and any known allergenic proteins. One ORF, GMB151_ORF.516, matched with a contiguous eight amino acid sequence (SSPTTTTS) present in the Cas s 5 allergen. No match with Cas s 5 protein was found in the overall search for an allergen match, and an 8-mer match in isolation is unlikely to indicate any potential cross-reactivity.

In addition, no biologically relevant identities were found with any toxic protein from the NCBI non-redundant database.

In conclusion, there are neither allergenic nor toxicological *in silico* findings associated with the potential ORF polypeptides.

B.2 New proteins

If it can be shown the new protein(s) is identical to one previously assessed by FSANZ, the only other safety information that must be provided is an updated bioinformatics comparison of the amino acid sequence to known protein toxins, anti-nutrients and allergens.

Where the new protein is not identical to one previously assessed by FSANZ, the following must be provided:

(a) Information on the potential toxicity of any new proteins, including:

(i) A bioinformatics comparison of the amino acid sequence of each of the new proteins to known protein toxins and anti-nutrients (e.g. protease inhibitors, lectins)

The Cry14Ab-1 and HPPD-4 proteins have not previously been assessed for food safety by FSANZ. Information has been provided above under Section B.1 (a) to show equivalence of Cry14Ab-1 and HPPD-4 proteins expressed by GMB151 soybean to the microbially-produced proteins based on the known amino acid sequences of Cry14Ab-1 and HPPD-4. Therefore, in this section, the only supplementary information on the proteins provided is bioinformatics analysis for these known proteins based on the amino acid sequences associated with the proteins.

<u>Cry14Ab-1 protein</u>

The potential amino acid sequence homology of the *Bacillus thuringensis* derived crystal protein, Cry14Ab-1 with known allergens and known toxins was evaluated by using several *in silico* approaches ([redacted] 2018; TXKIS002; Appendix 19).

This search evaluated the potential amino acid sequence identity of the query protein with known allergens by using two *in silico* approaches.

- An overall identity search was carried out to compare the complete query sequence with all protein sequences present in the public allergen database COMPARE (www.comparedatabase.org). The FASTA algorithm was used, with the BLOSUM50 scoring matrix and an E-value threshold of 10. The criterion indicating potential allergenicity was ≥35% identity over at least 80 consecutive amino acids with an allergenic protein.
- An 8-mer search was carried out to identify any short sequences of eight amino acids or longer that share 100% identity to an allergenic protein. This search was performed using SeqMatchAll from the EMBOSS suite, which compared the query sequence with all known allergens present in the allergen database.

In addition, two *in silico* approaches based on the FASTA algorithm associated with the BLOSUM50 scoring matrix were used to evaluate the potential amino acid sequence identity of the query protein with known toxins:

- An overall identity search with all protein sequences present in the NCBI non-redundant database. An E-value threshold of 0.1 was used for pre-selecting the most similar proteins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the familiarity and potential toxic properties of the query protein.
- An overall identity search with all protein sequences present in the in-house Bayer toxin database. An E-value threshold of 10 was used for pre-selecting the most identical toxins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the potential toxic properties of the query protein.

The overall identity search showed no biologically relevant identity between the query protein and any known allergenic proteins. In addition, the 8-mer search showed no 100% identity with known allergenic proteins.

As expected, the overall identity search against the general protein database showed that in most cases, the Cry14Ab-1 protein matched with Cry family sequences from *Bacillus thuringiensis* which are considered safe. Furthermore, no biologically relevant identities were found with any toxic proteins from the Bayer toxin database.

In conclusion, there are neither allergenic nor toxicological *in silico* findings associated with the Cry14Ab-1 protein.

HPPD-4 protein

The potential amino acid sequence homology of the HPPD-4 protein with known allergens and known toxins was evaluated by using several *in silico* approaches ([redacted] 2018; TXFAS015; Appendix 20).

This search evaluated the potential amino acid sequence identity of the query protein with known allergens by using two *in silico* approaches.

- An overall identity search was carried out to compare the complete query sequence with all protein sequences present in the public allergen database COMPARE (www.comparedatabase.org). The FASTA algorithm was used, with the BLOSUM50 scoring matrix and an E-value threshold of 10. The criterion indicating potential allergenicity was ≥35% identity over at least 80 consecutive amino acids with an allergenic protein.
- An 8-mer search was carried out to identify any short sequences of eight amino acids or longer that share 100% identity to an allergenic protein. This search was performed using

SeqMatchAll from the EMBOSS suite, which compared the query sequence with all known allergens present in the allergen database.

In addition, two *in silico* approaches based on the FASTA algorithm associated with the BLOSUM50 scoring matrix were used to evaluate the potential amino acid sequence identity of the query protein with known toxins:

- An overall identity search with all protein sequences present in the NCBI non-redundant database. An E-value threshold of 0.1 was used for pre-selecting the most similar proteins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the familiarity and potential toxic properties of the query protein.
- An overall identity search with all protein sequences present in the in-house Bayer toxin database. An E-value threshold of 10 was used for pre-selecting the most identical toxins. The biological relevance of the matches was further assessed. Biologically relevant matches provide insight on the potential toxic properties of the query protein.

The overall identity search showed no biologically relevant identity between the query protein and any known allergenic proteins. In addition, the 8-mer search showed no 100% identity with known allergenic proteins.

As expected, the overall identity search against the general protein database showed that in most cases, the HPPD-4 protein matched with other HPPD proteins from various origins. In addition, no significant similarities were found with any toxic protein from the Bayer toxin database.
In conclusion, there are neither allergenic nor toxicological *in silico* findings associated with the HPPD-4 protein.

(ii) Information on the stability of the protein to proteolysis in appropriate gastrointestinal model systems

Cry14Ab-1 protein

(a) In vitro digestibility in human simulated gastric fluid

The Cry14Ab-1 protein was tested for digestibility in human simulated gastric fluid (SGF) containing pepsin at pH 1.2 for incubation times from 0.5 to 60 minutes ([redacted] 2014; M-478215-01, Appendix 21).

The Cry14Ab-1 protein was incubated at 37°C in SGF and samples were taken for analysis at time-points of 0, 0.5, 1, 2, 5, 10, 20, 30 and 60 minutes. The resulting protein solution was analyzed for presence of the test proteins and potential stable protein fragments by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining (Figure 22) and by western blot (Figure 23).

The Cry14Ab-1 protein was digested very rapidly, within 30 seconds of incubation in SGF, in the presence of pepsin, at pH 1.2.

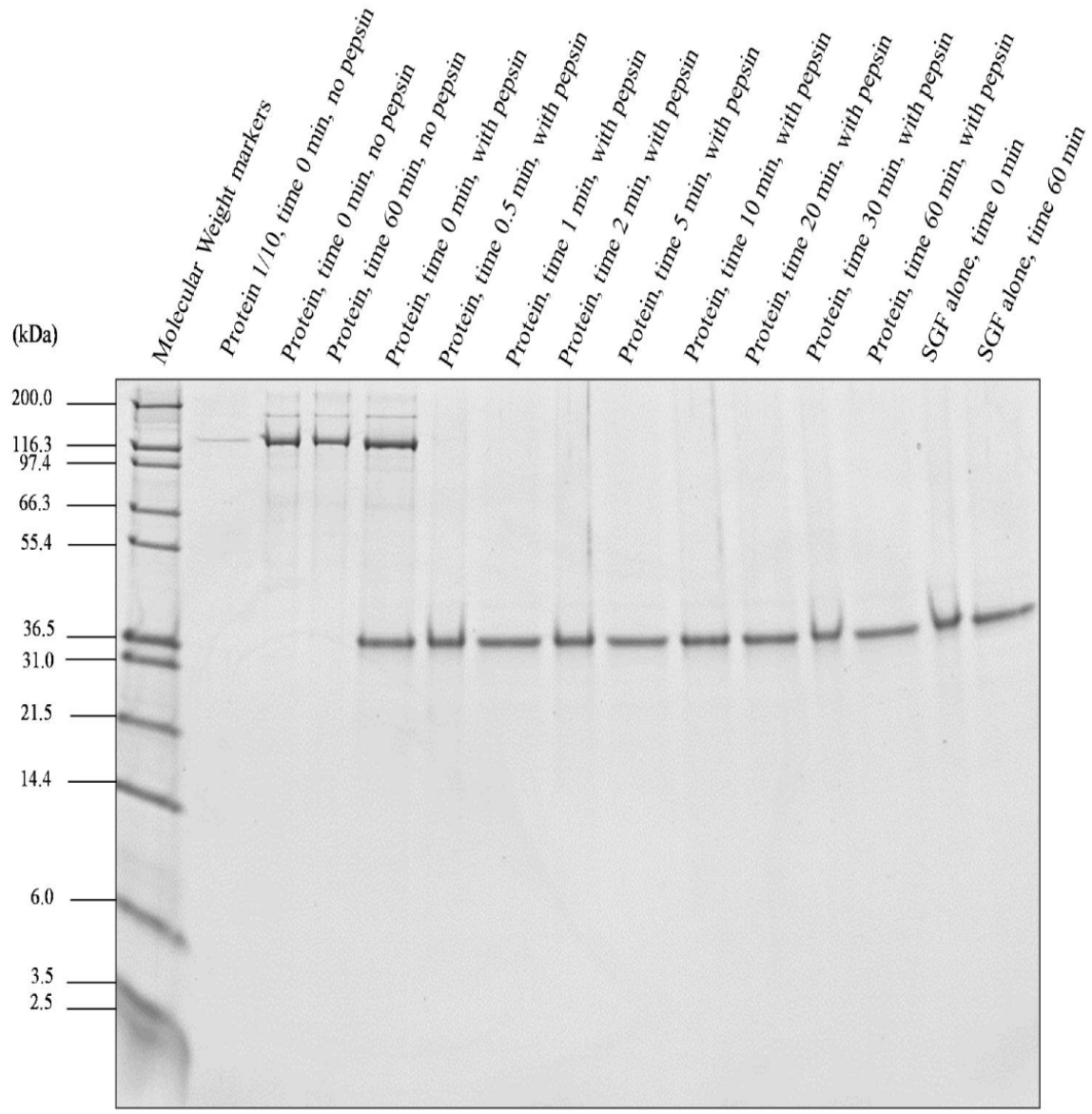


Figure 22. Coomassie blue stained SDS-PAGE of Cry14Ab-1 protein after incubation in human simulated gastric fluid

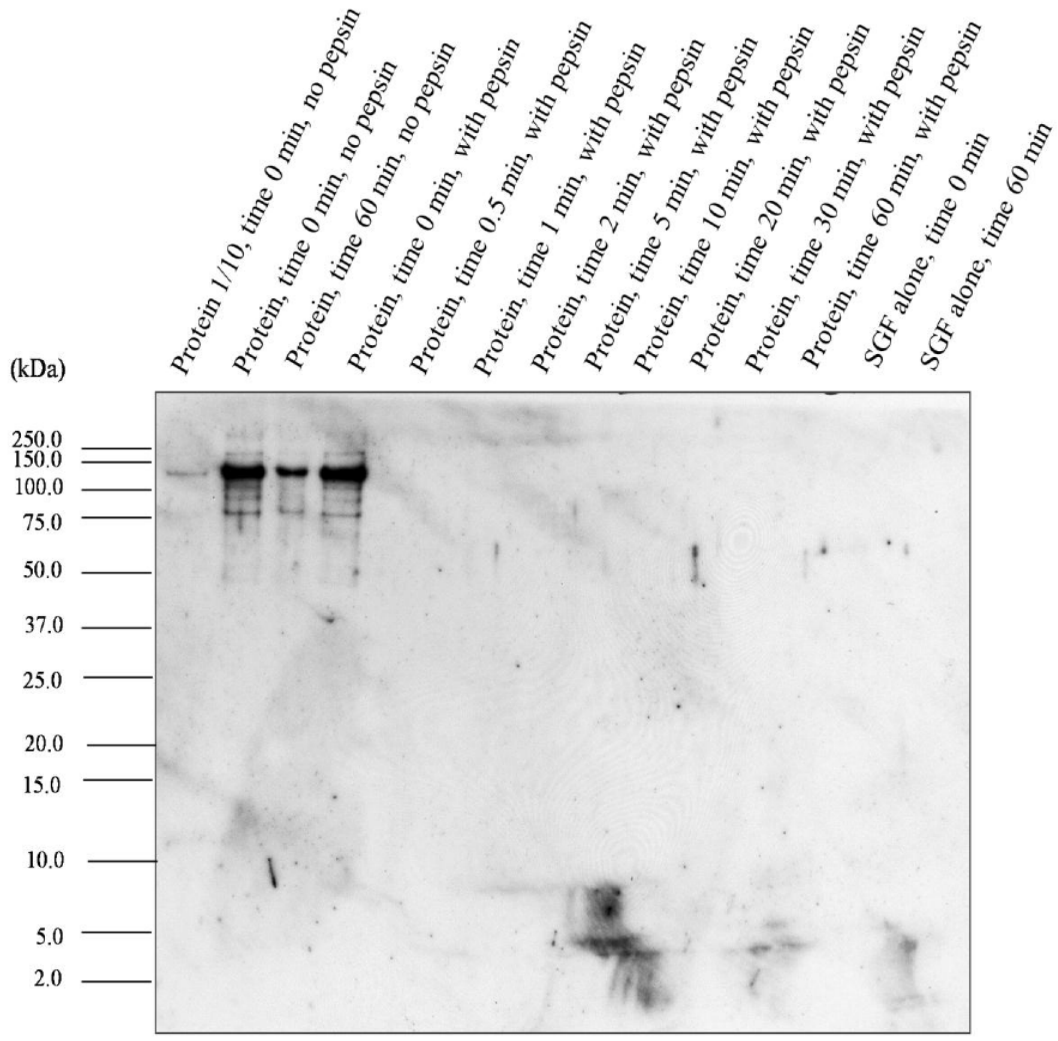


Figure 23. Western blot of Cry14Ab-1 protein after incubation in human simulated gastric fluid

Note: The observed signals below the 10 kDa molecular weight marker correspond to unspecific background which was generated during the chemiluminescence detection step. As no degradation band was visible at these molecular weights in the lanes 0.5 to 5 minutes, this background was considered not to compromise interpretation of the results.

In vitro digestibility in human simulated intestinal fluid

The Cry14Ab-1 protein was tested for digestibility in human simulated intestinal fluid (SIF) with pancreatin at pH 7.5 for incubation times from 0.5 to 60 minutes ([redacted] 2014; M-478845-01, Appendix 22).

The Cry14Ab-1 protein was incubated at 37°C with SIF and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resulting protein solutions were analyzed for presence of the Cry14Ab-1 protein and potential stable protein fragments by Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 24) and by western blot (Figure 25).

The Cry14Ab-1 protein was partially digested within 60 minutes of incubation with SIF, in the presence of pancreatin, at pH 7.5.

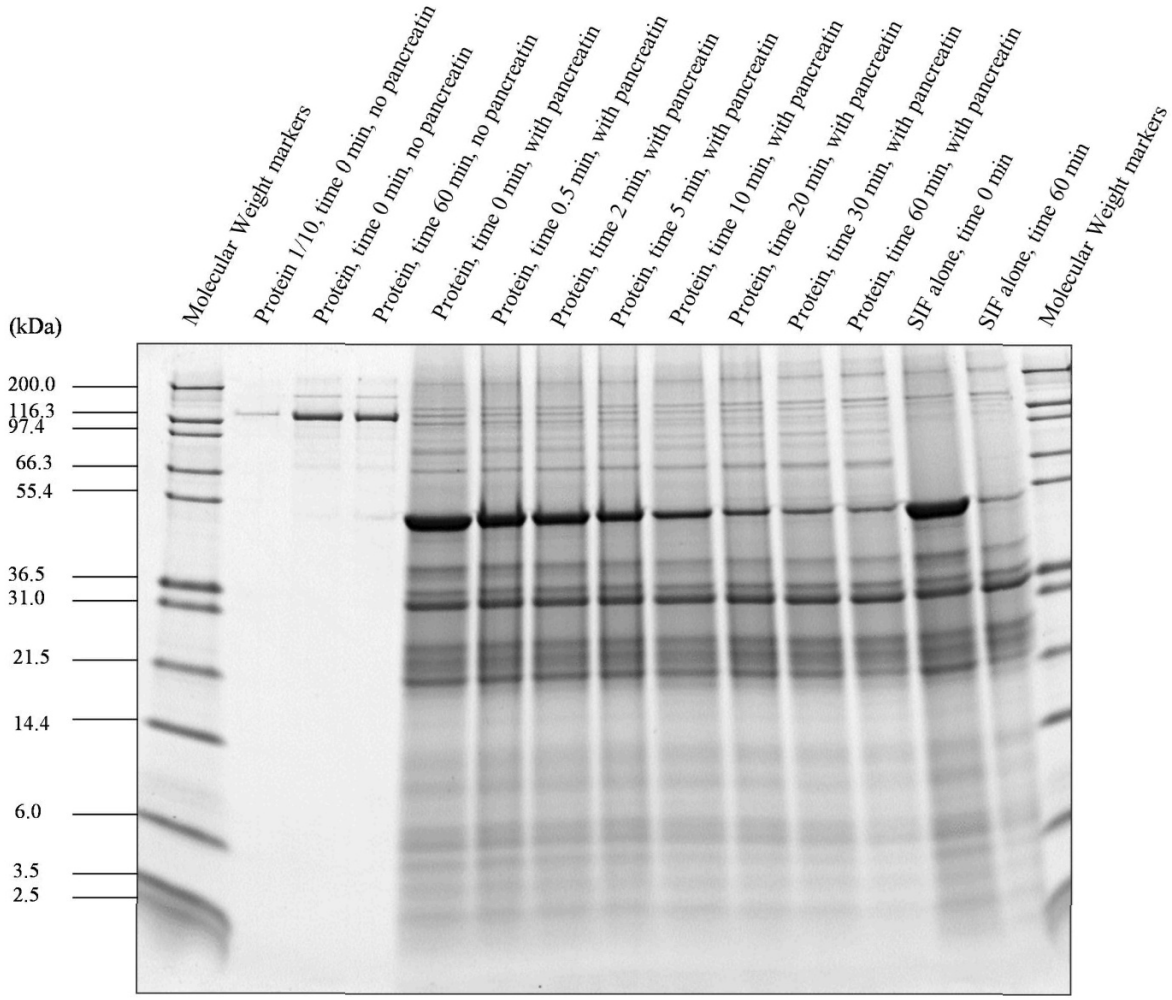


Figure 24. Coomassie blue stained SDS-PAGE of Cry14Ab-1 protein after incubation in human simulated intestinal fluid

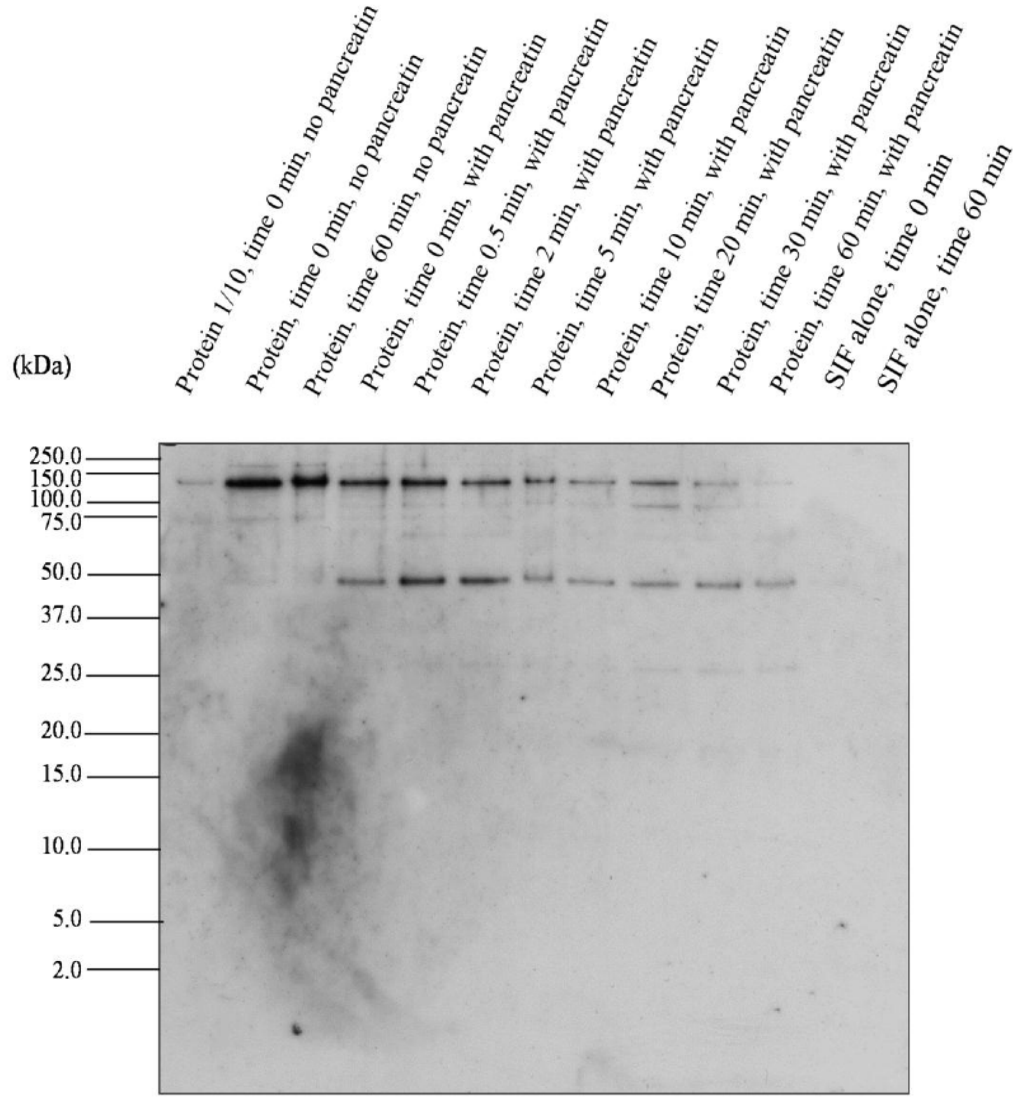


Figure 25. Western blot of Cry14Ab-1 protein after incubation in simulated human intestinal fluid

Note: The observed signals between the 20 and 5 kDa molecular weight markers correspond to unspecific background which was generated during the chemiluminescence detection step. As no degradation band was expected in the control lanes 0 and 60 minutes without pancreatin, this background was considered not to compromise interpretation of the results.

HPPD-4 protein

(a) In vitro digestibility in human simulated gastric fluid

The HPPD-4 protein was tested for digestibility in human simulated gastric fluid (SGF) containing pepsin at pH 1.2 for incubation times from 0.5 to 60 minutes ([redacted]; 2014; M-476249-01, Appendix 24).

The HPPD-4 protein was incubated at 37°C in SGF and samples were taken for analysis at time-points of 0, 0.5, 1, 2, 5, 10, 20, 30 and 60 minutes. The resulting protein solution was analyzed for presence of the test proteins and potential stable protein fragments by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining (Figure 28) and by western blot (Figure 29).

The HPPD-4 protein was digested very rapidly, within 30 seconds of incubation in SGF, in the presence of pepsin, at pH 1.2.

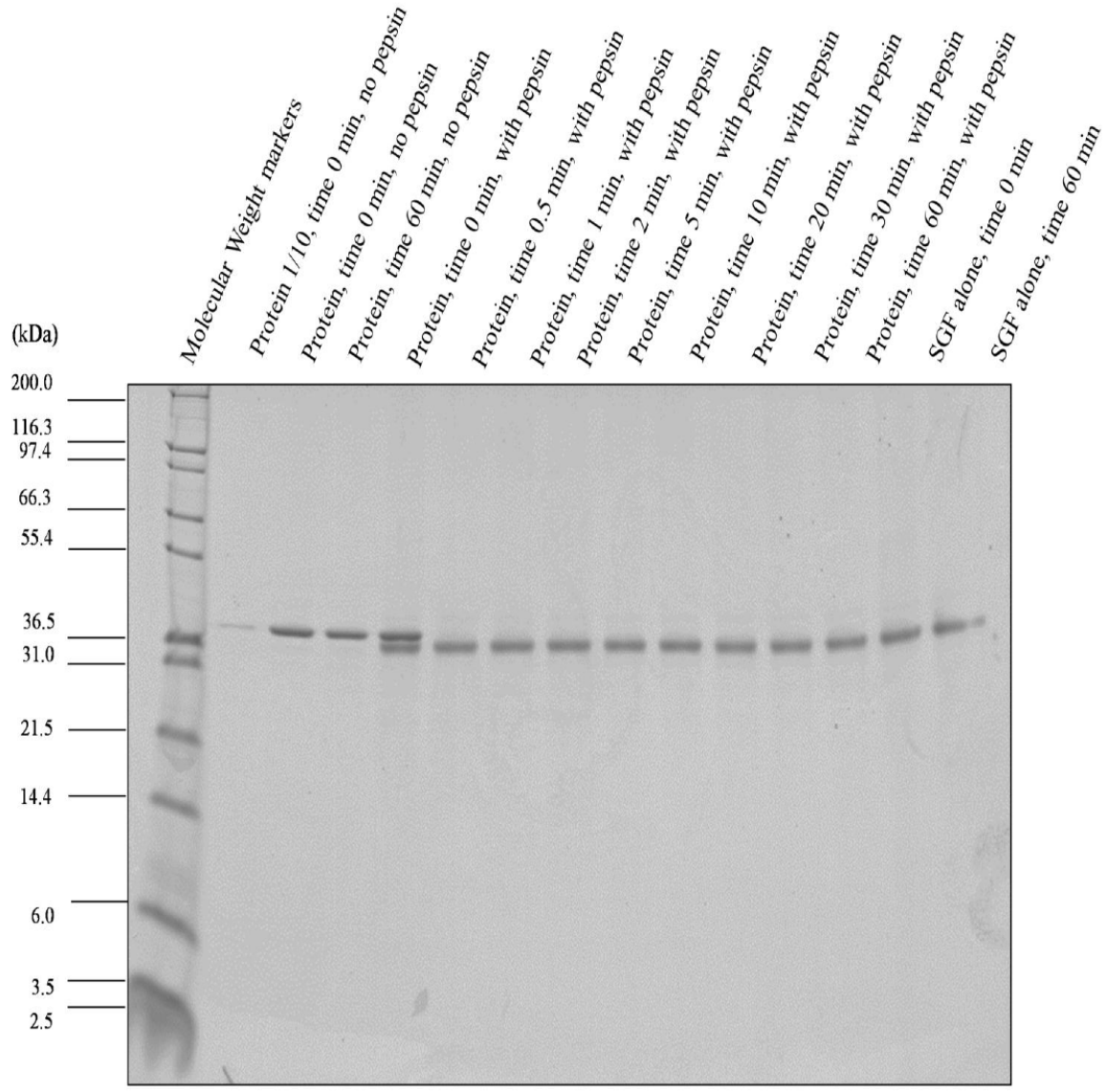


Figure 26. Coomassie blue stained SDS-PAGE of HPPD-4 protein after incubation in human simulated gastric fluid

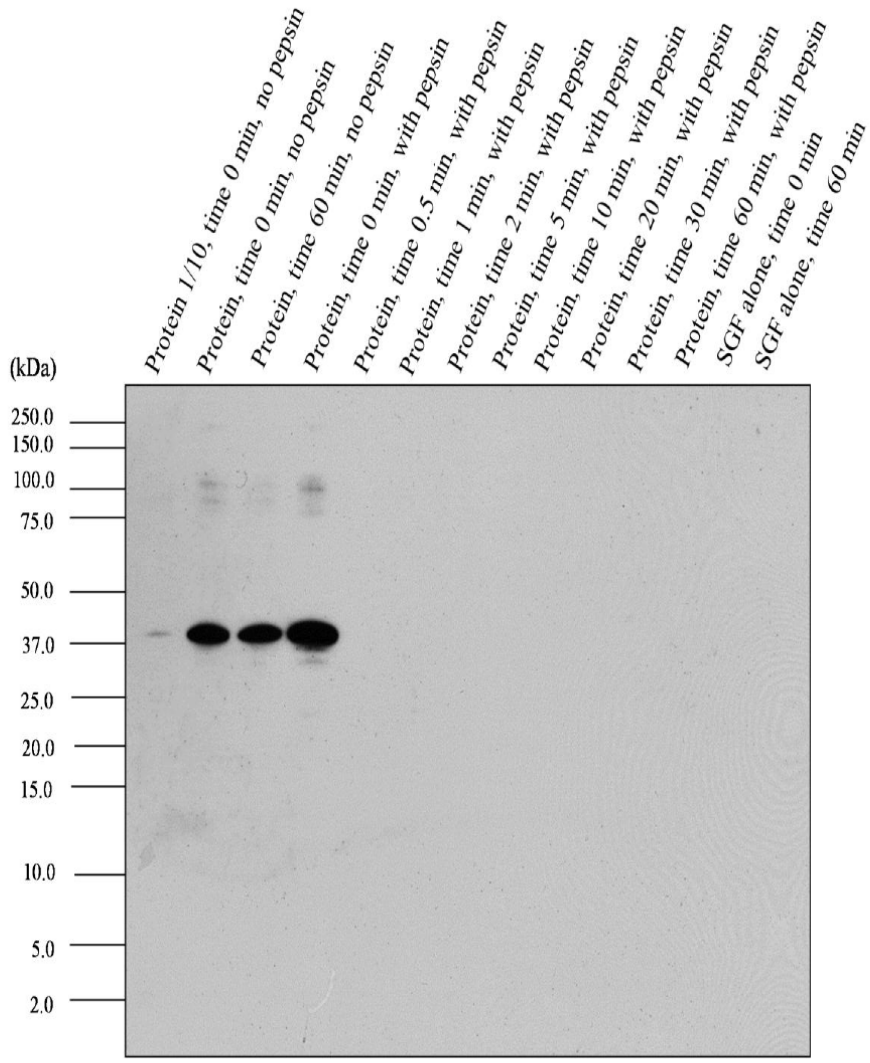


Figure 27. Western blot of HPPD-4 protein after incubation in human simulated gastric fluid

In vitro digestibility in human simulated intestinal fluid

The HPPD-4 protein was tested for digestibility in human simulated intestinal fluid (SIF) with pancreatin at pH 7.5 for incubation times from 0.5 to 60 minutes ([redacted] 2014; M-476906-01, Appendix 25).

The HPPD-4 protein was incubated at 37°C with SIF and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resulting protein solutions were analyzed for presence of the HPPD-4 protein and potential stable protein fragments by Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 30) and by western blot (Figure 31).

The HPPD-4 protein was partially digested within 5 minutes and completely digested within 10 minutes of incubation with SIF, in the presence of pancreatin, at pH 7.5.

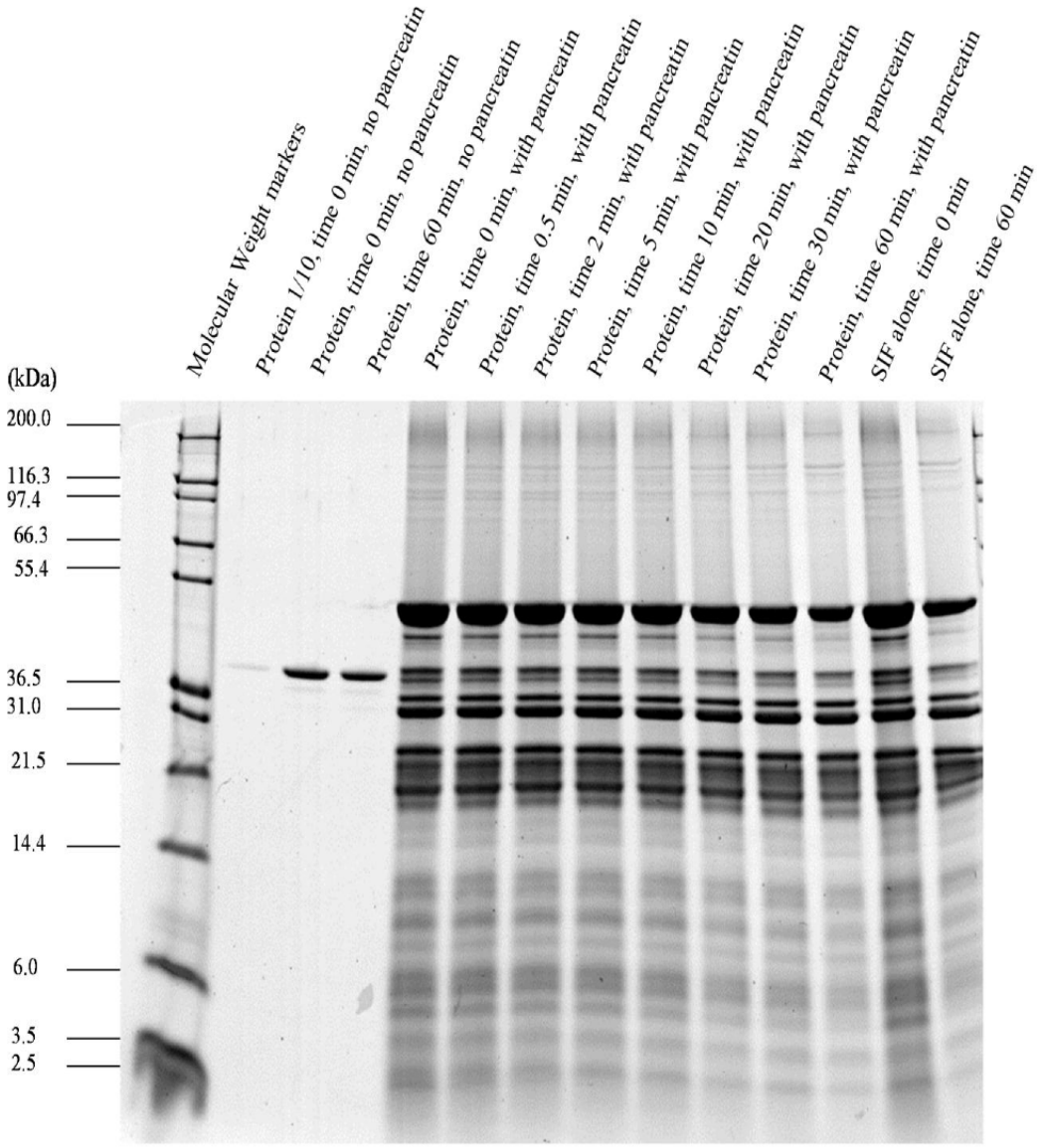


Figure 28. Coomassie blue stained SDS-PAGE of HPPD-4 protein after incubation in human simulated intestinal fluid

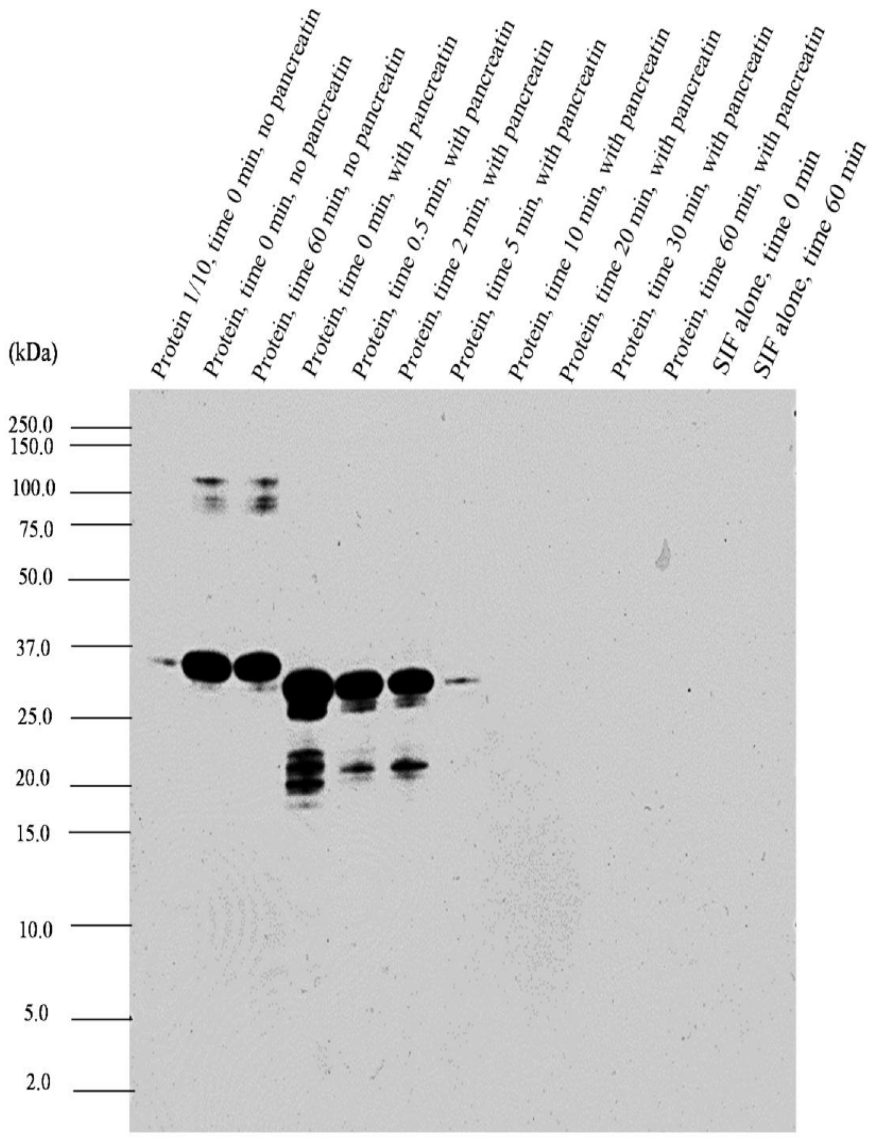


Figure 29. Western blot of HPPD-4 protein after incubation in human intestinal gastric fluid

An animal toxicity study if the bioinformatics comparison and biochemical studies indicate either a relationship with known protein toxins/anti-nutrients or resistance to proteolysis.

Given the digestive lability of both proteins combined with their lack of similarity to known protein toxins or allergens and the loss of stabilty with heating provides strong evidence that toxicological concerns are not raised by these proteins. In addition to this information, and acute toxicity study for each of the proteins is provided. These data confirm the absence of toxicity in animals for both the CryAb-1 and HPPD-4 proteins.

Cry14Ab-1 protein

The Cry14Ab-1 protein was evaluated for acute oral toxicity in male and female C57BL/6J mice (2016; M-538392-02, Appendix 27).

Groups of 6 male and 6 female C57BL/6J mice were administered the Cry14Ab-1 protein (batch number 1506_Cry14Ab-1) by oral gavage at the limit dose level of 2000 mg/kg body weight. Similarly constituted groups of 6 male and 6 female mice received vehicle alone, administered in the same manner, and acted as controls. All animals were observed for clinical signs daily for fifteen days while their body weights and food consumption were measured weekly. At the termination of the study period, the animals were weighed and subjected to a necropsy, including a macroscopic examination. The tissues were retained for possible microscopic examinations.

There were no mortalities, no treatment-related clinical signs, no effects on the body weight and food consumption parameters as well as no macroscopic changes at necropsy, in C57BL/6J mice after an acute oral administration of the Cry14Ab-1 protein at 2000 mg/kg body weight.

In conclusion, the treatment with the Cry14Ab-1 protein at 2000 mg/kg body weight via the oral route did not produce any signs of systemic toxicity in the male and female C57BL/6J mice.

HPPD-4 protein

The HPPD-4 protein was evaluated for acute oral toxicity in male and female C57BL/6J mice (.; 2016; M-496627-03, Appendix 28).

Groups of 10 male and 10 female C57BL/6J mice were administered the HPPD-4 protein (batch number 1338_HPPD-4) by oral gavage at the limit dose level of 2000 mg/kg body weight. Similarly constituted groups of 10 male and 10 female mice received vehicle alone, administered in the same manner, and acted as controls. All animals were observed for clinical signs daily for fifteen days while their body weights and food consumption were measured weekly. At the termination of the study period, the animals were weighed and subjected to a necropsy, including a macroscopic examination. The tissues were retained for possible microscopic examinations.

There were no mortalities, no treatment-related clinical signs, no effects on the body weight and food consumption parameters as well as no macroscopic changes at necropsy, in C57BL/6J mice after an acute oral administration of the HPPD-4 protein at 2000 mg/kg body weight.

In conclusion, the treatment with HPPD-4 protein at 2000 mg/kg body weight via the oral route did not produce any signs of systemic toxicity in the male and female C57BL/6J mice.

(b) Information on the potential allergenicity of any new proteins, including:

(i) Source of the new protein

Cry14Ab-1 protein

The Cry14Ab-1 protein is from the well known and characterized class of Cry proteins derived from *Bacillus thuringiensis*. Although it has not been assessed previously for food safety, a large number of Cry proteins from *Bacillus thuringensis* have been assessed for food safety in the past and this class of proteins is well established as having a good food safety record including established safety in terms of potential allergenicity (OECD, 2007) (refer to Table 3, Section A.2 (a) (i)).

HPPD-4 protein

The HPPD-4 protein is a new protein, from the same class as the known and characterized HPPD W336 protein which has established food safety. The HPPD W336 protein has been previously assessed by FSANZ.

The coding sequence of the 4-hydroxyphenylpyruvate dioxygenase (HPPD) protein was isolated from the *Pseudomonas fluorescens* strain A32. Four amino acids were substituted [redacted] to reduce HPPD inhibitors binding efficacy. The modified protein is designated as HPPD-4 (Porée F. *et al*, 2014, M-486300-01, Appendix 10).

The *hppd* gene was isolated from the bacterium *Pseudomonas fluorescens*, strain A32. *P. fluorescens* is a Gram-negative, rod-shaped, motile, asporogenous, aerobic bacterium. *P. fluorescens*, is ubiquitous in the environment, including soil, water and food (OECD, 1997a). It has many beneficial uses in agriculture, human health and bioremediation. It is not described as allergenic, toxic or pathogenic to healthy humans and animals and has an overall history of safe use.

HPPD proteins are ubiquitous in nature across all kingdoms: bacteria, fungi, plants and animals including mammals. HPPD amino acid sequences have been determined in bacteria such as *Streptomyces avermitilis* (Accession number Q53586), in fungi such as *Aspergillus fumigatus* (Accession number Q4WPV8), in plants such as *Arabidopsis thaliana* (Accession number P93836), and in animals such as *Caenorhabditis elegans* (Accession number Q22633), mouse (*Mus musculus*, Accession number P49429), and human (*Homo sapiens*, Accession number P32754).

In particular, HPPD proteins have been characterized in organisms present in human food, such as carrot (*Daucus carota*, Accession number O23920), barley (*Hordeum vulgare* Accession number O48604; Falk *et al*., 2002), pork (*Sus scrofa*, Accession number Q02110)) and beef (*Bos Taurus*, Accession number Q5EA20).

In conclusion, HPPD proteins are present in food from plant, fungal or animal origin, with good safety records. Therefore, HPPD proteins have a history of safe use.

(ii) A bioinformatics comparison of the amino acid sequence of the novel protein to known allergens

Cry14Ab-1 protein

The potential amino acid sequence homology of the *Bacillus thuringiensis* derived crystal protein (Cry14Ab-1) with known allergens and known toxins was evaluated by using several *in silico* approaches ([redacted] 2018; TXKIS002; Appendix 19). The nature of the bioinformatics comparison with known allergens was discussed in further detail above in Section B.2 (a) (i).

HPPD-4 protein

The potential amino acid sequence homology of the mutated 4-hydroxyphenylpyruvate dioxygenase (HPPD-4) protein with known allergens and known toxins was evaluated by using several *in silico* approaches ([redacted] 2018; TXFAS015; Appendix 20). The nature of the bioinformatics comparison with known allergens was discussed in further detail above in Section B.2 (a) (i).

(iii) The new protein's structural properties, including, but not limited to, its susceptibility to enzymatic degradation (e.g. proteolysis), heat and/or acid stability

Cry14Ab-1 protein

The effect of temperature as assessed by SDS-PAGE and Western Blot

The effect of temperature was assessed on Cry14Ab-1 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses ([redacted] 2015; M-515776-01, Appendix 23).

Samples of Cry14Ab-1 were incubated for 30 minutes at 4°C, 25°C, 37°C, 55°C, 75°C and 95°C, followed by SDS-PAGE and western blot analysis. The sample treated at 4°C was used for comparison of the other temperature-treated samples.

The SDS-PAGE (Figure 26) and western blot analyses (Figure 27) produced similar results. After temperature-treatments at 25°C, 37°C, 55°C and 75°C, the majority of Cry14Ab-1 remained in the supernatant as soluble protein. After temperature-treatments at 75°C, the intensity of the Cry14Ab-1 band decreased and a smearing pattern became more pronounced in the higher molecular weight region of the lanes. After temperature-treatment at 95°C, the Cry14Ab-1 band was no longer visible and only a faint smearing pattern remained visible. The SDS-PAGE and western blot results indicate that Cry14Ab-1 loses its stability after temperature-treatment of 75°C.

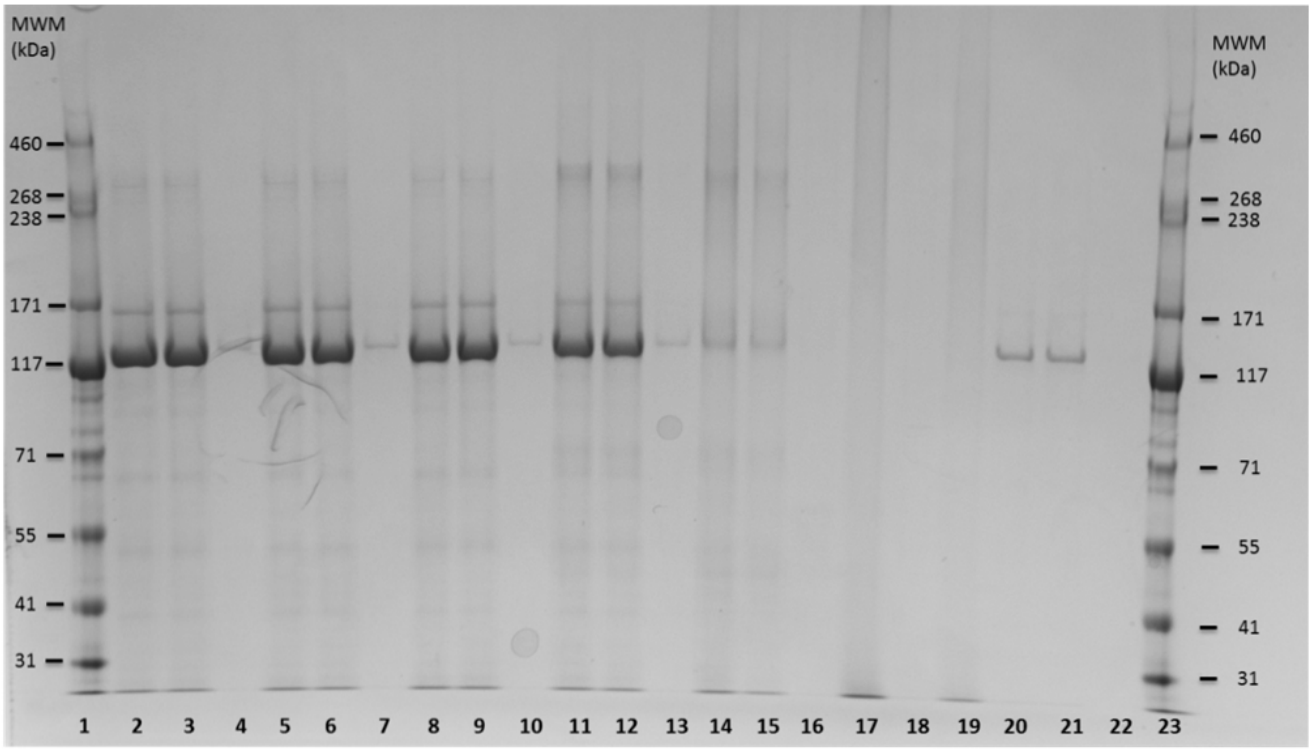


Figure 30. Coomassie blue stained SDS-PAGE analysis of the Cry14Ab-1 protein after temperature treatment

Lane 1: HiMark Pre-Stained Protein Standard Molecular Weight Marker (MWM)
Lane 2: 2 µg Cry14Ab-1, 4°C – Uncentrifuged (UC)
Lane 3: 2 µg Cry14Ab-1, 4°C - Supernatant (S)
Lane 4: 2 µg Cry14Ab-1, 4°C – Pellet (P)
Lane 5: 2 µg Cry14Ab-1, 25°C - UC
Lane 6: 2 µg Cry14Ab-1, 25°C - S
Lane 7: 2 µg Cry14Ab-1, 25°C - P
Lane 8: 2 µg Cry14Ab-1, 37°C - UC
Lane 9: 2 µg Cry14Ab-1, 37°C - S
Lane 10: 2 µg Cry14Ab-1, 37°C - P
Lane 11: 2 µg Cry14Ab-1, 55°C - UC
Lane 12: 2 µg Cry14Ab-1, 55°C - S
Lane 13: 2 µg Cry14Ab-1, 55°C - P
Lane 14: 2 µg Cry14Ab-1, 75°C - UC
Lane 15: 2 µg Cry14Ab-1, 75°C - S
Lane 16: 2 µg Cry14Ab-1, 75°C - P
Lane 17: 2 µg Cry14Ab-1, 95°C - UC
Lane 18: 2 µg Cry14Ab-1, 95°C - P
Lane 19: 2 µg Cry14Ab-1, 95°C - S
Lane 20: 0.2 µg Cry14Ab-1, 4°C (10% Control) - UC
Lane 21: 0.2 µg Cry14Ab-1, 4°C (10% Control) - S
Lane 22: 0.2 µg Cry14Ab-1, 4°C (10% Control) - P
Lane 23: HiMark Pre-Stained Protein Standard Molecular Weight Marker

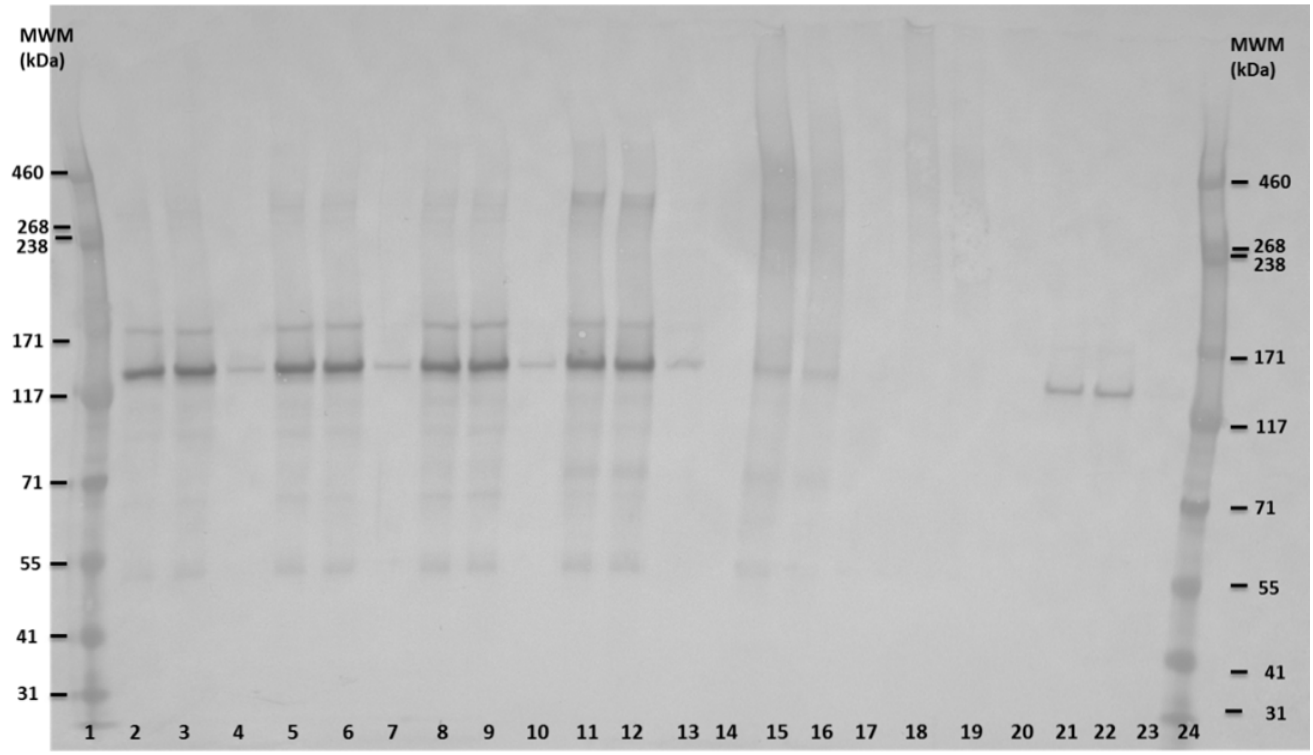


Figure 31. Western blot analysis of the Cry14Ab-1 protein after temperature treatment

Lane 1: HiMark Pre-Stained Protein Standard Molecular Weight Marker (MWM)
Lane 2: 50 ng Cry14Ab-1, 4°C - Uncentrifuged (UC)
Lane 3: 50 ng Cry14Ab-1, 4°C - Supernatant (S)
Lane 4: 50 ng Cry14Ab-1, 4°C – Pellet (P)
Lane 5: 50 ng Cry14Ab-1, 25°C - UC
Lane 6: 50 ng Cry14Ab-1, 25°C - S
Lane 7: 50 ng Cry14Ab-1, 25°C - P
Lane 8: 50 ng Cry14Ab-1, 37°C - UC
Lane 9: 50 ng Cry14Ab-1, 37°C - S
Lane 10: 50 ng Cry14Ab-1, 37°C - P
Lane 11: 50 ng Cry14Ab-1, 55°C - UC
Lane 12: 50 ng Cry14Ab-1, 55°C - S
Lane 13: 50 ng Cry14Ab-1, 55°C – P
Lane 14: Blank lane
Lane 15: 50 ng Cry14Ab-1, 75°C - UC
Lane 16: 50 ng Cry14Ab-1, 75°C - S
Lane 17: 50 ng Cry14Ab-1, 75°C – P
Lane 18: 50 ng Cry14Ab-1, 95°C - UC
Lane 19: 50 ng Cry14Ab-1, 95°C - S
Lane 20: 50 ng Cry14Ab-1, 95°C – P
Lane 21: 5 ng Cry14Ab-1, 4°C (10% Control) – UC
Lane 22: 5 ng Cry14Ab-1, 4°C (10% Control) - S
Lane 23: 5 ng Cry14Ab-1, 4°C (10% Control) - P
Lane 24: HiMark Pre-Stained Protein Standard Molecular Weight Marker

HPPD-4 protein

The effect of temperature as assessed by SDS-PAGE and Western Blot

The effect of temperature was assessed on the HPPD-4 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses ([redacted] 2015; M-515772-01, Appendix 26).

The HPPD-4 protein was incubated at 4°C, 25°C, 37°C, 55°C, 75°C and 95°C for 30 minutes. The sample treated at 4°C was used for comparison of the other temperature-treated samples.

The SDS-PAGE results (Figure 32) and the western blot results (Figure 33) produced similar results. After temperature-treatments at 25°C and 37°C, the majority of HPPD-4 remained in the supernatant as soluble protein. After temperature-treatments at 55°C and above, HPPD-4 began to appear as insoluble protein. The SDS-PAGE and western blot results indicate that HPPD-4 begins to lose its solubility upon temperature-treatments at 55°C and greater.

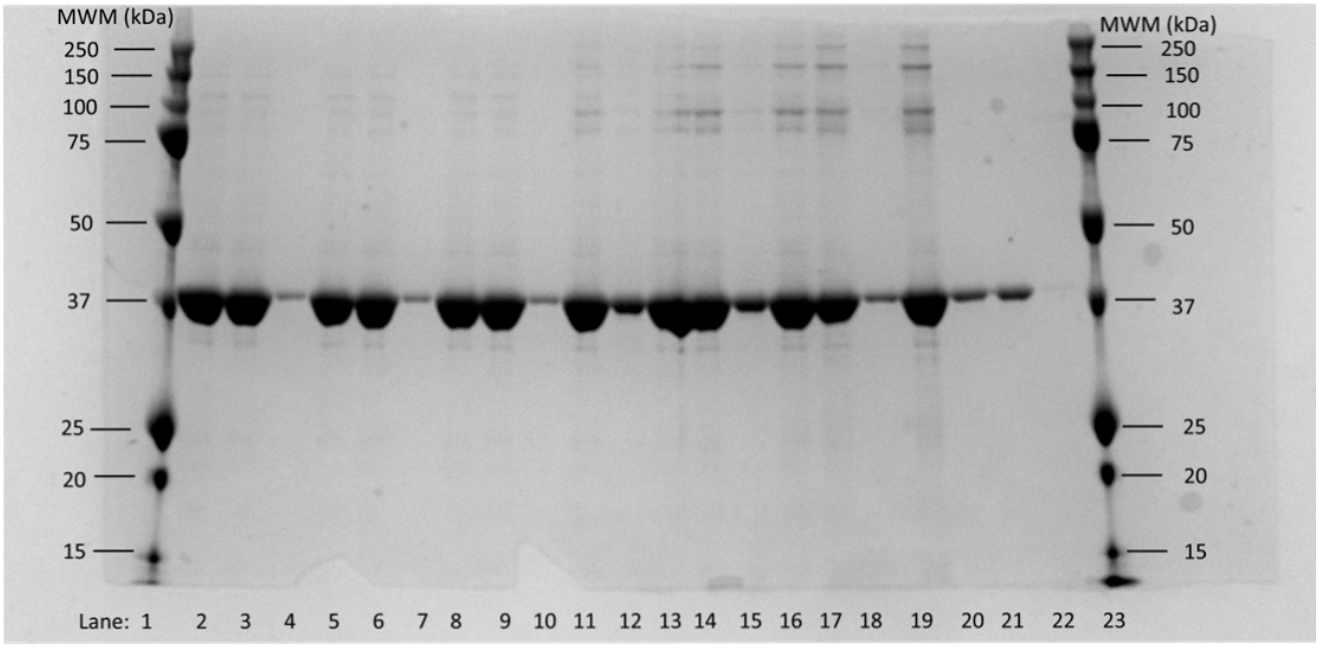


Figure 32. Coomassie blue stained SDS-PAGE analysis of HPPD-4 protein after temperature treatment

Lane 1: Precision Plus Protein™ Dual Xtra Standards Molecular Weight Marker
Lane 2: 5 µg HPPD-4, 4°C – Uncentrifuged (UC)
Lane 3: 5 µg HPPD-4, 4°C - Supernatant (S)
Lane 4: 5 µg HPPD-4, 4°C – Pellet (P)
Lane 5: 5 µg HPPD-4, 25°C - UC
Lane 6: 5 µg HPPD-4, 25°C - S
Lane 7: 5 µg HPPD-4, 25°C - P
Lane 8: 5 µg HPPD-4, 37°C - UC
Lane 9: 5 µg HPPD-4, 37°C - S
Lane 10: 5 µg HPPD-4, 37°C - P
Lane 11: 5 µg HPPD-4, 55°C - UC
Lane 12: 5 µg HPPD-4, 55°C - S
Lane 13: 5 µg HPPD-4, 55°C - P
Lane 14: 5 µg HPPD-4, 75°C - UC
Lane 15: 5 µg HPPD-4, 75°C - S
Lane 16: 5 µg HPPD-4, 75°C – P
Lane 17: 5 µg HPPD-4, 95°C - UC
Lane 18: 5 µg HPPD-4, 95°C - S
Lane 19: 5 µg HPPD-4, 95°C – P
Lane 20: 0.5 µg HPPD-4, 4°C (10% Control) - UC
Lane 21: 0.5 µg HPPD-4, 4°C (10% Control) - S
Lane 22: 0.5 µg HPPD-4, 4°C (10% Control) - P
Lane 23: Precision Plus Protein™ Dual Xtra Standards Molecular Weight Marker

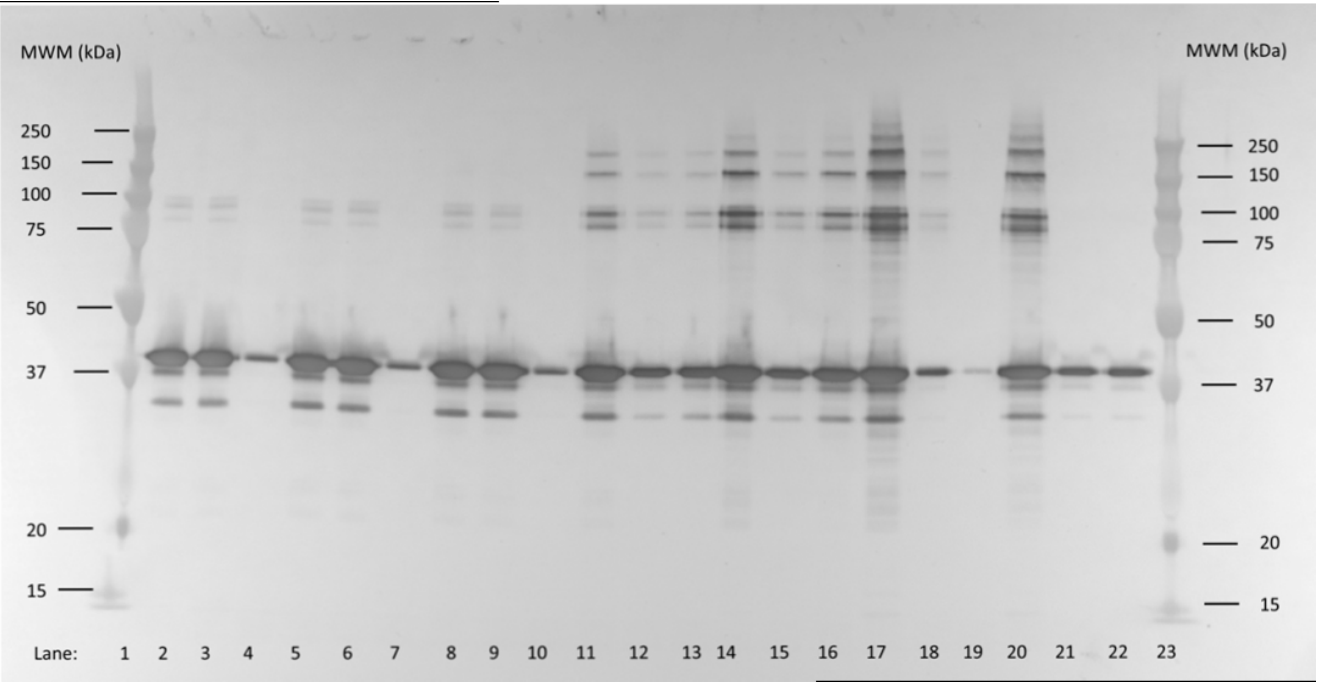


Figure 33. Western blot analysis of HPPD-4 protein after temperature treatment

Lane 1: Precision Plus Protein™ Dual Xtra Standards Molecular Weight Marker
Lane 2: 250 ng HPPD-4, 4°C - Uncentrifuged (UC)
Lane 3: 250 ng HPPD-4, 4°C - Supernatant (S)
Lane 4: 250 ng HPPD-4, 4°C – Pellet (P)
Lane 5: 250 ng HPPD-4, 25°C - UC
Lane 6: 250 ng HPPD-4, 25°C - S
Lane 7: 250 ng HPPD-4, 25°C - P
Lane 8: 250 ng HPPD-4, 37°C - UC
Lane 9: 250 ng HPPD-4, 37°C - S
Lane 10: 250 ng HPPD-4, 37°C - P
Lane 11: 250 ng HPPD-4, 55°C - UC
Lane 12: 250 ng HPPD-4, 55°C - S
Lane 13: 250 ng HPPD-4, 55°C - P
Lane 14: 250 ng HPPD-4, 75°C - UC
Lane 15: 250 ng HPPD-4, 75°C - S
Lane 16: 250 ng HPPD-4, 75°C – P
Lane 17: 250 ng HPPD-4, 95°C - UC
Lane 18: 250 ng HPPD-4, 95°C - S
Lane 19: 0.25 ng HPPD-4, 4°C (10% Control) - P
Lane 20: 250 ng HPPD-4, 95°C – P
Lane 21: 0.25 ng HPPD-4, 4°C (10% Control) - UC
Lane 22: 0.25 ng HPPD-4, 4°C (10% Control) - S

Lane 23: Precision Plus Protein™ Dual Xtra Standards Molecular Weight Marker

(iv) Specific serum screening where a new protein is derived from a source known to be allergenic or has sequence homology with a known allergen

The Cry14Ab-1 protein is derived from *Bacillus thuringiensis.* Overall, Cry proteins derived from *Bacillus thuringiensis* have proven to be safe for human consumption and uncommon sources of allergenicity (OECD, 2007). In the most recent bioinformatics analysis performed on the Cry14Ab-1 protein ([redacted] 2018; TXKIS002; Appendix 19), it was not found to display amino acid sequence homology with known allergens.

The HPPD-4 protein is not from a source known to be allergenic nor does it display sequence homology with known allergens.

(v) Information on whether the new protein(s) have a role in the elicitation of gluten-sensitive enteropathy, in cases where the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains

Not applicable. The introduced genetic materail is not obtained from wheat, rye, barley, oats or related cereal grains.

Where the new protein has been produced from an alternative source (e.g. microbial expression system) in order to obtain sufficient quantities for analysis, information must be provided to demonstrate that the protein tested is biochemically, structurally and functionally equivalent to that expressed in the food produced using gene technology.

Cry14Ab-1 and HPPD-4 proteins

Refer to Section B.1 (a), under equivalence of proteins expressed *in planta* with those produced microbially for evidence that proteins that have been tested are biochemically, structurally and functionally equivalent to that expressed in the food produced using gene technology.

Information on the potential toxicity and potential allergenicity of a newly expressed protein is also not required if:

(a) The protein is expressed from a transferred gene that is derived from the same species as the host or a species that is cross-compatible with the host, provided evidence is provided to demonstrate the following:

(i) The gene donor belongs to a species that is commonly used as food and has a history of safe use

The *cry14Ab-1.b* gene is derived from *Bacillus thuringiensis*. For the majority of *Bacillus thuringiensis* derived proteins expressed *in planta* that are currently registered, the source bacterium has been a registered microbial pesticide previously approved for use on food crops

without specific restrictions. In each case the source bacterium and genetically modified *Bt* crop, have a history of safe use as a food (Mendelsohn *et al*., 2003).

The gene donor for the *hppdPf-4Pa* gene is *Pseudomonas fluorescens*. Although *Pseudomanas fluorescens* is a species commonly used as food, HPPD proteins have been characterized from organisms present in human food, such as carrot (*Daucus carota*, Accession number O23920), barley (*Hordeum vulgare* Accession number O48604; Falk *et al*., 2002), pork (*Sus scrofa*, Accession number Q02110)) and beef (*Bos Taurus*, Accession number Q5EA20).

(ii) The protein is expressed at levels in the new food produced using gene technology that are consistent with the levels in the gene donor.

Not applicable.

(b) Evidence is provided to demonstrate the absence of the newly expressed protein from the parts of the host organism consumed as food.

The concentration of Cry14Ab-1 and HPPD-4 proteins in seed and processed fractions of treated and untreated GMB151 soybean were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA) ([redacted], 2018, 16-RSBS0011, Appendix 17).

The concentration of the Cry14Ab-1 protein in grain and hull samples ranged from 24.24 to 92.84 µg/g DW (Table 19). The highest concentration of Cry14Ab-1 was observed in grain. The Cry14Ab-1 protein concentration was below the lower limit of quantitation (LLOQ) in toasted meal, RBD oil, and protein isolate.

The concentration of the HPPD-4 protein in grain, protein isolate, and hull samples ranged from 0.27 to 5.57 µg/g DW (Table 20). The highest concentration of HPPD-4 was observed in grain. The HPPD-4 protein concentration was below the LLOQ in toasted meal and RBD oil.

Table 17. Concentrations of Cry14Ab-1 in GMB151 Soybean grain and Processed Fractions

Matrix	Trait-Specific Herbicide Treatment	Cry14Ab-1 (µg/g FW)[1]	Cry14Ab-1 (µg/g DW)[1]
Grain	Not Treated	71.79	81.48
	Treated	81.89	92.84
Toasted meal	Not Treated	<LLOQ	<LLOQ
	Treated	<LLOQ	<LLOQ
RBD oil	Not Treated	<LLOQ	NA
	Treated	<LLOQ	NA
Protein isolate	Not Treated	<LLOQ	<LLOQ
	Treated	<LLOQ	<LLOQ
Hulls	Not Treated	20.90	24.24
	Treated	23.50	26.99

[1]Values were calculated to full precision and rounded to 2 decimal places for reporting consistency.
LLOQ = Lower Limit of Quantitation
NA = No Applicable. DW calculations for oil samples were not performed since oil samples were analyzed as received and moisture data for these samples were not obtained.

Table 18. Concentrations of HPPD-4 in GMB151 Soybean Grain and Processed Fractions

Matrix	Trait-Specific Herbicide Treatment	HPPD-4 (µg/g FW)[1]	HPPD-4 (µg/g DW)[1]
Grain	Not Treated	3.34	3.79
	Treated	4.91	5.57
Toasted meal	Not Treated	<LLOQ	<LLOQ
	Treated	<LLOQ	<LLOQ
RBD oil	Not Treated	<LLOQ	<LLOQ
	Treated	<LLOQ	<LLOQ
Protein isolate	Not Treated	0.78	0.80
	Treated	0.47	0.48
Hulls	Not Treated	0.23	0.27
	Treated	0.36	0.42

[1]Values were calculated to full precision and rounded to 2 decimal places for reporting consistency.
LLOQ = Lower Limit of Quantitation
NA = No Applicable. DW calculations for oil samples were not performed since oil samples were analyzed as received and moisture data for these samples were not obtained.

B.3 Other (non-protein) new substances

If other (non-protein) substances are produced as a result of the introduced DNA, information must be provided on the following:

(a) The identity and biological function of the substance

Non-protein substances cannot be created from DNA. The central maxim of molecular biology is that DNA makes RNA and RNA makes protein. Therefore, no non-protein substances could be created from the introduction of the DNA insert.

(b) Whether the substance has previously been safely consumed in food

Not relevant.

(c) Potential dietary exposure to the substance

Not relevant.

(d) Where RNA interference has been used:

(i) The role of any endogenous target gene and any changes to the food as a result of silencing that gene

Not applicable. RNA interference has not been used.

(ii) The expression levels of the RNA transcript

Not applicable. RNA interference has not been used.

(iii) The specificity of the RNA interference

Not applicable. RNA interference has not been used.

B.4 Novel herbicide metabolites in GM herbicide-tolerant plants

Data must be provided on the identity and levels of herbicide and any novel metabolites that may be present in the food produced using gene technology.

If novel metabolites are present then the application should address the following, where appropriate:

(a) Toxicokinetics and metabolism

(b) Acute toxicity

(c) Short-term toxicity

(d) Long-term toxicity and carcinogenicity

(e) Reproductive and developmental toxicity

(f) Genotoxicity

4-Hydroxyphenylpyruvate dioxygenase (HPPD) is an Fe(II)-dependent, non-heme oxygenase. HPPD is a key enzyme involved in the catabolism of tyrosine which catalyzes the conversion of 4-hydroxyphenylpyruvate to homogentisate. In plants, the HPPD enzyme is also involved in several anabolic pathways; its reaction product homogentisate (2,5-dihydroxyohenylacetate) being the aromatic precursor of tocopherol and plastoquinone, which are essential to the photosynthetic transport chain and antioxidative systems (Fritze, I. M.; et al.; 2004; M-359884-01). Figure 34 shows a diagram of the different metabolic pathways in which HPPD is involved in plants and non-photosynthetic organisms.

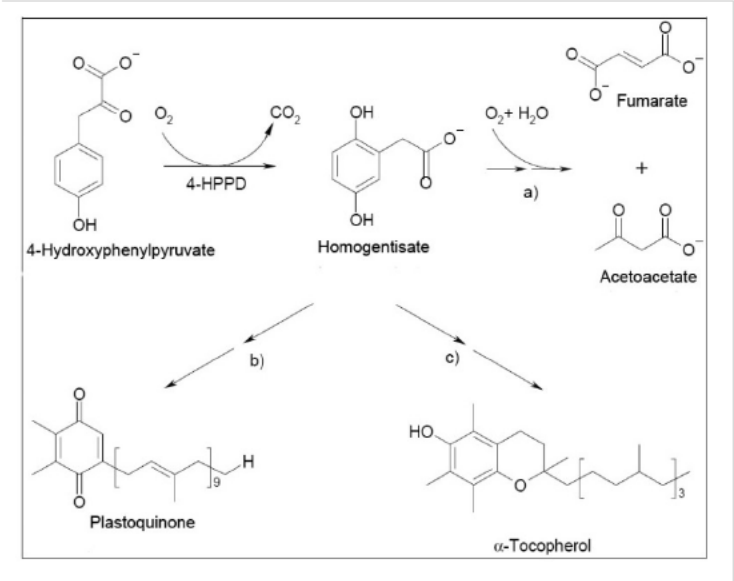


Figure 34 Biochemical pathways of HPPD proteins

a) catabolism of tyrosine, b) biosynthesis of plastoquinone (plants)

c) biosynthesis of tocopherol and tocotrienols (plants)

The activity of HPPD is suppressed by HPPD-inhibiting herbicides (Figure 35). HPPD enzyme inhibition results in the disruption of the biosynthesis of carotenoids. Plants lacking carotenoids cannot protect themselves from the radicals generated by the light activation of chlorophyll, causing bleaching, necrosis, and death.

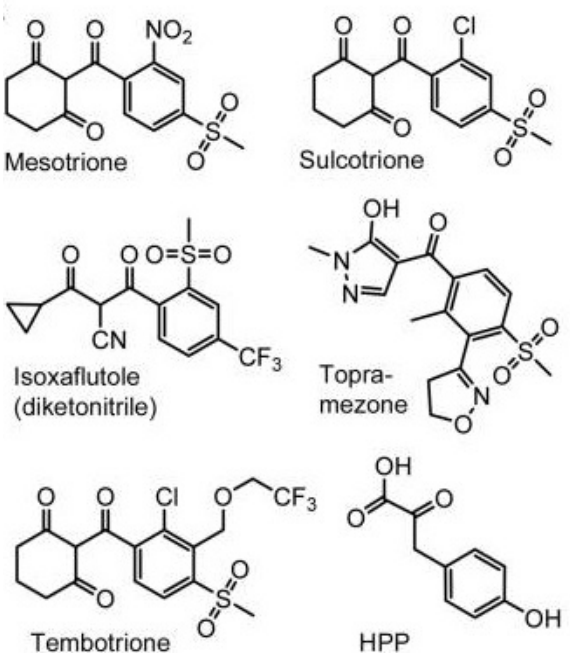


Figure 35. Structures of HPPD-inhibiting herbicides and the substrate 4-hydroxyphenylpyruvate. (Siehl D. L. et al., 2014)

GMB151 soybean expresses the HPPD-4 protein, a modified HPPD enzyme which reduces the binding efficacy of HPPD inhibitors, thereby conferring tolerance to HPPD-inhibitor herbicides, such as isoxaflutole (IFT). There are no novel metabolites produced in GMB151.

We propose that current maximum residue limits for IFT in/on soybean commodities remain appropriate for consideration with this application for approval of GMB151 soybean.

B.5 Compositional Analyses of the Food Produced Using Gene Technology

(a) The levels of key nutrients, toxicants and anti-nutrients in the food produced using gene technology compared with the levels in appropriate comparator (usually the non-GM counterpart). A statistical analysis of the data must be provided.

Composition analyses were conducted to determine the levels of nutrients and anti-nutrients in soybean grain and select nutrients in soybean forage samples from multiple field trials and conduct a comparative assessment between GMB151 soybean (not treated and treated with trait-specific herbicide), the non-genetically modified (non-GM) counterpart, and non-GM reference varieties ([redacted]. 2018; 17-RSSB0044-C, Appendix 29).

Field production

Composition analysis was conducted on forage and grain samples from eight field trials conducted in the 2017 continental U.S. season. Selected sites are representative of likely

environments where GMB151 soybean will be commercially grown. The field trial site information for the eight sites selected is presented in the table below.

Table 19. Field Trial Sites

Site Code	Nearest Town or City	Site County	State
01	Richland	Keokuk	Iowa
02	York	York	Nebraska
03	Elk Horn	Shelby	Iowa
04	Stewardson	Shelby	Illinois
05	Germansville	Lehigh	Pennsylvania
06	Fisk	Butler	Missouri
07	Larned	Pawnee	Kansas
11	Carlyle	Clinton	Illinois

In addition to the GMB151 soybean and conventional counterpart, nine different non-GM commercial reference varieties were included to provide reference ranges for the composition assessment. The non-GM reference varieties were provided by Schillinger Genetics Inc. (Des Moines, Iowa, U.S.) and NuPride Genetics Network, LLC (Lincoln, Nebraska, U.S.). Each field trial location planted only three of the nine commercial reference varieties. The entries included are presented below.

Table 20. Description of Soybean Entries

Entry ID	Material Name	Other Specification	Site Code
A	Thorne	Non-GM Counterpart	All
D	GMB151	GM not treated	All
E	GMB151	GM treated	All
F	E3494	Non-GM Reference	06, 07, 11
G	NGN 3121STS	Non-GM Reference	06, 07, 11
H	NGN 3347C	Non-GM Reference	06, 07, 11
I	E2282	Non-GM Reference	01, 03, 05
J	E2692	Non-GM Reference	01, 03, 05
K	NGN 3292C	Non-GM Reference	01, 03, 05
L	E3066	Non-GM Reference	02, 04
M	E2993	Non-GM Reference	02, 04
N	E3192	Non-GM Reference	02, 04

Each entry in each field trial was replicated four times in a randomized complete block design (RCBD).

Conventional herbicide management was applied to all entries. The HPPD inhibitor herbicide, isoxaflutole (Balance Pro) was applied to Entry E at a target rate of 70.1 g a.i./ha at growth stage BBCH 00-03 (pre-emergence).

Forage samples were harvested at BBCH 71 – BBCH 78 (pod formation) and grain samples were harvested at BBCH 89 – BBCH 99 (grain maturity), from all plots for subsequent composition analysis. Forage samples were shipped frozen and grain samples were shipped at ambient temperatures to [redacted]

Composition Analysis

Composition analysis of the soybean forage and grain samples was conducted at to [redacted]. Forage samples were analyzed for proximates, fiber, calcium, and phosphorus. Grain samples were analyzed for proximates, fiber, amino acids, fatty acids, minerals, vitamins, and anti-nutrients (Table 23). The analytical methods employed and the reference standards used are detailed in the appendix of the composition analytical report ([redacted]; 2018; 17-RSSB0044-C, Appendix 29).

Table 21. Composition Analytes, Units, and Methods for Soybean Forage and Grain

Matrix	Parameter	Units
	Proximates and Fiber	
Grain, Forage	Moisture	% FW
	Ash	% FW, DW
	Carbohydrates	
	Crude Fat	
	Crude Protein	
	Acid Detergent Fiber	
	Neutral Detergent Fiber	
Grain	Total Dietary Fiber	
	Amino Acids	
Grain	Alanine, Arginine, Aspartic Acid, Glutamic Acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Phenylalanine, Proline, Serine, Threonine, Tyrosine, Valine	% FW, DW
	Cystine, Methionine	
	Tryptophan	
	Fatty Acids	
Grain	C8:0, C10:0, C12:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C18:3, C18:4, C19:0, C20:0, C20:1, C20:2, C20:3, C20:4, C20:5, C22:0, C22:1,C22:5 n-3, C22:5 n-6, C22:6, C24:0	% FW, DW, % Total Fatty Acids
	Minerals	
Grain, Forage	Calcium, Phosphorus	mg/kg FW, DW
Grain	Copper, Iron, Magnesium, Manganese, Potassium, Sodium, Zinc	
	Vitamins	
Grain	Alpha Tocopherol (Vitamin E)	mg/kg FW, DW
	Vitamin B1(Thiamine)	
	Vitamin B2 (Riboflavin)	
	Vitamin B3 (Niacin)	
	Vitamin B5 (Pantothenic Acid)	
	Vitamin B6 (Pyridoxine)	
	Vitamin B9 (Folic Acid)	
	Vitamin K1	
	Anti-Nutrients	
Grain	**Isoflavones**	
	Total Daidzein	mg/kg FW, DW
	Total Genistein	
	Total Glycitein	
	Total Isoflavones	
	Lectins	mg/g FW, DW
	Phytic Acid	% FW, DW
	Raffinose	
	Stachyose	
	Trypsin Inhibitor	TIU/mg FW, DW

FW = Fresh Weight; DW = Dry Weight; TIU = Trypsin Inhibitor Unit

Statistical Analysis

Composition data for 192 samples collected from eight sites were statistically analyzed using SAS version 9.4 (SAS, 2002-2012).

Analytes with more than one third of sample values below the limit of quantification (LOQ) were excluded from further statistical evaluation (i.e., ANOVA and mean comparisons) and discussion. These analytes are presented in Table 24. Minimum and maximum values for analytes where some values are above LOQ, but there is insufficient data for statistical analysis are presented Table 29 and Table 30.

For several grain analytes; C17:1 Heptadecenoic Acid, C24:0 Lignoceric Acid, and Vitamin B1, (Table 24) some samples had values less than the LOQ but there were sufficient (more than two thirds) sample values above LOQ for statistical evaluation. In these cases, the below LOQ value was substituted by a value equal to half the LOQ in order to be included in the statistical analysis.

Soybean forage and grain samples from eight sites generated a total of 192 observations for each analyte for each matrix. There were 32 observations for Entry A, 32 observations each for Entry D and Entry E, and 96 observations for the reference variety entries (Entries F, G, H, I, J, K, L, M and N), which were statistically analyzed collectively. Observations for the non-GM counterpart (Entry A) were compared to GMB151 soybean not treated (Entry D), and GMB151 soybean treated with trait-specific herbicide (Entry E).

Combined-Site Analysis

Composition data were statistically analyzed for each analyte combined over all sites with a mixed model ANOVA. The additive model for the design is:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \delta_{k(j)} + \varepsilon_{ijk}$$

where Y_{ijk} is the individual value measurement, μ the overall mean, α_i the fixed effect associated with entry, β_j the random effect associated with site, $\alpha\beta_{ij}$ the random effect for the interaction of entry by site, $\delta_{k(j)}$ the random effect associated with block nested within site and ε_{ijk} the random error. The degrees of freedom were estimated using the method specified by Kenward and Roger (1997).
Based on the mixed model, entry differences (Entry A vs Entry D and Entry A vs Entry E) were estimated and presented along with the p-values (t-test) for the entry differences. Statistical significance was evaluated at $p<0.05$ level.

Descriptive Statistics

Descriptive statistics for each of the nine forage analytes and 64 quantifiable grain analytes were calculated, including mean and standard deviation for Entries A, D, and E and the minimum and maximum values for the reference varieties. Additionally, tolerance intervals for each analyte, specified to contain 99% of the population with 95% confidence, were calculated based on the reference varieties. Tolerance interval values that were negative were set to zero.

By-Site Analysis

For each composition analyte, a by-site analysis was performed using a mixed model ANOVA with the fixed entry effect and the random block effect, followed by pairwise t-tests comparing

Entry A vs Entry D and Entry A vs Entry E. A summary of the composition analytes, by analyte category, is presented in the report ([redacted]. 2018; 17-RSSB0044-C, Appendix 29).

Table 22. Soybean Grain Analytes with Samples Below the Limit of Quantitation (LOQ)

	Number of Values		Excluded from
Parameter	**>= LOQ**	**< LOQ**	**analysis**
C8:0 Caprylic Acid	0	192	Yes
C10:0 Capric Acid	0	192	Yes
C12:0 Lauric Acid	0	192	Yes
C14:1 Myristoleic Acid	0	192	Yes
C15:0 Pentadecanoic Acid	0	192	Yes
C15:1 Pentadecenoic Acid	0	192	Yes
C17:1 Heptadecenoic Acid	191	1	No
C18:4 Stearidonic Acid	2	190	Yes
C19:0 Nonadecanoic Acid	18	174	Yes
C20:2 Eicosadienoic Acid	106	86	Yes
C20:3 Eicosatrienoic Acid	0	192	Yes
C20:4 Arachidonic Acid	0	192	Yes
C20:5 Eicosapentaenoic Acid	66	126	Yes
C22:1 Erucic Acid	0	192	Yes
C22:5 N3 Docosapentaenoic Acid	0	192	Yes
C22:5 N6 Docosapentaenoic Acid	3	189	Yes
C22:6 Docosahexaenoic Acid	15	177	Yes
C24:0 Lignoceric Acid	155	37	No
Sodium	112	80	Yes
Vitamin B1 (Thiamine)	149	43	No

Results

Proximates, Fiber and Minerals in Soybean Forage (Table 25 and Table 26).

No significant differences were observed between the non-GM conventional counterpart (Entry A) and GMB151 not treated or treated with trait-specific herbicides (Entry D and E) for any of the proximate, fibers (moisture, ash, carbohydrates, crude fat, crude protein, acid detergent fiber, and neutral detergent fiber), or selected minerals (calcium and phosphorus).

Proximates and Fiber in Soybean Grain (Table 27).

No significant differences were observed between the non-GM counterpart (Entry A) and GMB151 soybean not treated or treated with trait-specific herbicide (Entry D and Entry E) for, ash, crude fat, acid detergent fiber, and total dietary fiber.

No significant difference was observed between the non-GM counterpart (Entry A) and GMB151 soybean not treated with trait-specific herbicide (Entry D) for neutral detergent fiber. However a statistically significant difference ($p < 0.05$) was observed between the non-GM counterpart (Entry A) and GMB151 soybean treated with trait-specific herbicide (Entry E) for this analyte.

Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GMB151 soybean not treated or treated with trait-specific herbicide (Entry D and Entry E) for moisture, carbohydrates, and crude protein.

However, the GMB151 soybean mean values, for all proximates and fiber, were within the range of the reference varieties and the tolerance intervals. Therefore, the statistically significant differences are not considered biologically relevant.

Amino Acids in Soybean Grain (Table 28).

No significant differences were observed between the non-GM counterpart (Entry A) and GMB151 soybean not treated or treated with trait-specific herbicide (Entry D and Entry E) for any of the amino acids.

Fatty Acids in Soybean Grain (Table 29).

No significant differences were observed between the non-GM counterpart (Entry A) and GMB151 soybean not treated or treated with trait-specific herbicide (Entry D and Entry E) for C14:0 myristic acid, C16:1 palmitoleic acid, C17:0 heptadecanoic acid, C18:0 stearic acid, C18:3 linolenic acid, and C20:0 arachidic acid.

No significant differences were observed between the non-GM counterpart (Entry A) and GMB151 soybean not treated with trait-specific herbicide (Entry D) for C24:0 lignoceric acid, however a statistically significant difference ($p < 0.05$) was observed between the non-GM counterpart (Entry A) and GMB151 treated with trait-specific herbicide (Entry E) for this analyte.

Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GMB151 soybean not treated or treated with trait-specific herbicide (Entry D and Entry E) for C16:0 palmitic acid, C17:1 heptadecenoic acid, C18:1 oleic acid, C18:2 linoleic acid, C20:1 eicosenoic acid, and C22:0 behenic acid.

The GMB151 soybean mean values, for all fatty acids, were within the range of the reference varieties except for C16:0 palmitic acid for GMB151 soybean not treated, which was slightly lower than the minimum reference variety value. However, the GMB151 soybean mean values were all within the tolerance intervals; therefore, the statistically significant differences are not considered biologically relevant.

Minerals in Soybean Grain (Table 30).

Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GMB151 soybean treated with trait-specific herbicide (Entry E) for calcium, copper, and zinc.

However, the GMB151 soybean mean values for calcium, copper, and zinc were within the mean range of the reference varieties and tolerance intervals. Therefore, the statistically significant differences are not considered biologically relevant.

Vitamins in Soybean Grain (Table 31).

Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GMB151 soybean treated with trait-specific herbicide (Entry E) for vitamin B1 (thiamine), vitamin B5 (pantothenic acid) and vitamin B9 (folic acid).

However, the GMB151 soybean mean values for vitamin B1 (thiamine), vitamin B5 (pantothenic acid) and vitamin B9 (folic acid) were within the mean range of the reference varieties and the tolerance intervals. Therefore, the statistically significant differences are not considered biologically relevant.

Anti-nutrients in Soybean Grain (Table 32).

A statistically significant difference ($p < 0.05$) was observed between the non-GM counterpart (Entry A) and GMB151 soybean treated with trait-specific herbicide (Entry E) for phytic acid. Statistically significant differences ($p < 0.05$) were observed between the non-GM counterpart (Entry A) and GMB151 soybean not treated or treated with trait-specific herbicide (Entry D and Entry E) for total daidzein, total genistein, total glycitein, and total isoflavones.

However, the GMB151 soybean mean values for phytic acid, total daidzein, total genistein, total glycitein, and total isoflavones were within the mean range of the reference varieties and the tolerance intervals. Therefore, the statistically significant differences are not considered biologically relevant.

Summary and Conclusions

All of the nine composition analytes for forage were above LOQ and were statistically analyzed. No statistically significant differences ($p < 0.05$) were observed for any of the forage analytes.

Of the 81 composition analytes for grain, 64 had sufficient levels above LOQ for statistical analysis. Of the 64 analytes that were statistically analyzed, statistically significant differences ($p < 0.05$) were observed for 22 analytes, 13 of which were statistically different between both the non-GM counterpart (Entry A) and GMB151 soybean not treated or treated with trait-specific herbicide (Entry D and Entry E), and nine of which were statistically different between the non-GM counterpart (Entry A) and GMB151 soybean treated with trait-specific herbicide (Entry E). However, the mean values of GMB151 soybean, not treated and treated with trait-specific herbicide, for all grain analytes were within the range of the reference varieties and/or the tolerance intervals. Therefore, the statistically significant differences are not considered relevant.

Based on the comparative assessment, the composition of GMB151 soybean forage and grain is comparable to that of the non-GM counterpart and reference varieties.

Table 23. Comparison of Proximates and Fiber in Forage of GMB151 Soybean with its Non-GM Counterpart[a]

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GMB151 Not Treated (Entry D) Mean ± SD	GMB151 Treated (Entry E) Mean ± SD	Non-GM Reference Varieties Range (Entries F–N) [b] (Min–Max)	Tolerance Interval Non-GM Reference Varieties (Entries F–N) [c] (Lower–Upper)	Comparison t-test A vs D [d] p-value	Comparison t-test A vs E [d] p-value
Moisture (% FW)	79.2 ± 3.95	79.2 ± 3.41	79.1 ± 4.04	70.9 - 85.6	65.9 - 88.7	0.960	0.906
Ash (% DW)	8.02 ± 2.91	8.60 ± 3.48	8.14 ± 2.82	5.15 - 29.10	0 - 21.43	0.285	0.826
Carbohydrates (% DW)	68.7 ± 3.96	66.3 ± 4.94	67.1 ± 4.64	48.2 - 76.3	51.5 - 82.7	0.053	0.182
Crude Fat (% DW)	3.78 ± 1.58	4.18 ± 1.82	4.31 ± 1.54	0.26 - 11.40	0 - 11.37	0.410	0.279
Crude Protein (% DW)	19.6 ± 2.50	20.9 ± 3.71	20.5 ± 2.86	15.2 - 30.6	11.5 - 28.3	0.099	0.246
Acid Detergent Fiber (% DW)	32.4 ± 5.19	33.7 ± 7.27	30.3 ± 5.59	20.7 - 58.1	11.9 - 54.4	0.428	0.170
Neutral Detergent Fiber (% DW)	37.4 ± 6.05	37.1 ± 8.56	34.0 ± 5.21	25.2 - 58.0	17.0 - 55.8	0.889	0.093

[a] Composition samples were derived from eight field trials conducted in USA in 2017.
[b] Range of results from nine reference varieties (entries F–N).
[c] 99% Tolerance Interval: Range of reference varieties based on tolerance intervals specified to contain 99% of the population with 95% confidence.
[d] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

Table 24. Comparison of Select Minerals in Forage of GMB151 Soybean with its Non-GM Counterpart[a] (mg/kg DW)

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GMB151 Not Treated (Entry D) Mean ± SD	GMB151 Treated (Entry E) Mean ± SD	Non-GM Reference Varieties Range (Entries F–N) [b] (Min–Max)	Tolerance Interval Non-GM Reference Varieties (Entries F–N) [c] (Lower–Upper)	Comparison t-test A vs D [d] p-value	Comparison t-test A vs E [d] p-value
Calcium	1054 ± 297	1115 ± 277	1068 ± 261	522 - 1843	201.3 - 1870.9	0.255	0.799
Phosphorus	319 ± 77.1	336 ± 75.3	309 ± 73.2	164 - 463	122.0 - 479.7	0.416	0.606

[a] Composition samples were derived from eight field trials conducted in USA in 2017.
[b] Range of results from nine reference varieties (entries F–N).
[c] 99% Tolerance Interval: Range of reference varieties based on tolerance intervals specified to contain 99% of the population with 95% confidence.
[d] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

Table 25. Comparison of Proximates and Fiber in Grain of GMB151 Soybean with its Non-GM Counterpart[a]

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GMB151 Not Treated (Entry D) Mean ± SD	GMB151 Treated (Entry E) Mean ± SD	Non-GM Reference Varieties Range (Entries F–N) [b] (Min–Max)	Tolerance Interval Non-GM Reference Varieties (Entries F–N) [c] (Lower–Upper)	Comparison t-test A vs D [d] p-value	Comparison t-test A vs E [d] p-value
Moisture (% FW)	12.2 ± 2.70	11.5 ± 2.33	11.3 ± 2.30	8.9 - 19.4	4.0 - 19.7	<0.001	<0.001
Ash (% DW)	4.93 ± 0.23	4.91 ± 0.19	4.94 ± 0.24	4.09 - 5.63	4.27 - 5.60	0.705	0.814
Carbohydrates (% DW)	35.2 ± 0.85	34.4 ± 0.97	34.5 ± 1.14	32.3 - 39.1	32.1 - 40.0	0.005	0.013
Crude Fat (% DW)	20.2 ± 0.77	20.3 ± 0.99	20.1 ± 0.92	17.5 - 23.8	15.8 - 24.2	0.624	0.721
Crude Protein (% DW)	39.7 ± 1.07	40.4 ± 1.37	40.4 ± 1.37	33.6 - 44.1	32.6 - 45.5	0.013	0.009
Acid Detergent Fiber (% DW)	16.2 ± 2.10	15.5 ± 1.63	15.7 ± 1.41	10.6 - 21.2	9.8 - 20.9	0.168	0.271
Neutral Detergent Fiber (% DW)	16.3 ± 1.52	16.1 ± 0.97	15.7 ± 1.00	13.1 - 19.1	11.7 - 20.1	0.439	0.019
Total Dietary Fiber (% DW)	17.5 ± 1.64	17.4 ± 1.68	17.4 ± 2.07	12.1 - 21.9	12.2 - 23.6	0.846	0.766

[a] Composition samples were derived from eight field trials conducted in USA in 2017.
[b] Range of results from nine reference varieties (entries F–N).
[c] 99% Tolerance Interval: Range of reference varieties based on tolerance intervals specified to contain 99% of the population with 95% confidence.
[d] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

Table 26. Comparison of Amino Acids in Grain of GMB151 Soybean with its Non-GM Counterpart[a] (% DW)

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GMB151 Not Treated (Entry D) Mean ± SD	GMB151 Treated (Entry E) Mean ± SD	Non-GM Reference Varieties Range (Entries F–N) [b] (Min–Max)	Tolerance Interval Non-GM Reference Varieties (Entries F–N) [c] (Lower–Upper)	Comparison t-test A vs D [d] p-value	Comparison t-test A vs E [d] p-value
Alanine	1.74 ± 0.066	1.75 ± 0.071	1.76 ± 0.083	1.48 - 2.00	1.43 - 1.98	0.379	0.230
Arginine	2.72 ± 0.182	2.77 ± 0.217	2.78 ± 0.245	2.05 - 3.48	1.90 - 3.50	0.167	0.111
Aspartic Acid	4.75 ± 0.149	4.84 ± 0.283	4.82 ± 0.286	3.93 - 5.66	3.68 - 5.80	0.148	0.243
Cystine	0.514 ± 0.053	0.529 ± 0.051	0.527 ± 0.055	0.314 - 0.645	0.285 - 0.692	0.278	0.368
Glutamic Acid	7.49 ± 0.25	7.68 ± 0.43	7.64 ± 0.46	6.15 - 8.81	5.85 - 9.21	0.054	0.121
Glycine	1.76 ± 0.09	1.79 ± 0.10	1.79 ± 0.11	1.41 - 1.96	1.40 - 2.06	0.209	0.137
Histidine	1.08 ± 0.067	1.09 ± 0.076	1.08 ± 0.091	0.845 - 1.25	0.815 - 1.32	0.553	0.741
Isoleucine	1.88 ± 0.081	1.90 ± 0.088	1.90 ± 0.100	1.52 - 2.13	1.50 - 2.19	0.207	0.213
Leucine	3.08 ± 0.12	3.12 ± 0.14	3.11 ± 0.16	2.53 - 3.51	2.46 - 3.59	0.195	0.235
Lysine	2.89 ± 0.24	2.92 ± 0.23	2.92 ± 0.19	2.33 - 3.41	2.11 - 3.56	0.632	0.635
Methionine	0.513 ± 0.039	0.516 ± 0.039	0.528 ± 0.035	0.376 - 0.583	0.361 - 0.625	0.706	0.105
Phenylalanine	2.06 ± 0.15	2.09 ± 0.16	2.09 ± 0.20	1.58 - 2.56	1.50 - 2.61	0.386	0.517
Proline	2.07 ± 0.088	2.11 ± 0.100	2.11 ± 0.118	1.66 - 2.46	1.61 - 2.46	0.119	0.109
Serine	2.11 ± 0.11	2.13 ± 0.12	2.13 ± 0.13	1.71 - 2.35	1.67 - 2.46	0.330	0.350
Threonine	1.63 ± 0.071	1.64 ± 0.085	1.64 ± 0.095	1.33 - 1.79	1.30 - 1.88	0.347	0.487
Tryptophan	0.542 ± 0.025	0.527 ± 0.041	0.531 ± 0.022	0.370 - 0.603	0.398 - 0.624	0.083	0.199
Tyrosine	1.24 ± 0.10	1.26 ± 0.10	1.25 ± 0.12	0.92 - 1.46	0.88 - 1.56	0.310	0.613
Valine	1.90 ± 0.076	1.92 ± 0.084	1.93 ± 0.104	1.55 - 2.21	1.52 - 2.23	0.342	0.208

[a] Composition samples were derived from eight field trials conducted in USA in 2017.
[b] Range of results from nine reference varieties (entries F–N).
[c] 99% Tolerance Interval: Range of reference varieties based on tolerance intervals specified to contain 99% of the population with 95% confidence.
[d] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

Table 27. Comparison of Fatty Acids in Grain of GMB151 Soybean with its Non-GM Counterpart[a] (% Total Fatty Acids)

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GMB151 Not Treated (Entry D) Mean ± SD	GMB151 Treated (Entry E) Mean ± SD	Non-GM Reference Varieties Range (Entries F–N) [b] (Min–Max)	Tolerance Interval Non-GM Reference Varieties (Entries F–N) [c] (Lower–Upper)	Comparison t-test A vs D [d] p-value	Comparison t-test A vs E [d] p-value
C14:0 Myristic Acid	0.0701 ± 0.0072	0.0690 ± 0.0072	0.0702 ± 0.0084	0.0574 - 0.0873	0.0520 - 0.0895	0.578	0.934
C16:0 Palmitic Acid	11.0 ± 0.24	10.6 ± 0.27	10.7 ± 0.27	10.7 - 12.4	10.2 - 12.8	<.001	0.002
C16:1 Palmitoleic Acid	0.0833 ± 0.0054	0.0812 ± 0.0044	0.0847 ± 0.0047	0.0648 - 0.1010	0.0593 - 0.1075	0.128	0.300
C17:0 Heptadecanoic Acid	0.0913 ± 0.0053	0.0904 ± 0.0070	0.0902 ± 0.0062	0.0725 - 0.1080	0.0643 - 0.1160	0.498	0.368
C17:1 Heptadecenoic Acid	0.0618 ± 0.0038	0.0653 ± 0.0041	0.0652 ± 0.0050	0.0257 - 0.0673	0.0433 - 0.0724	0.005	0.006
C18:0 Stearic Acid	4.38 ± 0.32	4.34 ± 0.32	4.38 ± 0.34	3.79 - 5.24	3.41 - 5.36	0.540	0.996
C18:1 Oleic Acid	21.8 ± 1.37	23.6 ± 2.39	23.4 ± 2.05	17.2 - 28.0	15.3 - 28.8	0.003	0.006
C18:2 Linoleic Acid	53.7 ± 1.18	52.6 ± 1.80	52.6 ± 1.51	49.4 - 56.9	47.2 - 58.3	0.007	0.009
C18:3 Linolenic Acid	7.81 ± 0.43	7.49 ± 0.52	7.52 ± 0.51	6.26 - 10.40	5.00 - 10.98	0.064	0.096
C18:4 Stearidonic Acid	<LOQ	<LOQ - 0.113	<LOQ	<LOQ - 0.0859	NA	NA	NA
C19:0 Nonadecanoic Acid	<LOQ - 0.0943	<LOQ - 0.449	<LOQ - 0.0653	<LOQ - 0.307	NA	NA	NA
C20:0 Arachidic Acid	0.315 ± 0.018	0.320 ± 0.015	0.324 ± 0.019	0.277 - 0.393	0.250 - 0.403	0.289	0.062
C20:1 Eicosenoic Acid	0.182 ± 0.019	0.189 ± 0.022	0.191 ± 0.019	0.135 - 0.256	0.115 - 0.260	0.010	0.003
C20:2 Eicosadienoic Acid	<LOQ - 0.0546	<LOQ - 0.0516	<LOQ - 0.0579	<LOQ - 0.0579	NA	NA	NA
C20:5 Eicosapentaenoic Acid	<LOQ - 0.104	<LOQ - 0.0534	<LOQ - 0.0657	<LOQ - 0.0856	NA	NA	NA
C22:0 Behenic Acid	0.331 ± 0.010	0.378 ± 0.013	0.382 ± 0.012	0.320 - 0.390	0.294 - 0.401	<0.001	<0.001
C22:5 N6 Docosapentaenoic Acid	<LOQ - 0.0515	<LOQ	<LOQ	<LOQ - 0.081	NA	NA	NA
C22:6 Docosahexaenoic Acid	<LOQ - 0.0567	<LOQ - 0.0415	<LOQ - 0.109	<LOQ - 0.0654	NA	NA	NA
C24:0 Lignoceric Acid	0.111 ± 0.021	0.101 ± 0.024	0.098 ± 0.032	0.038 - 0.180	0 - 0.200	0.080	0.024

[a] Composition samples were derived from eight field trials conducted in USA in 2017.
[b] Range of results from nine reference varieties (entries F–N).

[c] 99% Tolerance Interval: Range of reference varieties based on tolerance intervals specified to contain 99% of the population with 95% confidence.
[d] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).
NA = Not Applicable because more than 1/3 of the values are <LOQ. Minimum and maximum are reported instead of mean and standard deviation.

Table 28. Comparison of Minerals in Grain of GMB151 Soybean with its Non-GM Counterpart[a] (mg/kg DW)

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GMB151 Not Treated (Entry D) Mean ± SD	GMB151 Treated (Entry E) Mean ± SD	Non-GM Reference Varieties Range (Entries F–N) [b] (Min–Max)	Tolerance Interval Non-GM Reference Varieties (Entries F–N) [c] (Lower–Upper)	Comparison t-test A vs D [d] p-value	Comparison t-test A vs E [d] p-value
Calcium	2547 ± 362	2435 ± 345	2343 ± 334	1480 - 3689	1004 - 3987	0.172	0.017
Copper	14.8 ± 4.50	14.8 ± 3.44	13.3 ± 2.61	6.8 - 23.2	3.2 - 20.7	0.939	0.040
Iron	118 ± 29.2	118 ± 41.1	115 ± 45.3	59 - 364	0 - 233	0.959	0.786
Magnesium	2580 ± 201	2497 ± 204	2498 ± 268	1954 - 3177	1758 - 3278	0.163	0.164
Manganese	39.2 ± 9.72	40.4 ± 9.79	38.2 ± 10.46	23.8 - 74.7	3.7 - 68.1	0.389	0.520
Phosphorus	6135 ± 762	6170 ± 673	6090 ± 730	4789 - 7773	4088 - 8109	0.788	0.723
Potassium	19545 ± 1214	19937 ± 1601	19862 ± 1425	16973 - 22831	15691 - 24021	0.292	0.392
Sodium	<LOQ - 45.7	<LOQ - 51.4	<LOQ - 51.2	<LOQ - 55.5	NA	NA	NA
Zinc	58.4 ± 11.47	57.7 ± 13.47	54.2 ± 9.90	33.0 - 85.6	18.0 - 80.6	0.692	0.024

[a] Composition samples were derived from eight field trials conducted in USA in 2017.
[b] Range of results from nine reference varieties (entries F–N).
[c] 99% Tolerance Interval: Range of reference varieties based on tolerance intervals specified to contain 99% of the population with 95% confidence.
[d] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).
NA = Not Applicable because more than 1/3 of the values are <LOQ. Minimum and maximum are reported instead of mean and standard deviation.

Table 29. Comparison of Vitamins in Grain of GMB151 Soybean with its Non-GM Counterpart[a] (mg/kg DW)

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GMB151 Not Treated (Entry D) Mean ± SD	GMB151 Treated (Entry E) Mean ± SD	Non-GM Reference Varieties Range (Entries F–N) [b] (Min–Max)	Tolerance Interval Non-GM Reference Varieties (Entries F–N) [c] (Lower–Upper)	Comparison t-test A vs D [d] p-value	Comparison t-test A vs E [d] p-value
Alpha Tocopherol (Vitamin E)	27.6 ± 9.59	27.4 ± 9.67	28.1 ± 9.36	12.3 - 53.2	0 - 57.2	0.835	0.717
Vitamin B1 (Thiamine)	1.80 ± 0.72	1.67 ± 0.75	1.46 ± 0.74	0.41 - 2.54	0 - 2.93	0.128	<.001
Vitamin B2 (Riboflavin)	4.44 ± 0.74	4.51 ± 0.70	4.33 ± 0.88	2.26 - 5.92	2.32 - 6.03	0.675	0.507
Vitamin B3 (Niacin)	26.7 ± 6.57	29.2 ± 6.34	29.3 ± 6.58	13.7 - 46.9	5.7 - 47.0	0.084	0.075
Vitamin B5 (Pantothenic Acid)	8.64 ± 1.76	9.27 ± 2.23	9.50 ± 2.22	5.42 - 14.60	4.01 - 16.05	0.098	0.026
Vitamin B6 (Pyridoxine)	4.59 ± 1.18	4.63 ± 1.13	4.81 ± 1.20	2.12 - 7.69	0.85 - 8.43	0.878	0.461
Vitamin B9 (Folic Acid)	5.50 ± 1.64	6.43 ± 2.02	6.77 ± 2.50	2.19 - 13.30	0 - 12.57	0.084	0.021
Vitamin K1	0.703 ± 0.330	0.700 ± 0.334	0.707 ± 0.335	0.344 - 1.440	0 - 1.592	0.937	0.895

[a] Composition samples were derived from eight field trials conducted in USA in 2017.
[b] Range of results from nine reference varieties (entries F–N).
[c] 99% Tolerance Interval: Range of reference varieties based on tolerance intervals specified to contain 99% of the population with 95% confidence.
[d] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

Table 30. Comparison of Anti-nutrients in Grain of GMB151 Soybean with its Non-GM Counterpart[a]

Parameter	Non-GM Counterpart (Entry A) Mean ± SD	GMB151 Not Treated (Entry D) Mean ± SD	GMB151 Treated (Entry E) Mean ± SD	Non-GM Reference Varieties Range (Entries F–N) [b] (Min–Max)	Tolerance Interval Non-GM Reference Varieties (Entries F–N) [c] (Lower–Upper)	Comparison t-test A vs D [d] p-value	Comparison t-test A vs E [d] p-value
Isoflavones (mg/kg DW)							
Total Daidzein	850 ± 217	712 ± 199	709 ± 203	380 - 1696	0 - 1788	<0.001	<0.001
Total Genistein	808 ± 211	690 ± 192	703 ± 211	428 - 1498	236 - 1582	<0.001	0.002
Total Glycitein	160 ± 29.0	136 ± 32.2	134 ± 18.2	102 - 350	24 - 320	0.004	0.002
Total Isoflavones	1818 ± 436	1538 ± 396	1546 ± 421	947 - 3455	364 - 3506	<0.001	<0.001
Lectins (mg/g DW)	2.54 ± 0.71	2.44 ± 0.67	2.43 ± 0.76	0.78 - 4.64	0.23 - 4.41	0.587	0.579
Phytic Acid (% DW)	1.45 ± 0.25	1.53 ± 0.23	1.60 ± 0.25	0.88 - 2.11	0.77 - 2.26	0.079	0.001
Raffinose (% DW)	1.02 ± 0.23	1.03 ± 0.26	0.99 ± 0.21	0.40 - 1.38	0.39 - 1.40	0.798	0.583
Stachyose (% DW)	3.18 ± 0.18	3.18 ± 0.18	3.19 ± 0.25	2.59 - 4.50	2.40 - 4.53	0.970	0.890
Trypsin Inhibitor (TIU/mg DW)	35.8 ± 5.66	35.9 ± 4.74	36.3 ± 5.88	18.1 - 41.8	15.1 - 44.4	0.949	0.657

[a] Composition samples were derived from eight field trials conducted in USA in 2017.
[b] Range of results from nine reference varieties (entries F–N).
[c] 99% Tolerance Interval: Range of reference varieties based on tolerance intervals specified to contain 99% of the population with 95% confidence.
[d] t-Test p-value: Pairwise comparison to the non-GM counterpart (Entry A).

(b) Information on the range of natural variation for each constituent measured to allow for assessment of biological significance should any statistically significant differences be identified

The OECD "Revised consensus document on compositional considerations for new varieties of soybean (Glycine max L. Merr.): Key food and feed nutrients, anti-nutrients, toxicants and allergens" (2012) provides the required information on natural variation for each constituent measured within the compositional studies to allow assessment of biological significance should any statistically significant differences be identified in the above studies by FSANZ.

(c) The levels of any other constituents that may potentially be influenced by the genetic modification, as a result, for example, of downstream metabolic effects, compared with the levels in an appropriate comparator as well as the range of natural variation.

Other than the intended presence of the Cry14Ab-1 and HPPD-4 proteins in GMB151 soybean, food products derived from GMB151 soybean have been shown to be compositionally and nutritionally similar to products derived from commercial varieties of non-transgenic soybean (see Section B.5(a) directly above).

(d) The levels of any naturally occurring allergenic proteins in the GM food compared with the levels in an appropriate comparator. Particular attention must be paid to those foods that are required to be declared when present as an ingredient, and where significant alterations to protein content could be reasonably anticipated.

Refer to Section A.2 (b) (i) above for information on endogenous allergenic proteins within soybean that are relevant to GMB151 soybean.

Part C Information Related to the Nutritional Impact of the Food Produced Using Gene Technology

The application must contain the following information if the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the food produced using gene technology compared to the non-GM counterpart food:

(a) Data are required on the anticipated dietary intake of the GM food in relation to the overall diet, together with any information which may indicate a change to the bioavailability of the nutrients from the GM food

Based on the composition analysis, where nutrient and anti-nutrient levels were found to be similar between the non-GM conventional counterpart and the GMB151 soybean grain and processed fraction samples, no analysis of dietary intake in relation to the overall diet is required as bioavailability of the nutrients from GMB151 soybean derived foods is expected to be similar to bioavailability of nutrients from non-GM soybean derived foods.

(b) Where the GM food contains an intended nutritional change, information, such as clinical trial data, must be provided to determine the nutritional impact of the GM food.

Not applicable.

Part D Other Information

There is no requirement to conduct animal feeding or whole food toxicity studies on the food produced using gene technology. However, if a 90-day (or longer) whole food toxicity study in rodents has been provided to satisfy the data and information requirements of another jurisdiction, this should also be provided to FSANZ as additional supporting information.

A 90-day whole food toxicity study in rodents is available for GMB151 soybean ([redacted] 2018, 00021233, Appendix 30). It has been provided within this dossier as additional supporting information.

LITERATURE CITED

Copies of the documents listed below can be provided on request.

Agaisse, H. and Lereclus, D., 1995, How Does *Bacillus thuringiensis* Produce So Much Insecticidal Crystal Protein? *J. of Bacteriology*, pp. 6027–6032.

Allison RF., Dougherty WG., Parks TD., Willis L., Johnston RE., Kelly M., Armstrong FB. (1985). Biochemical analysis of the capsid protein gene and capsid protein of tobacco etch virus: N-terminal amino acids are located on the virion's surface. Virology, 147(2), 309-316.

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

Aronson, A. I. 1993. The two faces of *Bacillus thuringiensis*: insecticidal proteins and post-exponential survival. Mol. Microbiol. 7:489–496.

Astwood, J.D. (1996) Stability of food allergens to digestion *in vitro*. *Nature Biotechnology* 14, 1269-1273.

Ballmer-Weber, B.K. and Vieths, S. (2008) Soy allergy in perspective. *Current opinion in allergy and clinical immunology* 8(3):270-275.

Beltrán, E., Fenet, H., Cooper, F. and Coste, C.-M. (2003) Fate of isoxaflutole in soil under controlled conditions. *Journal of Agricultural and Food Chemistry* 51:146-151.

Bernstein, I.L., Bernstein, J.A., Miller, M., Tierzieva, S., Bernstein, D.I., Lummus, Z., Selgrade, M.K., Doerfler, D.L., Seligy, V.L. (1999) Immune responses in farm workers after exposure to *Bacillus thuringiensis* pesticides. *Environmental Health Perspectives* 107, 575-582.

Besler, M., Helm, R.M. and Ogawa, T. (2000) Allergen data collection update: soybean (*Glycine max*). Internet Symposium on Food Allergens, 2000, 2 (Suppl 3) 435. Available at: http://www.food-allergens.de/symposium-2-2/soybean/soy-abstract.htm

Betz, F.S., Hammond, B.G., Fuchs, R.L. (2000) Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. *Regulatory Toxicology and Pharmacology* 32, 156-173.

Bjorksten, B., Crevel, R., Hischenhuber, C., Lovik, M., Samuels, F., Strobel, S., Taylor, S.L., Wal, J.M., Ward, R. 2008. Criteria for identifying allergenic foods of public health importance. *Regul. Toxicol. Pharmacol*. 51, 42–52.

Bossis, E., Lemanceau, P., Latour, X. and Gardan, L. (2000) The taxonomy of *Pseudomonas fluorescens* and *Pseudomonas putida*: current status and need for revision. *Agronomie* 20:51-63.

Bradshaw, R.A.; Brickley, W.W. and Walker, K.W. (1998) N-Terminal processing: the methionine aminopeptidase and Nα-acetyl transferase families. *Trends Biochem. Sci*. 23, 263-267.

Bravo A., Gill S.S. and Soberón M. (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 49, 423–435.

Bravo A., Gómez I., Porta H., García-Gómez B.I., Rodriguez-Almazan C., Pardo L. and Soberón M. (2013) Evolution of *Bacillus thuringiensis* Cry toxins insecticidal activity. Microbial Biotechnology 6, 17–26.

Bundock, P., Hooykaas, P. (1998). Interactions between *Agrobacterium tumefaciens* and Plant Cells. Phytochemical Signals and Plant-Microbe Interaction. Phytochemical Signals and Plant-Microbe Interaction/Recent Advances in Phytochemistry. Chapter 11: 207-229.

Chae, J-H., Ha, B-K., Chung, G., Park, J-E., Park, E., Ko, J-M., Shannon, G., Song, J. T., Lee, J-D. (2015) Identification of environmentally stable wild soybean genotypes with high

alpha-linolenic acid concentration, *Crop Science*, Vol 55, 1629-1636, doi:10.2135/cropsci2014.09.0635.

Codex Alimentarius Commission (CAC) (2009) *Codex Alimentarius* Foods derived from modern biotechnology. *Codex Alimentarius* Commission, Rome. 85 pages. ftp://ftp.fao.org/codex/Publications/Booklets/Biotech/Biotech_2009e.pdf

Codex Alimentarius Commission (CAC) (1985, rev. 2018) General Standard for the Labelling of Prepackaged Foods. CXS 1-1985. http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCODEX%2BSTAN%2B1-1985%252FCXS_001e.pdf

Compant, S., Duffy, B., Nowak, J., Clement, C. and Barka, E.A. (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied Environmental Microbiology* 71:4951-4959.

Cooporative Research Centres (CRC) (1983) Handbook of processing and utilization in agriculture, Volume 2, Part 2 - Plant Products. Department of Innovation, Industry, Science and Research, Canberra.

Cornelissen M. and Vandewiele M. (1989). Nuclear transcriptional activity of the tobacco plastid *psb*A promoter. Nucleic Acids Research, **17**, 19-25.

Crickmore N. (2005) Using worms to better understand how *Bacillus thuringiensis* kills insects. Trends Microbiol 13:347–350.

Crickmore N., Zeigler D.R., Feitelson J., Schnepf E., Van Rie J., Lereclus D., Baum J. and Dean D.H. (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiology and Molecular Biology Reviews 62, 807–813.
Cromwell, G. L., 2012. Soybean meal-An exceptional protein source. Soybean Meal InfoCenter, Ankeny, IA.

Damgaard, P.H., Granum, P.E., Bresciani, J., Torregrossa, M.V., Eilenberg, J., Valentino, L. (1997) Characterization of *Bacillus thuringiensis* isolated from infections in burn wounds. *FEMS Immunology and Medical Microbiology* 18, 47-53.

De Groot, M.J.A., Bundock, P., Hooykaas, P.J.J. and Beijersbergen, A.G.M. (1998) *Agrobacterium*-mediated transformation of filamentous fungi. *Nature Biotechology* 16:839–842.

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P., Goodman, H.M. (1982) Nopaline synthase: Transcript mapping and DNA sequence. *Journal of Molecular and Applied Genetics* 1, 561-573. Document no. M-131630-01-2.

Doekes, G., Larsen, P., Sigsgaard, T., Baelum, J. (2004) IgE sensitization to bacterial and fungal biopesticides in a cohort of Danish greenhouse workers: the BIOGART study. *American Journal of Industrial Medicine* 46, 404-407.

Douglas, J.S. (1996) Recommended compositional and nutritional parameters to test in soybean. Technical assessment Services, Washington DC, USA.

Downing, K.J, Leslie, G. and Thomson, J.A. (2000) Biocontrol of the sugarcane borer *Eldana saccharina* by expression of the *Bacillus thuringiensis cry1Ac7* and *Serratia marcescens chiA* genes in sugarcane-associated bacteria. *Applied Environmental Microbiology* 66:2804–2810.

Erdman J.W. 1979. Oilseed phytate: nutritional implications. *J. Am. Oil Chem. Soc*. Vol 56, Pp 736-741.
EFSA (European Food Safety Authority), 2010. EFSA panel on Genetically Modified Organisms (GMO). Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. EFSA J. 8, 1–168.

European Food Safety Agency (EFSA) (2011) Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. EFSA Journal 2011;9(6):2193. 54 pages.
http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/2193.pdf

European Parliament. DIRECTIVE 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. Official Journal of the European Union, pp. L 308/15 – L 308/18, Annex IIIa. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32003L0089&qid=1552450907924&from=EN

Fasina, YO, Classen, HL, Garlich, JD, Swaisgood, HE, Clare, DA, 2003, Investigating the possibility of monitoring lectin levels in commercial soybean meals intended for poultry feeding using steam-heated soybean meal as a model, *Poultry Science,* Vol 82, Issue 4, 1 April, 648–656, https://doi.org/10.1093/ps/82.4.648

Federici, B.A. and Siegel, J.P. (2008) Safety assessment of *Bacillus thuringiensis* and Bt crops used in insect control. In: Food Safety of Proteins in Agricultural Biotechnology. Ed Hammond BG, CRC Press, Boca Raton, FL, 45–102.

Feitelson, J.S., Payne, J., Kim, L. (1992) *Bacillus thuringiensis* insects and beyond. Bio/Technol 10: 271–275.

Fisher, R., Rosner, L. (1959) Toxicity of the microbial insecticide, Thuricide. *Journal of Agricultural Food Chemistry* 7, 686-688.

Fliessbach, A., Winkler, M., Lutz, M.P., Oberholzer, H.R. and Mader, P. (2009) Soil amendment with *Pseudomonas fluorescens* CHA0: lasting effects on soil biological properties in soils low in microbial biomass and activity. *Microbial Ecology* 1-13.

Food and Agriculture Organization/World Health Organization (FAO/WHO) (2001) Evaluation of allergenicity of genetically modified foods. Report of a joint Food and Agriculture Organisation of the United Nations (FAO)/World Health Organisation (WHO) expert consultation on foods derived from biotechnology. Rome, Italy. 35 pages. ftp://ftp.fao.org/es/esn/food/allergygm.pdf

Food Standards Australia New Zealand (FSANZ) (2011) Application A1051 – Food derived from Herbicide-tolerant Soybean line FG72. Safety Assessment Report (Approval). http://www.foodstandards.gov.au/code/applications/pages/applicationa1051food4902.aspx

Fretzdorff, B. and Brümmer, J.-M. (1992) Reduction of phytic acid during breadmaking of whole-meal breads. *Cereal Chemistry* 69, 266–270.

Fritze, I.M., L. Linden, J. Freigang, G. Auerbach, R. Huber, and S Steinbach. 2004. The crystal structures of Zea mays and arabidopsis 4-hydroxyphenylpyruvate dioxygenase. Plant Physiology. 134: 1388-1400.

Fuchs, R.L., Astwood, J.D. (1996) Allergenicity assessment of foods derived from genetically modified plants. *Food Technology* 9, 83-88.

Gershman, M.D., Kennedy, D.J., Noble-Wang, J., Kim, C., Gullion, J., Kacica, M., Jensen, B., Pascoe, N., Saiman, L., McHale, J., Wilkins, M., Schoonmaker-Bopp, D., Clayton, J., Arduino, M. and Srinivasan, A. (2008) Multistate outbreak of *Pseudomonas fluorescens* bloodstream infection after exposure to contaminated heparinized saline flush prepared by a compounding pharmacy. *Clinical Infectious Diseases* 47:1372-1379.

Gibb, A.P., Martin, K.M., Davidson, G.A., Walker, B. and Murphy, W.G. (1995) Rate of growth of *Pseudomonas fluorescens* in donated blood. *Journal of Clinical Pathology* 48:717-718.

Green, M., Heumann, M., Sokolow, R., Foster, L.R., Bryant, R., Skeels, M. (1990) Public health implications of the microbial pesticide *Bacillus thuringiensis*: an epidemiological study, Oregon, 1985-86. *American Journal of Public Health* 80, 848-852.

Grefen C., Donald N., Hashimoto K., Kudla J., Schumacher K., Blatt MR. (2010). A ubiquitin-10 promoter-based vector set for fluorescent protein tagging facilitates temporal stability and native protein distribution in transient and stable expression studies. *The Plant Journal for Cell and Molecular Biology*, 64(2), 355-365.

Griffitts J.S., Huffman D.L., Whitacre J.L., Barrows B.D., Marroquin L.D., Müller R., Brown J.R., Hennet T., Esko J.D. and Aroian R.V. (2003) Resistance to a bacterial toxin is mediated by removal of a conserved glycosylation pathway required for toxin-host interactions. Journal of Biological Chemistry 278, 45594–45602.

Griffitts J.S. and Aroian R.V. (2005) Many roads to resistance: How invertebrates adapt to Bt toxins. Bioessays 27, 614–624.

Haas, D. and Defago, G. (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Review Microbiology* 3:307-319.

Hamid, M., Siddiqui, I.A. and Shahid Shaukat, S. (2003) Improvement of *Pseudomonas fluorescens* CHA0 biocontrol activity against root-knot nematode by the addition of ammonium molybdate. *Letters in Applied Microbiology* 36:239-244.

Hartman, G. L., West, E. D., Herman, T. K. (2011) Crops that feed the World 2. Soybean worldwide production, use, and constraints caused by pathogens and pests, Food Security, Vol 3, Issue 1, 5-17.

Harwood, C. R. (1992) *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. Trends Biotechnol. 10:247–256.

Hernandez, E., Ramisse, F., Ducoureau, J.P., Cruel, T., Cavallo, J.D. (1998) *Bacillus thuringiensis* subsp. *konkukian* (serotype H34) superinfection: case report and experimental evidence of pathogenicity in immunosuppressed mice. *Journal of Clinical Microbiology* 36, 2138-2139.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature, 303, 179.

Höfte, H., and H. R. Whiteley (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53:242–255.

Hothersall, J., Wu, J., Rahman, A.S., Shields, J.A., Haddock, J., Johnson, N., Cooper, S.M., Stephens, E.R., Cox, R.J., Crosby, J., Willis, C.L., Simpson, T.J. and Thomas, C.M. (2007) Mutational analysis reveals that all tailoring region genes are required for production of polyketide antibiotic mupirocin by *Pseudomonas fluorescens*: pseudomonic acid B biosynthesis precedes pseudomonic acid A. *Journal of Biological Chemistry* 282:15451-15461.

Huby, R.D., Dearman, R.J., Kimber, I. (1995) Why are some proteins allergens? *Toxicological Sciences* 55, 235-246.

Hui, Y.H. (1992) Encyclopedia of food science and technology. Vol 4. John Wiley and Sons Inc., New York. pp: 2389-2396.
Hungria, Mariangela; Chang, Woo-Suk; Lee, Hae-In. 2015, Chapter 41 Soybean production in the Americas, In Lugtenberg B. (Ed.), Principles of Plant-Microbe Interactions, 393-400, Springer International Publishing Switzerland, DOI 10.1007/978-3-319-08575-3_41

Hymowitz, T. and Newell, C.A. (1981) Taxonomy of the genus Glycine, domestication and uses of soybeans. *Economic Botony* 35:272-288.

Hymowitz, T. 2008, The history of the soybean. In Johnson, L. A., White, P. J., Galloway, R. (Eds.), Soybeans: Chemistry, Production, Processing, and Utilization, 1-31, AOCS Press. https://doi.org/10.1016/B978-1-893997-64-6.50004-4.

Johnson, Lawrence A. and Myers, Deland J. (1995) Chapter 21-Industrial Uses for Soybeans, In Erickson, David R. (Ed.), Practical Handbook of Soybean Processing and Utilization,380-427, Academic Press and AOCS Press.

Joshi, T., Valliyodan, B., Wu, J-H., Lee, S-H., Xu, D., Nguyen, H-T. (2013) Genomic differences between cultivated soybean, G. max and its wild relative G. soja, BMC Genomics, 14 (Suppl 1), S5.

Kaufman H.W., Kleinberg L. (1971) Effect of pH on calcium binding by phytic acid and its inositiol phosphoric acid derivatives and on the solubility of their calcium salts. Archs. Oral Biol. Vol 16, Pp 445-460.

Kay R., Chan A., Daly M., McPherson J. (1987). Duplication of CaMV 35S Promoter Sequences Creates a Strong Enhancer for Plant Genes. *Science* (New York, N.Y.), 236(4806), 1299-1302.

Koch M.S., Ward J.M., Levine S.L., Baum J.A., Vicini J.L. and Hammond B.G. (2015) The food and environmental safety of *Bt* crops. Frontiers in Plant Science 6, 283.

Ladics G.S., Budziszewski G.J., Herman R.A., Herouet-Guicheney C., Joshi S., Lipscomb E.A., McClain S., Ward J.M. (2014) Measurement of endogenous allergens in genetically modified soybeans – Short communication. Reg. Tox. Pharm. 70, 75–79.

Lambert, B. and Peferoen, M. (1992) Insecticidal promise of *Bacillus thuringiensis*. *BioScience* 42:112–122.

Kuo, W-S., Chak, K-F. (1996) Identification of novel *cry*-type genes from *Bacillus thuringiensis* strains on the basis of restriction fragment length polymorphism of the PCR-amplified DNA. *Applied and Environmental Microbiology* 62, 1369-1377.

Lereclus, D., A. Dele´cluse, and M.-M. Lecadet (1993) Diversity of *Bacillus thuringiensis* toxins and genes, p. 37–69. *In* P. Entwistle, J. S. Cory, M. J. Bailey, and S. Higgs (ed.), *Bacillus thuringiensis*, an environmental biopesticide: theory and practice, John Wiley & Sons, Ltd., Chichester, U.K.

Levin, D.B. (2005) Human Health Impact Assessment after Exposures to *Bacillus thuringiensis* subspecies *kurstaki*. In *6th Pacific Rim Conference on the Biotechnology of Bacillus thuringiensis and its Environmental Impact*, Côté, J.C., Otvos, I.S, Schwartz, J.L., Vincent, C. (Eds). Victoria BC, p 61-63.

Li, F.S., 1990, Catalogue of Chinese Wild Soybean Resources. China Agricultural Press, Beijing, China.

Liener, I.E. (1994) Implications of antinutritional components in soybean foods. *Critical reviews in food science and nutrition* 34(1):31-67.
Liu, P.V. (1964) Pathogenicity of *Pseudomonas fluorescens* and related Pseudomonads to warm-blooded animals. *American Journal of Clinical Pathology* 41:150-153.

Maga, J.A. (1982) Phytate: its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis. *Journal of Agricultural and Food Chemistry* 30:1-9.

Matringe M., Sailland A., Pelissier B., Rolland A. and Zink O. (2005) p-hydroxyphenylpyruvate dioxygenase inhibitor-resistant plants. *Pest Management Science*, **61**, 269-273.

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

McKellar, R.C. (1982) Factors influencing the production of extracellular proteinase by *Pseudomonas fluorescens*. *Journal of Applied Bacteriology* 53:305-316.

Mendelsohn, M., Kough, J., Vaituzis, Z., Matthews, K. (2003) Are *Bt* crops safe? *Nature Biotechnology* 21, 1003-1009.

Messina, M. and Messina, V. (1991) Increasing use of soybeans and their potential role in cancer prevention. *Journal of the American Dietary Association* 91:836–840.

Mohankuman, S., Balakrishnan, N. and Samiyappan, R. (2012) Biotechnological and Molecular Approaches in the Management of Pests and Diseases of Crop Plants. In: *Integrated Pest Management: Principles and Practice, D. P. Abrol and U. Shankar, Eds*., pp. 92 – 118.

Munro, I.C., Harwood, M., Hlywka, J.J., Stephen, A.M., Doull, J., Flamm, G.W., Adlercreutz, H. (2003) Soy Isoflavones: a Safety Review, *Nutrition Reviews*, Volume 61, Issue 1, 1 January 2003, Pages 1-33, https://doi.org/10.1301/nr.2003.janr.1-33

National Biodiesel Board, Sustainability Fact Sheet, April 2008.

NC Soybean Producers Association, http://ncsoy.org/media-resources/uses-of-soybeans/, accessed in June 2018.

Nwokolo, E. (1996) Soybean (*Glycine max* (L.) Merr.), In Nwokolo, E., Smartt, J. (Eds.), Food and Feed from Legumes and OilSeeds, 90-102, Springer, Boston, MA.

O'Dell B.L. (1969) Effect of dietary components upon zinc availability. A review with original data. Am. J. Clin. Nutr. Vol 22, Pp 1315-1322.

Organisation for Economic Co-operation and Development (OECD) (1997a) Series on harmonization of regulatory oversight Nº6. Consensus document on information used on the assessment of environmental applications involving *Pseudomonas*. March 4, 1997.

Organisation for Economic Co-operation and Development (OECD) (1997b) Series on harmonization of regulatory oversight in biotechnology No15. Consensus document on the biology of Glycine max (L.) Merr. (soybean). November 14, 2000.

Organisation for Economic Co-operation and Development (OECD) (2001c) Series on the safety of novel foods and feeds No2. Consensus document on compositional considerations for new varieties of soybean: key food and feed nutrients and anti-nutrients. November 30, 2001.

Organization for Economic Co-operation and Development (OECD) (2007) Series on Harmonization of Regulatory Oversight in Biotechnology No. 42. Consensus document on safety information on transgenic plants expressing *Bacillus thuringiensis* - derived insect control proteins. 109 pp.
Organization for Economic Co-operation and Development (OECD) (2012) Revised Consensus Document on Compositional Considerations for New Varieties of Soybean [*Glycine max* (L.) Merr.]: Key Food and Feed Nutrients, Anti-nutrients, Toxicants and Allergens, ENV/JM/MONO(2012)24.

Padgette S. R., Biest-Taylor N., Nida D. L., Bailey M. R., MacDonald J., Holden L. R. and Fuchs R. L. (1996). The Composition of Glyphosate-Tolerant Soybean Seeds Is Equivalent to That of Conventional soybeans. American Institute of Nutrition, 702-716.

Palleroni, N.J. (1981) Introduction to the family *Pseudomonadaceae*. In: Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schegel, H.G. (Eds) *The Prokaryotes - A Handbook on Habitats, Isolation and Identification of Bacteria* Chapter 59. Springer-Verlag, Berlin. pp. 655-665.

Pallett, K.E., Little, J.P., Sheekey, M. and Veerasekaran, P. (1998) The mode of action of isoxaflutole. I. Physiological effects, metabolism and selectivity. *Pesticide Biochemistry and Physiology* 62(2):113-124.

Pallett, K.E., Cramp, S.M., Little, J.P., Veerasekaran, P., Crudace, A.J. and Slater, A.E. (2001) Isoxaflutole: the background to its discovery and the basis of its herbicidal properties. *Pest Management Science* 57:133-142.

Palma L, Muñoz D, Berry C, Murillo J and Caballero P. (2014) *Bacillus thuringiensis* toxins: An overview of their biocidal activity. Toxins 6, 3296–3325.

Pardun, H. (1989). Vegetable lecithins - valuable auxiliary and active substances? *Fat Science Technology* 91(2):45-58.

Pearce, M., Behie, G., Chappel, M. (2002) The effects of aerial spraying with *Bacillus thuringiensis Kurstaki* on area residents. *Environmental Health Review* 1, 19-22.

Peariso, A.M.; Nicholson, K.M.; Jones, R.B.; Green-Church, K.B. and Funk, M.O.Jr. 2007. Electrospray ionization mass spectrometry of soybean lipoxygenases: N-terminal acetylation, chemical modification, and solution conformation. Proteins 70, 650-658.

Peng, R., Xiong, A., Li, X., Fuan, H. and Yao, Q. (2003) A delta-endotoxin encoded in *Pseudomonas fluorescens* displays a high degree of insecticidal activity. *Applied Microbiology and Biotechnology* 63:300-306.

Polevoda, B. and Sherman, F. 2000. N-terminal acetylation of eukaryotic proteins. J.Biol.Chem. 275, 36479-36482.

Polevoda, B. and Sherman, F. 2003. N-terminal Acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. J.Mol.Biol. 325, 595-622
Puckett, A., Davison, G., Entwistle, C.C. and Barbara, J.A. (1992) Post transfusion septicaemia 1980-1989: importance of donor arm cleansing. *Journal of Clinical Pathology* 45:155-157.

Qiu, L-J, Chang, R-Z, 2010, The origin and history of soybean, In Singh G. (Ed.), The soybean: botany, production and uses, 1-23, CAB International, doi:10.1079/9781845936440.0000

Raaijmakers, J.M., de Bruijn, I. and de Kock, M.J. (2006) Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. *Molecular Plant-Microbe Interactactions* 19:699-710.

Ravindran, V., Ravindran, G. and Sivalogan, S. (1994) Total and phytate phosphorus contents of various food and feedstuffs of plant origin. *Food Chemistry* 50:133-136.

Reddy, N. R. and Pierson, M. D., 1994, Reduction in antinutritional and toxic components in plant foods by fermentation. Food Research International 27: 281 – 285.

Rosenquist, H., Smidt, L., Andersen, S.R., Jensen, G.B., Wilcks, A. (2005) Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. *FEMS Microbiology Letters* 250, 129-136.

Rupprecht, J.K., Liu, A., Kelly, I. and Allen, R. (2004) Degradation of RPA 202248 [U-14C-phenyl]alpha(-(cyclopropylcarbonyl)-2-(methylsulfonyl)-beta-oxo-4-(trifluromethyl)benzenepropanenitrile), the primary degradation product of isoxaflutole, in an outdoor aquatic microcosm system. *Journal of Environmental Science and Health B* 39(5-6):725-736.

Samples, J.R., Buettner, H. (1983) Ocular infection caused by a biological insecticide. *Journal of Infectious Disease* 148, 614.

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., Christou, P. (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnology Journal*, 9(3), pp. 283-300.
https://doi.org/10.1111/j.1467-7652.2011.00595.x

Sanfaçon H., Brodmann P., Hohn T. (1991). A dissection of the cauliflower mosaic virus polyadenylation signal. *Genes & Development*, 5(1), 141-149.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J.R. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev*. 62, 705–806.

Schnepf, H. E., Whiteley, H. R. (1981) Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. *Proceedings of the National Academy of Sciences USA* 78**,** 2893-7.

Schoelz, J.E., Bourque, J.E. Plant pararetroviruses (Caulimoviridae). In: Granoff A, Webster RB, eds. Encyclopedia of Virology. San Diego: Academic Press, 1999: 1275–81.

Selb R, Wal JM, Moreno FJ, Lovik M, Mills C, Hoffmann-Sommergruber K, Fernandez A. 2017. Assessment of endogenous allergenicity of genetically modified plants exemplified by soybean - Where do we stand? Food Chem Toxicol. 2017 Mar.101:139-148.

Serna Saldívar, S. O., Avilés‐Gaxiola, S., Chuck‐Hernández, C. 2018, Inactivation Methods of Trypsin Inhibitor in Legumes: A Review. Journal of Food Science, 83: 17-29. doi:10.1111/1750-3841.13985

Shutt, D.A. (1976) The effects of plant estrogens on animal reproduction. *Endeavour* 35**:**110–113.

Sicherer, S. H. and Sampson, H. A. (2006) Food Allergy. *Journal of Allergy and Clinical Immunology* 117:S470–S475.

Siegel, J.P. (2001) The mammalian safety of *Bacillus thuringiensis*-based insecticides. *Journal of Invertebrate Pathology* 77:13-21.

Siehl, D.L. et al. (2014) Broad 4-hydroxyphenylpyruvate dioxygenase inhibitor herbicide tolerance in soybean with an optimized enzyme expression cassette. *Plant Physiology* 166: 1162 – 1176.

Skerman, V.B.D., McGowan, V. And Sneath, P.H.A. (1980) Approved lists of bacterial names. *International Journal of Systematic Bacteriology* 30:225-420. Available at: http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=bacname

Soyconnection, by the United Soybean Board, http://www.soyconnection.com/soybeanoil/soybean-oil-overview, accessed in June 2018.

Stachel S.E., Zambryski P.C. (1986) virA and virG control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. *Cell* 46, 325-333.

Strickberger, M.W. (1976) In *Probability and statistical testing. Genetics*, 2nd ed. New York: Macmillan Publishing Company. pp. 140-163.

Tacconelli, E., Carmeli, Y., Aizer, A., Ferreira, G., Foreman, M.G. and D'Agata, E.M. (2003) Mupirocin prophylaxis to prevent *Staphylococcus aureus* infection in patients undergoing dialysis: a meta-analysis. *Clinical Infectious Disease* 37:1629-1638.

Thompson S.A., Weber C.W. 1979. Influence of pH on the binding of copper, zinc and iron in six fibre sources. J. Food Sci. Vol 44, Pp 752-754.

Thrane, C., Nielsen, M.N., Sorensen, J. and Olsson, S. (2001) *Pseudomonas fluorescens* DR54 reduces sclerotia formation, biomass development, and disease incidence of *Rhizoctonia solani* causing damping-off in sugar beet. *Microbial Ecology* 42:438-445.

Tomlinson, J.A. (1987) Epidemiology and control of vegetable diseases. *Ann. Appl. Biol.*; 110: 661–81.

US Soy, https://ussoy.org/uses-for-soybeans/, accessed in June 2018.

United States Environmental Protection Agency (US-EPA) (1994) *Pseudomonas fluorescens* - Tolerance Exemption 8/94. Available at: http://pmep.cce.cornell.edu/profiles/biopestbiocont/biofung/pseudomo_fluor/pseuflu_exm_0894.html

United States Environment Protection Agency (US-EPA) (2007) Biopesticide active ingredients and products containing them. 39 pages.

United States Environmental Protection Agency (US-EPA) (2008a) Frost-preventing bacteria: *Pseudomonas fluorescens* A506 (006438); *Pseudomonas fluorescens* 1629RS (006439); *Pseudomonas syringae* 742RS (006411) Fact Sheet.

United States Environmental Protection Agency (US-EPA) (2008b) Frost-Preventing Bacteria: *Pseudomonas fluorescens* A506 (006438); *Pseudomonas fluorescens* 1629RS (006439); *Pseudomonas syringae* 742RS (006411) Product pages.

United States Food and Drug Administration (US-FDA) (2012) Guidance for industry. Recommendations for the early food safety evaluations of new non-pesticidal proteins produced by new plant varieties intended for food use. http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Biotechnology/ucm096156.htm#paper

United States Food and Drug Administration (US-FDA) (2004) Food Allergen Labeling and Consumer Protection Act of 2004. https://www.fda.gov/downloads/Food/GuidanceRegulation/UCM179394.pdf
Vaidehi, M.P. and Kadam, S.S. (1989) Soybean. In: Salunkhe, D.K. and Kadam, S.S. (Eds) *Handbook of World Food Legumes: Nutritional Chemistry,Processing Technology and Utilization*. Volume III. CRC Press Inc. Boca Ratón, Florida.

Valadares De Amorim, G., Whittome, B., Shore, B., Levin, D.B. (2001) Identification of *Bacillus thuringiensis* subsp. *kurstaki* strain HD1-Like bacteria from environmental and human samples after aerial spraying of Victoria, British Columbia, Canada, with Foray 48B. *Applied and Environmental Microbiology* 67, 1035-1043.

Vasconcelos, Ilka M. and Oliveira, José Tadeu A., 2004, Antinutritional properties of plant lectins, Toxicon, Vol 44, Issue 4, 385 - 403.

Vohra P., Kratzer F.H. 1966. Influence of various phosphates and other complexing agents on the availability of Zinc for Turkey Poults. J. Nutr. Vol 89, Pp 106-112.

Voisard, C., Keel, C., Haas, D. and Defago, G. (1989) Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. *EMBO Journal* 8:351-358.

Wei J.Z., Hale K., Carta L., Platzer E., Wong C., Fang S.C., Aroian R.V. (2003) *Bacillus thuringiensis* crystal proteins that target nematodes. PNAS 100: 2760–2765.

WHO/IPCS, 1999. Environmental Health Criteria 217. *Bacillus thuringiensis*. World Health Organization, Geneva.

Wilson, M. and Lindow, S.E. (1993) Release of recombinant microorganisms. *Annual Review of Microbiology* 47:913-944.

Zambryski, P. (1988) Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Annual Review of Genetics* 22, 1-30.

Zhang, Z., Schwartz, S., Wagner, L., Miller, W. – 2000 - A greedy algorithm for aligning DNA Sequences, J Comput Biol 2000; 7(1-2):203-14.

Zhu J., Oger P.M., Schrammeijer B., Hooykaas P.J.J., Farrand S.K, Winans S.C. (2000) The Bases of Crown Gall Tumorigenesis. *Journal of Bacteriology* 182, 3885-3895.
Y. L. Zhu, Q. J. Song, D. L. Hyten, C. P. Van Tassell, L. K. Matukumalli, D. R. Grimm, S. M. Hyatt, E. W. Fickus, N. D. Young and P. B. Cregan (2003) Single-Nucleotide Polymorphisms in Soybean, Genetics 163: 1123–1134.