Application to Food Standards Australia New Zealand for the Inclusion of Glyphosate-Tolerant Canola DP-Ø73496-4 in Standard 1.5.2 - Food Produced Using Gene Technology

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OECD Unique Identifier: DP-Ø73496-4

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Release of Information

Pioneer Hi-Bred International, Inc. ("Pioneer") is submitting the information in this assessment for review by the Food Standards Australia New Zealand (FSANZ) as part of the regulatory process. Pioneer holds proprietary rights to the extent allowable by law to all such information and by submitting this information, Pioneer does not authorize its release to any third party except to the extent it is duly requested under the *Freedom of Information Act 1982* (*FOI Act*) or in compliance with the responsibility of FSANZ to publish documents required under sections 8, 8(A), 8(C) and 8(D) of the *FOI Act*; and this information is responsive to the specific aforementioned request. Accordingly, except as specifically stated above, Pioneer does not authorize the release, publication or other distribution of this information (including website posting or otherwise), nor does Pioneer authorize any third party to use, obtain, or rely upon any such information, directly or indirectly, as part of any other application or for any other use, without Pioneer's prior notice and written consent. Submission of this information does not in any way waive Pioneer's rights (including rights to exclusivity and compensation) to such information.

Confidential Commercial Information (CCI)

Indicate any information considered to be confidential commercial information. This information is to be separated from the other parts of the application (both electronically and in hard copy). Submit a formal request according to the requirement of section 4 of the *FSANZ Act*.

The following reports (in the List of Supporting Studies, page 104) is marked as Confidential Commercial information (CCI) and attached as a separate CCI report:

Study 2: PHI-2010-086/040: Sequence Characterization of Insert and Flanking Genomic Regions of Canola Event DP-Ø73496-4 (CCI)

Study 5: PHI-2010-127: Development and Validation of Event-Specific qPCR Detection Method for DP-Ø73496-4 (CCI)

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Abbreviations, Acronyms, and Definitions[a]

~	approximately
1822B	canola donor line used for transformation
1822R	non-transgenic canola line used in breeding
5300B	non-transgenic canola line used in breeding
5536F	non-transgenic canola line used in breeding
6393B	non-transgenic canola line used in breeding
6395B	non-transgenic canola line used in breeding
AACC	American Association of Cereal Chemists
ACY1	Aminoacylase I
ADF	acid detergent fiber
ALS	Acetylactate synthase
AOAC	Association of Official Analytical Chemists
AOF	Australian Oilseeds Federation
APHIS	Animal and Plant Health Inspection Service of USDA
ATCC	American Type Culture Collection
B. licheniformis	*Bacillus licheniformis*
*Bam*H I	Restriction enzyme from *Bacillus amyloliquefaciens*
BAR	phosphinothricin acetyltransferase
B. napus	*Brassica napus* L.
BBCH	Bundesanstalt, Bundessortenamt and Chemical industry
bla (Ap^R)	Ampicillin resistance gene
bp	base pair
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
CI	Confidence interval
CNS	Central nervous system
CPSI	Carbamyl phosphate synthetase I
C_T	threshold cycle
CS	Compound symmetry
Da	Dalton
DDE	Daily dietary exposure
df	degree of freedom
DIG	digoxygenin
DP-Ø73496-4	OECD identifier for herbicide-tolerant canola event
DP-Ø9814Ø-6	OECD identifier for approved herbicide-tolerant maize event
DM	Dry matter
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
E score	expectation score

[a] Note: Abbreviations of units of measurement and of physical and chemical quantities are used according to the standard format described in Instructions to Authors in the Journal of Biological Chemistry (http://www.jbc.org/).

ELISA	enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
EPSPS	enolpyruvylshikimate-3-phosphate synthase
EU	European Union
FAO	Food and Agriculture Organization
FARRP	Food Allergy Research and Resource Program
FDA	Food and Drug Administration
FDR	false discovery rate
FOIA	Freedom on Information Act
GAT	glyphosate acetyltransferase
GAT4621	specific GAT protein
GM canola line 73496	canola lines containing the DP-Ø73496-4 event
GNAT	GCN 5-related N-acetyltransferases
GRAS	Generally recognized as safe
*Hae*III	Restriction enzyme from *Haemophilus aegyptius*
Hind III	Restriction enzyme from Haemophilus influenzae
HPLC	High performance liquid chromatography
HRP	horseradish peroxidase
ICP-OED	Inductively coupled plasma optical emission spectroscopy
IgG	immunoglobulin G
ISAAA	International Service for the Acquisition of Agri-biotech Applications
ISO	International Organization for Standardization
kb	kilobase pair
kDa	kilodalton
LLOQ	lower limit of quantitation
LS-Means	Least squares mean
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
mRNA	Messenger ribonucleic acid
NAA	N-acetylaspartate
NAG	N-acetylglutamate
NAGly	N-acetylglycine
NAGS	N-acetylglutamate synthase
NAS	N-acetylserine
NAT	N-acetylthreonine
NCBI	National Center for Biotechnology Information
Nco I	Restriction enzyme from Nocardia corallina
NDF	neutral detergent fiber
NOAEL	No observed adverse effect level
Not I	Restriction enzyme from Nocardia otitidis-caviarum
OD	optical density
OECD	Organisation for Economic Co-operation and Development
OMAFRA	Ontario Ministry of Agriculture, Food and Rural Affairs
PAT	phosphinothricin acetyltransferase
PBN	Premarket Biotechnology Notice

PCR	polymerase chain reaction
pinII	proteinase inhibitor II
ppm	parts per million
REML	Residual maximum likelihood
SAS	Statistical Analysis Software
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ssp I	Restriction enzyme from Sphaerotilus species
UBQ10	polyubiquitin gene from Arabidopsis thaliana
UPLC	Ultra performance liquid chromatography
USDA	United States Department of Agriculture
USDA-ERS	Economic Research Service of the U.S. Department of Agriculture
USDA-NASS	National Agricultural Statistics Service of the U.S. Department of Agriculture
UV	Ultraviolet

Note: Abbreviations of units of measurement and of physical and chemical quantities are used according to the standard format described in Instructions to Authors in the Journal of Biological Chemistry (http://www.jbc.org/).

Part 1 General Information

Executive Summary

Pioneer Hi-Bred International, Inc. has developed a transgenic canola product that provides tolerance to glyphosate herbicides. This event has an OECD identifier of DP-Ø73496-4 and is hereafter referred to as GM canola line 73496. GM canola line 73496 will provide a market alternative to currently available herbicide-tolerant canola lines. Pioneer is submitting information for review to the FSANZ about the food safety of GM canola line 73496. GM canola line 73496 has been genetically modified to express the GAT4621 (glyphosate acetyltransferase) protein. The *gat4621* gene is a variant of three *gat* genes from the common soil bacterium *Bacillus licheniformis*. The GAT4621 protein is equivalent to the protein expressed in maize event DP-Ø9814Ø-6, approved by FSANZ in May 2010, and is encoded by the *gat4621* gene which confers tolerance to glyphosate-containing herbicides by acetylating glyphosate and thereby rendering it non-phytotoxic. In GM canola line 73496, the expression of the *gat4621* gene is driven by the *Arabidopsis* polyubiquitin constitutive promoter.

GM canola line 73496 was generated using biolistic transformation of canola microspores with a gel-purified fragment isolated from plasmid PHP28181 containing the *gat4621* gene cassette. Molecular characterization of GM canola line 73496 by Southern blot analysis confirmed that a single, intact PHP28181A DNA fragment was inserted into the genome. Southern blot analysis verified the absence of plasmid backbone DNA. Segregation analysis of GM canola line 73496 confirmed Mendelian inheritance of the *gat4621* gene.

The potential for allergenicity and toxicity of GM canola line 73496 was evaluated by examining the allergenic potential of canola as a crop and by assessing the allergenic and toxic potential of the GAT4621 protein. Canola is not a common allergenic food and the modification in GM canola line 73496 is not expected to alter the allergenic potential of canola. The allergenic potential of the GAT4621 protein was assessed using a weight-of-evidence approach using guidance from the Codex Alimentarius Commission. Bioinformatic analyses revealed no biologically significant identities to known or putative protein allergens or toxins for the GAT4621 protein sequences. The GAT4621 protein is non-glycosylated and is rapidly digested (within 30 seconds) in simulated gastric fluid. In simulated intestinal fluid, the GAT4621 protein hydrolyzed within five minutes. There was no evidence of acute toxicity in mice for GAT4621 at a dose of 1640 mg protein per kg of body weight. Based on the GAT4621 protein levels in 73496, exposure levels would be exponentially lower than the tested doses. These data support the conclusion that the GAT4621 protein is unlikely to cause an allergic reaction in humans or be a toxin in humans and therefore support the food safety of GAT4621. Finally, the applications of soybean event DP-356Ø43-5 and maize event DP-Ø9814Ø-6 which also express GAT4621 protein were assessed by FSANZ and concluded no potential health and safety concern in 2009 and 2010, respectively (FSANZ, 2009; FSANZ, 2010).

Extensive nutrient composition analysis of seed was conducted to compare the composition of GM canola line 73496 to that of a near-isoline control and to commercial canola lines. Compositional analysis of GM canola line 73496 was used to evaluate any changes in the concentrations of key nutrients, anti-nutrients and secondary metabolites. Compositional comparability is a general indicator that GM canola line 73496 does not exhibit characteristics of relevance that impact the food safety of these canola lines in comparison to commercially available canola. Based on the compositional evaluation, the seed of GM canola line 73496 was considered to be nutritionally comparable to conventional canola.

Although the primary substrate of the GAT4621 protein is glyphosate, GAT4621 is also known to N-acetylate certain free amino acids (L-aspartate, L-glutamate, glycine, L-serine, and L-threonine) resulting in production of *N*-acetylaspartate (NAA), *N*-acetylglutamate (NAG), *N*-acetylglycine (NAGly), *N*-acetylserine (NAS), and *N*-acetylthreonine (NAT). The efficiency of acetylation of free amino acids by GAT proteins is considerably lower than the activity displayed toward glyphosate. Therefore, the concentrations of these five acetylated amino acids were measured in seed, whole plant, and processed product samples derived from GM canola line 73496. Findings demonstrated elevated concentrations of NAA and NAG, and in some instances NAGly, NAS, and NAT when compared with the corresponding concentrations of these substances in unmodified canola. Low but quantifiable amounts of each *N*-acetylated amino acid were found in each sample type except refined, bleached, deodorized (RBD) oil, where levels were either not detectable or below the limit of quantification. These five acetylated amino acids are not novel substances as they are present in conventional canola as well as in other plants.

Commercial canola seed for consumption is processed into oil and meal fractions. Oil is the major fraction consumed by humans.Processed fractions derived from GM canola line 73496 were analyzed for the presence of NAA, NAG, NAS, NAT, and NAGly. None of these substances were detected in refined, bleached, deodorized oil (the major fraction that is consumed by humans) obtained from GM canola line 73496 ; therefore, the increased concentration of these acetylated amino acids in GM canola line 73496 will not affect human exposure to any of these substances.

Based on the food safety assessment of GM canola line 73496, Pioneer concludes there will be no adverse effects to human health resulting from the consumption of GM canola line 73496. Commercialization of GM canola line 73496 and consumption of oil derived from GM canola line 73496 would be fully consistent with FSANZ's Food Policy and in compliance with all applicable requirements of the Standard 1.5.2 of the*Australia New Zealand Food Standards Code*.

Applicant Details

(a)	Applicant's name/s	[redacted]
(b)	Company/organisation name	Pioneer Hi-Bred Australia
(c)	Address (street and postal)	LMB 9001 204 Wyreema Road, Toowoomba QLD 4350, Australia
(d)	Telephone number	[redacted]
(e)	Email address	[redacted]
(f)	Nature of applicant's business	Pioneer Hi-Bred International, Inc. (Pioneer) is the world's leading developer and supplier of advanced plant genetics and biotechnology products to farmers worldwide. DuPont is a science company, delivering science-based solutions that make a difference in people's lives in food and nutrition, health care, apparel, home and construction, electronics and transportation
(g)	Details of other individuals, companies or organisations associated with the application	Not applicable.

Purpose of the Application

The purpose of this submission is to make an application to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of Glyphosate-tolerant canola DP-Ø73496-4 in **Standard 1.5.2** - *Food Produced Using Gene Technology*.

Pioneer is also seeking from FSANZ an amendment to **Standard 1.4.2** – *Maximum Residue Limits* to harmonise the MRL tolerance for glyphosate on canola food imports with the extend established in the Code of Federal Regulations of the United States of America and the Codex.

Pioneer has developed a transgenic canola product that provides tolerance to glyphosate herbicides. This event has an OECD identifier of DP-Ø73496-4 and is hereafter referred to as GM canola line 73496. This variation would permit the use of food derived from herbicide tolerant GM canola line 73496 developed by Pioneer.

Relevant Overseas Approvals

Food and feed safety assessment of GM canola line 73496 was submitted to the United States Food and Drug Administration (FDA) and Pioneer completed the consultation process on May 1, 2011. The food, feed and environment safety assessment of GM canola line 73496 was also submitted in Canada and approved by Health Canada and Canada Food Inspection Agency on May 4, 2012. Finally, the food and feed assessment of GM canola line 73496 was submitted in Mexico and approved by Department of Health on July 2, 2012.

A petition for the Determination of Non-regulated Status for Herbicide-Tolerant GM canola line 73496 was submitted to the USDA in August 2011. Submission of a tolerance petition and supporting residue data to the U.S. Environmental Protection Agency (EPA) to amend the glyphosate tolerance to include N-acetylglyphosate for canola was submitted in February 2011. In addition, submissions were made to the following regulatory authorities/agencies:

1. European Food Safety Authority (EFSA) for food/feed/import assessment on May 16, 2012.

2. Ministry of Agriculture, Forestry and Fisheries (MAFF) for environmental assessment in Japan on July 10, 2012. Ministry of Health, Labor and Welfare (MHLW) for internal review of food safety assessment in Japan on January 30, 2013; Ministry of Health, Labor and Welfare (MHLW) for internal review of feed safety assessment in Japan on April 26, 2013.

3. Rural Development Administration (RDA) and Korea Food and Drug Administration (KFDA) in Korea on November 29, 2012.

Throughout the remainder of 2013, the necessary submissions for import approvals in key international markets with functioning regualtory systems will continue to be made. To date, no approvals have been rejected or withdrawn. Furthermore, Pioneer is committed to robust product stewardship prior to and continuing after all relevant authorisations are granted.

Justification for the Application

Herbicide-tolerant canola varieties have provided Australian growers with a very useful tool to help manage weeds in their canola crops. Herbicide-tolerant canola varieties have helped to increase the control of weeds, especially herbicide tolerant weeds and reduced the volume of herbicides applied to canola crops for weed control (Brookes and Barfoot, 2010; Duke and Powles, 2009; Smyth *et al*., 2010). Additionally, the yield potential of canola can be optimized by the application of herbicides that remove weeds early in the crops development which compete for soil nutrients and moisture.

Since its introduction to Australia in 2008, the use of glyphosate in glyphosate tolerant canola has become an option as a post-emergence herbicide for canola to control herbicide tolerant weeds that reduce yields. As a broad-spectrum non-selective herbicide, glyphosate is effective

in controlling both annual and perennial grasses and broadleaf weeds, while possessing an excellent environmental profile and low mammalian toxicity.

The GM canola line proposed for this application is named 73496. This canola line was developed with glyphosate-tolerance to provide an alternative to existing herbicide-tolerant canola products currently available in the Australian market. The availability of GM canola line 73496 will provide growers with an additional market choice for herbicide-tolerant canola.

Assessment Procedure

Pioneer Hi-Bred Australia is anticipating that this application will be considered under *General Procedure* for Administrative Assessment process by FSANZ.

Cost-Benefit Effect

The primary objective for the introduction of GM canola line 73496 is to provide Australian canola growers with the choice of an alternative herbicide tolerant weed management system which is based on the GM canola line 73496 providing tolerance to the application of glyphosate herbicide when applied for non-selective post-emergent control of a range of annual and perennial grass and broadleaf weeds in canola.

If the draft variation to permit the sale and use of food derived from GM canola line 73496 is approved by FSANZ the stakeholders who maybe impacted by the release of the GM canola line 73496 are:

- Consumers
- Canola Growers
- Supply Chain
- Government

The potential cost-benefit impact on each of these stakeholders may be tangible and/or intangible, hence quantitative and qualitative considerations must be taken into account in such an assessment. Therefore in establishing these potential impacts an insight as to the quantum of the costs and benefits resulting from the introduction of GM canola line 73496 effects may be gleaned from the impacts resulting from the introduction of previous herbicide tolerant canola crops (GM and Non GM).

1. Consumers

   As Australia is a net exporter of canola, the introduction of GM canola line 73496 will not limit the supply of canola and canola products for the Australian supply chain participants

and the consumer. Following the introduction of the first GM herbicide tolerant canola to Australia in 2008 supply chain participants have retained their right for choice when choosing their source of canola (GM derived or Non GM derived) for export or for processing and consumption in the Australian market.

If the draft variation to permit the sale and use of food derived from GM canola line 73496 is approved by FSANZ supply chain participant and consumer choice would remain as there would be no restriction on the broader availability of canola products derived from GM canola line 73496. The Australia canola oilseed supply chain participants and consumer at all times will retain the right of choice as to the source of food or products which are derived from GM canola line 73496.

2. Canola Growers

Between 2008 and 2011, 1907 canola growers in New South Wales, Victoria and Western Australia have been accredited to grow the first GM herbicide tolerant canola. (AOF, 2007) Canola growers have chosen to plant GM herbicide tolerant canola either as a complimentary weed control system or as an alternate weed control system to current weed control systems because it provides more efficient, effective and economic control of weeds, especially weeds which have developed herbicide tolerance to alternate herbicides applied in canola and other crops within the crop rotation.

Apart from weed control GM herbicide tolerant canola provides increased flexibility in farmer management operations and the potential to increase yields and gross margins as a result of the choice to select GM herbicide tolerant canola in hybrid canola germplasm.

If the draft variation to permit the sale and use of food derived from GM canola line 73496 is approved by FSANZ canola grower's choice as to which weed control systems to adopt would remain given that the varieties containing the GM canola line 73496 would be made available to all canola growers in states where the growing of GM canola is permitted.

Australian canola growers at all times will retain the right of choice as to the source of their weed control system whether that be the current GM and Non GM weed control systems available or herbicide tolerant varieties which contain the GM canola line 73496.

3. Supply Chain

The grains industry recognises that choice must be a priority across the supply chain and that all customers – from farmers to consumers - can use or access the products of their choice.

To ensure continuity in the supply and operations of the canola industry supply chain 25 major grains industry stakeholders agreed to abide by the following principles which would underpin the commercialisation of GM canola are that it will be introduced in a manner that:

> Maintains or enhances trade in Australian canola

> Enables market choice along the supply chain

> Is open and transparent

> Provides confidence to all stakeholders, particularly to customers, consumers and governments.

Since the introduction of GM herbicide tolerant canola in 2008 the canola supply chain has continued to manage the supply, processing and delivery of canola grain and canola products to the domestic and export markets. Between 2008 and 2011 the area of GM canola in New South Wales, Victoria and Western Australia (2010 onwards) increased from 6,908 ha's to 133,333 ha's with the resulting production increasing from 9,936 mt's to 139,089 mt's. (AOF, 2007)

During this period canola industry supply chain stakeholders have provided the domestic and export market, the choice to source GM, Non GM or comingled GM and Non GM canola grain and/or products. The option of choice for sourcing these options of supply has allowed the canola supply chain to continue to operate in a manner which has not inhibited the market.

If the draft variation to permit the sale and use of food derived from GM canola line 73496 is approved by FSANZ the principle of choice on which the commercialisation of GM canola to the supply chain will be maintained. Sectors within the canola supply chain such as grain handlers and marketers, food importers and exporters, distributors, processors and manufacturers will continue to have the option of choice. For those that choose to source GM canola grain and/or products derived from GM canola line 73496 they would have the knowledge that the products were compliant with the FSANZ Code and that as a result market access for both supply and sale is not limited.

If the draft variation to permit the sale and use of food derived from GM canola line 73496 was rejected it would result in the requirement for segregation of any products containing canola GM canola line 73496 from those containing approved canola. This would be likely to increase the costs of domestically produced canola products (e.g. labelling) derived from GM canola line 73496.

The Australia canola supply chain stakeholders at all times will retain the right of choice as to the source of their canola grain and/or products whether that be the current GM and Non GM grain sources or grain from herbicide tolerant varieties which contain the GM canola line 73496.

4. Government

If the draft variation to permit the sale and use of food derived from GM canola line 73496 is approved by FSANZ then in the event that canola grain and/or canola products derived from GM canola line 73496 were detected it would ensure that there is compliance with the FSANZ Code and that there would be no disruption to the trade or the supply chain due to non-compliance with the FSANZ Code.

Part 2 Specific Data Requirements for Safety Assessment

A1 Technical information on the GM food

A1(a) A description of the new GM organism

Pioneer has developed a transgenic canola product that provides tolerance to glyphosate herbicides. This event has an OECD identifier of DP Ø73496-4 and is hereafter referred to as GM canola line 73496. GM canola line 73496 will provide a market alternative to currently available glyphosate-tolerant canola lines.

GM canola line 73496 has been genetically modified to express the GAT4621 (glyphosate acetyltransferase) protein. The *gat4621* gene is a variant of three *gat* genes from the common soil bacterium *Bacillus licheniformis*. The GAT4621 protein is equivalent to the protein expressed in maize event DP-Ø9814Ø-6, approved by FSANZ in May 2010, and is encoded by the *gat4621* gene which confers tolerance to glyphosate-containing herbicides by acetylating glyphosate and thereby rendering it non-phytotoxic. In GM canola line 73496, the expression of the *gat4621* gene is driven by the Arabidopsis polyubiquitin constitutive promoter.

A1(b) Name, number or other identifier of each new line or strain

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants", this event has an OECD identifier of DP-Ø73496-4.

A1(c) The name the food will be marketed under (if known)

In accordance with regulatory approvals, GM canola line 73496 will be marketed as Optimum GLY™ Canola.

A1(d) The types of products likely to include the food or food ingredient

GM canola line 73496 will be utilized for the same uses as currently commercialized canola varieties. Canola seed is grown to produce canola seed, oil and canola meal. Canola oil is a useful vegetable oil used in a range of cooking and manufacturing applications. Canola meal is the dry matter left after the oil is extracted and is a valuable high protein livestock feed supplement.

A2 Description of donor and host organisms

A2(a) Description of all donor organism(s)

A2(a)(i) Common and scientific names and taxonomic classification

B. licheniformis, the source organism for the *gat4621* gene, is used for the production of a number of enzymes such as proteases and amylases that have wide application in the detergent industry. *B. licheniformis* has been used in the United States, Canada, and Europe in the fermentation industry for production of food enzymes (e.g., alpha-amylase, cyclodextrin glycosyltransferase, hemicellulase, proteases, and pullulanase; Rey *et al*., 2004). All *B. licheniformis* cultures available from the American Type Culture Collection (ATCC) are classified as Biosafety Level 1. Items that the ATCC classifies at Biosafety Level 1 have no known history of causing disease in humans or animals based on their assessment of potential risk using U.S. Public Health Service guidelines, with assistance provided by ATCC scientific advisory committees. The taxonomy of *B. licheniformis* is:

Order – Bacillales

Family – Bacillaceae

Genus – *Bacillus*

Species – *B. licheniformis*

A2(a)(ii) Information on pathogenicity, toxicity, allergenicity

B. licheniformis is a bacterium that is commonly found in soil and bird feathers and is part of the subtilis group along with *Bacillus subtilis* and *Bacillus pumilus*. These bacteria are known to cause food poisoning and food spoilage and *B. licheniformis* is known for contaminating dairy products (Salkinoja-Salonen *et al*., 1999). Food borne outbreaks usually involve cases of cooked meats and vegetables, raw milk, and industrially produced baby food contaminated with *B. licheniformis*.

The complete nucleotide sequence of *B. licheniformis* consists of the ATCC 14580 genome that has a circular chromosome of 4,222,336 bp (base pairs) and contains 4,208 predicted protein-coding genes with an average size of 873 bp, 7 rRNA operons, and 72 tRNA genes. The GC content is 46.2% and no plasmids have been detected (Rey *et al*., 2004).

Although derived from *B. licheniformis,* the *gat4621* gene is inserted in isolation from other *B. licheniformis* genes. Further, a Safety Assessment for the *gat4621* gene has been conducted by FSANZ in association with assessment of the dual herbicide-tolerant maize 98140 that contains the *gat4621* gene concluding that no potential public health and safety concerns were identified and that on the basis of the data provided, and other available information, food derived from

maize event DP-Ø9814Ø-6 containing the *gat4621* gene is considered as safe for human consumption as food derived from conventional maize varieties.

The other construct sequences have all been used previously in field releases with negligible risks associated with them.

A2(a)(iii) History of use of the organism in food supply or human exposure

B. licheniformis, the source organism for the *gat4621* gene, is used for the production of a number of enzymes such as proteases and amylases that have wide application in the detergent industry. *B. licheniformis* has been used in the United States, Canada, and Europe in the fermentation industry for production of food enzymes (e.g., alpha-amylase, cyclodextrin glycosyltransferase, hemicellulase, proteases, and pullulanase; Rey *et al.*, 2004). All *B. licheniformis* cultures available from the American Type Culture Collection (ATCC) are classified as Biosafety Level 1. Items that the ATCC classifies at Biosafety Level 1 have no known history of causing disease in humans or animals based on their assessment of potential risk using U.S. Public Health Service guidelines, with assistance provided by ATCC scientific advisory committees.

A2(b) Description of the host organism

A2(b)(i) Phenotypic information

The common name of the parent organism is canola. This term refers to varieties of *Brassica napus* L. that meet specific standards on the levels of the chemicals erucic acid and glucosinolates. The taxonomy of canola is:

Order – Brassicales

Family – Brassicaceae (previously known as Crucifereae)

Genus – *Brassica* L.

Species – *B. napus* L.

A2(b)(ii) How the organism is propagated for food use

B. napus L. has an extended history of safe use in agriculture. It was cultivated by ancient civilisations in Asia and the Mediterranean. Its use has been recorded as early as 2000BC in India and has been grown in Europe since the 13th century (Colton and Sykes, 1992). Over the last 40 years, canola has become an important crop in most grain growing regions of Australia. Since the first commercial planting of canola in Australia in 1969, the areas sown has increased to an expected 2.39 million ha producing up to 3.13 million tonnes of seed at an average oil content of between 42-43% in 2012 (AOF, 2012).

The commercial propagation of canola is through either open pollinated or hybrid seed production techniques. The production of high purity canola seed can be classified into two catagories, certified seeds and basic seeds, and the certification process is based on the Rules and Directives of the OECD schemes and international Seed Test Association (Smith and Baxter, 2002). Alternatively, farmers may choose to retain seed where the crop has been grown from open pollinated seed. Farmers may then use this open pollinated seed for planting seed in the following year. Over a number of years experiments have demonstrated that canola grown by farmer retained open pollinated seeds may result genetic drift and lead to reductions in oil quality, yield and argonomic performance (Marcroft *et al*., 1999).

Within crop rotations, canola crops provide important weed management options and disease breaks for cereal crops such as wheat and barley. Other advantages of canola in crop rotations are increased machinery efficiency, as it is generally planted and harvested at different times to cereals, as well as providing revenue diversification as a “cash crop” within the rotation. Biology documents on the unmodified plant species, canola (*B. napus* L.), have been published by the OGTR (OGTR, 2011), Canadian Food Inspection Agency (CFIA, 1994) and by the Organization for Economic Co-operation and Development (OECD, 1997). These documents provide background on the biology of *B. napus* including:

- Information on use of canola as a crop plant;
- Taxonomic status of *Brassica;*
- Identification methods;
- Reproductive biology;
- Centres of origin and diversity;
- Crosses, including intra- and inter-specific/genus crosses and gene flow; and
- Agro-ecology, including information about cultivation, volunteers and weediness, soil ecology, and canola-insect interactions.

A2(b)(iii) What part of the organism is used for food

Canola seed is high in oil, which in Australia is typically between 40-42%. The oil component is extracted and used for a range of domestic and industrial uses including:

- Cooking oil
- Spreads and shortening
- Prepared foods
- Cosmetics
- Lubricants, fuels and other industrial applications

Following processing and extraction of the oil, the resulting meal contains protein, fibre, carbohydrates and ash components, together with any residual oil not removed through the oil extraction process. The meal is used for stockfeed rations primarily for pigs, poultry and dairy cattle

Wide-spread use of canola oil as a food is a recent trend. The high erucic acid and glucosinolate content of rapeseed oil and meal limited its use as food for humans and livestock until the 1970's when plant breeders developed low-erucic acid/low-glucosinolate rapeseed varieties and referred to this material as "canola". In 1985, the U.S. FDA granted these varieties with the status "Generally Recognized as Safe" (GRAS) (USDA-ERS, 2010). The first canola varieties with low euric acid and low glucosinolate content were released by the Victorian Dept of Agriculture in 1980.

In Australia, as with other countries, canola is primarily crushed and used as edible oil and livestock meal. Globally canola oil is the third most popular oil after soybean and palm oil (USDA-ERS, 2010). Within Australia Canola is the most sought-after soft oil, and represents approximately 45% of Australia's total soft oils usage. (AOF, 2007) The low saturated fat content of canola oil contributes to the commercial success of canola oil. More recently, the development of high-oleic acid varieties has enabled the use of this vegetable oil for high-temperature frying (USDA-ERS, 2010).

A2(b)(iv) Whether special processing is required to render food safe to eat

Traditional rapeseed (*B. napus* L) is unsuitable as a source of food for either humans or animals due to the presence of two naturally occurring toxicants in the seed, erucic acid and glucosinolates. However, intensive breeding programs in several countries, including Australia, have produced high quality varieties that are significantly lower in these two toxicants. The term 'canola' refers to those varieties of *B. napus* L. that meet specific standards on the levels of erucic acid and glucosinolates. Those cultivars must yield oil low in erucic acid (below 2 percent) and meal low in glucosinolates (total glucosinolates of 30 mmoles/g toasted oil free meal) (Codex, 2005).

A2(b)(v) The significance to the diet in Australia and NZ of the host organism

In 2012/13 the major oilseed rape producers globally were the European Union (18.80m mt), Canada (13.31m mt) and China (12.60m mt) (USDA Foreign Agricultural Service (FAS *Annual Report on World Agricultural Production,* www.fas.usda.gov). Canada is the largest exporter of canola followed by Australia with regular exports of over one million tonnes of canola seed (15-20% of the world's canola trade) to Japan, Europe, China, Pakistan and other international markets annually. (AOF, 2007)

In Australia, canola is an established crop in the medium and high rainfall (400 mm and above) areas of southern Australia, which represents the winter production cereal belt. However the

development of early maturing varieties is expanding growing areas of canola into the low rainfall areas of the wheat belt.

Following the introduction of the first canola varieties in 1980 and being a minor crop prior to the turn of the century, canola is now Australia‟s third largest broad-acre grain crop (after wheat and barley). In 2012 there was 2,390,000 hectares of canola planted in Australia, resulting in a harvest of 3,130,000 tonnes (AOF, 2007).

Australia uses over 600,000 tonnes of oils and fats annually with the vast majority utilised in edible applications. Australian consumer consumption of oils shows a growing preference for healthier products, with an increasing demand for canola and high oleic oils. Soft (or seed) oils are strengthening their share of the total fats and oils market, and now account for over half of all usage. Canola is the most sought-after soft oil, and represents approximately 45% of our total soft oils usage (AOF, 2007).

Australian consumers use canola oil for a range of domestic and industrial uses including:

- Cooking oil
- Spreads and shortening
- Prepared foods
- Meal for dairy, poultry and other livestock
- Cosmetics
- Lubricants, fuels and other industrial applications

A3 The nature of the genetic modification

A3(a) Method used to transform host organism

GM canola line 73496 was produced by biolistic transformation with a *Hind*III/*Not*I fragment, PHP28181A, from plasmid PHP28181.

Microspores were prepared from donor line 1822B and transformed essentially as described (Chen and Tulsieram, 2007). Gold particles coated with the PHP28181A DNA fragment were used for transformation. No additional carrier DNA was used during transformation. Biolistic transformation was carried out as described (Klein *et al*., 1987). Transformed embryogenic microspores were cultured in fresh medium in dark conditions for 10 to 12 days, then under dim light for one to three weeks. Green embryos were transferred to fresh medium and cultured for two weeks at 4℃ and then for 4 weeks at 25℃ in the presence of glyphosate (0.1 mM) to select for glyphosate-tolerant transformants. Germinated shoots or plants were transferred to growth medium supplemented with glyphosate for selection.

Plants that were regenerated from transformation and tissue culture (designated T0 plants) were selected for further characterization by molecular analyses, herbicide efficacy, and agronomic evaluations. Refer to Figure 1 for a schematic overview of the transformation and event development process for GM canola line 73496.

The subsequent breeding of GM canola line 73496 proceeded as indicated in Figure 2 to produce specific generations for the characterization and assessments conducted. Table 1 indicates the breeding generations used for each of the analyses described in this submission.

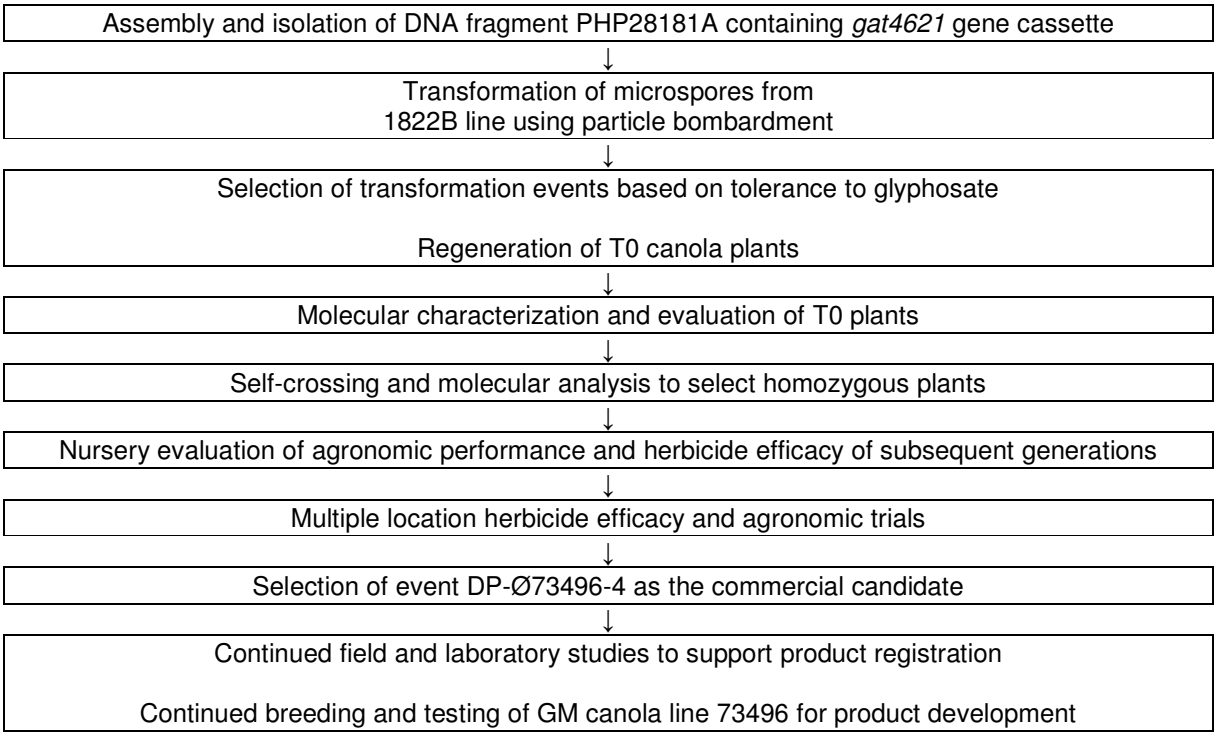

Figure 1. Schematic of the Development of GM canola line 73496

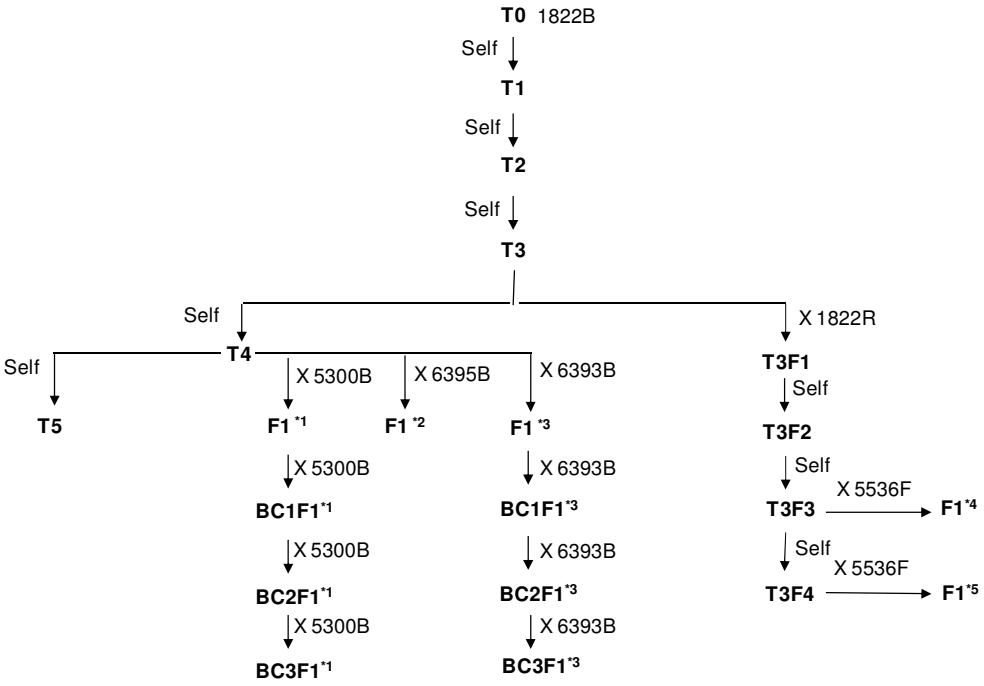

Figure 2. Breeding Diagram for GM canola line 73496 and Generations Used for Analyses

Table 1. Generations and Comparators Used for Analysis of GM canola line 73496

Analysis	**Generation**	**Comparators and Commercial Reference Lines**
Molecular Characterization (A3(d)(i), A3(d)(ii))	T2, T3, F1*2, T3F2, T3F3	1822B, 1822R, and 6395B
Genetic Inheritance (A3(f)(i))	F1*1, *3, BC1F1*1, *3, BC2F1*1, *3, BC3F1*1, *3, T3F2	Not applicable
Concentrations of GAT4621 and Acetylated Amino acids (B3(c) and B7(b))	F1*4	5536F x 1822R (near isoline)
Composition (B7(a))	F1*4	5536F x 1822R (near isoline) and Pioneer commercial lines 46A65, 45H72, 45H73, 46H02, and 44A89

A3(b) Intermediate hosts (eg. bacteria)

GM Canola line 73496 was produced by biolistic transformation with a *Hind*III/*Not*I fragment, PHP28181A, from plasmid PHP28181, without the use of bacteria as intermediate host organism.

A3(c)(i) Gene construct including size, source and function of all elements

gat4621 Gene expression cassette

GM canola line 73496 was produced by biolistic transformation with fragment PHP28181A (Figure 3) that was isolated from plasmid PHP28181 (Figure 4). The genetic elements of plasmid PHP28181 and the transformation fragment PHP28181A are described in Table 2 and Table 3, respectively.

The *gat4621* gene was optimized by a gene shuffling process of glyphosate acetyltransferase genes from *Bacillus licheniformis* (described in section A2(a)(iii)). The GAT4621 protein, encoded by the *gat4621* gene, confers tolerance to glyphosate-containing herbicides by acetylating glyphosate, thereby rendering it non-phytotoxic (Castle *et al*., 2004). The GAT4621 protein is comprised of 147 amino acids and has a molecular weight of approximately 17 kDa (Figure 5) (Castle *et al*., 2004; Siehl *et al*., 2007).

Regulatory elements

The expression of the *gat4621* gene is controlled by the *Arabidopsis thaliana* polyubiquitin promoter (*UBQ10*) (Norris *et al*., 1993). The terminator for the *gat4621* gene is the 3' terminator sequence from the proteinase inhibitor II gene of *Solanum tuberosum* (*pin*II terminator) (An *et al*., 1989; Keil *et al*., 1986).

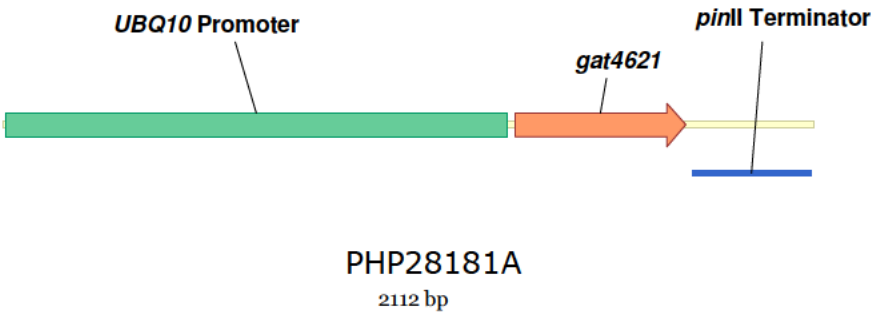

Figure 3. Schematic Diagram of Fragment PHP28181A
Schematic diagram of transformation fragment PHP28181A with the *gat4621* gene and its regulatory elements indicated. The size of the fragment is 2112 base pairs (bp).

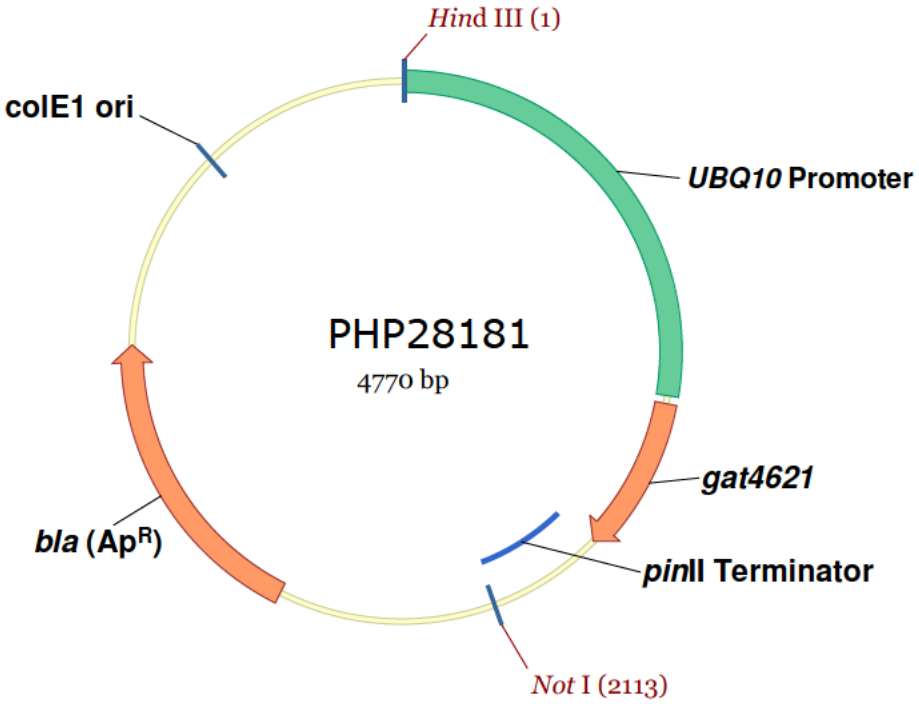

Figure 4. Schematic Diagram of Plasmid PHP28181
Schematic diagram of plasmid PHP28181 indicating the genetic elements. *Hind* III and *Not* I restriction enzyme sites flank the transformation fragment PHP28181A. The size of the plasmid is 4770 bp.

Table 2. Description of Genetic Elements in Plasmid PHP28181

Region	Location on plasmid (base pair position)	Known Genetic Element	Size (base pairs)	Description
Transformation Fragment PHP28181A	1 to 2112		2112	See Table 3 for information on the elements in this region
Plasmid Construct	2113 to 4770	includes elements below	2658	DNA from various sources for plasmid construction and plasmid replication
	2736 to 3596	*bla* (Ap^R)	861	β-lactamase gene coding for ampicillin resistance from *E. coli* (Sutcliiffe, 1978) (Yanisch-Perron, *et al.*, 1985)
	4170 to 4539	colE1 ori	370	*Hae* II fragment containing bacterial origin of replication region (colE1 derived) (Tomizawa *et al.*, 1977)

Table 3. Description of the Genetic Elements in Fragment PHP28181A

Location on Fragment (bp position)	Genetic Element	Size (base pairs)	Description
1 to 7	Polylinker Region	7	Region required for cloning genetic elements
8 to 1312	*UBQ10* Promoter	1305	Version of the promoter region from *Arabidopsis thaliana UBQ10* polyubiquitin gene (Norris *et al.*, 1993) developed by E. I. duPont de Nemours and Company
1313 to 1335	Polylinker Region	23	Region required for cloning genetic elements
1336 to 1779	*gat4621* Gene	444	Synthetic glyphosate *N*-acetyltransferase gene (Castle *et al.*, 2004; Siehl *et al.*, 2007)
1780 to 1796	Polylinker Region	17	Region required for cloning genetic elements
1797 to 2106	*pin*II Terminator	310	Terminator region from *Solanum tuberosum* proteinase inhibitor II gene (An *et al.*, 1989; Keil *et al.*, 1986)
2107 to 2112	Polylinker Region	6	Region required for cloning genetic elements

```
  1     MAIEVKPINA EDTYDLRHRV LRPNQPIEAC MFESDLTRSA FHLGGFYGGK
 51     LISVASFHQA EHSELQGKKQ YQLRGVATLE GYREQKAGSS LVKHAEEILR
101     KRGADMIWCN ARTSASGYYR KLGFSEQGEV FDTPPVGPHI LMYKRIT*
```

Figure 5. Deduced Amino Acid Sequence of the GAT4621 Protein
Deduced amino acid sequence from translation of the *gat4621* gene from plasmid PHP28181. The GAT4621 protein is 147 amino acids in length and has a molecular mass of approximately 17 kDa. The asterisk (*) indicates the translational stop codon.

A3(c)(ii) Detailed map of the location and orientation of all genetic elements

The map of plasmid PHP28181 and the transformation fragment PHP28181A are presented in Figure 3 and Figure 4, respectively. The genetic elements are described in Table 2 and Table 3, respectively.

A3(d)(i) Molecular characterisation including identification of GM elements

Molecular characterization of the inserted DNA evaluates the integrity of the introduced cassette and provides a confirmation that the elements of the expression cassette are intact. Genetic stability is evaluated to confirm the inheritance of the insertion and confirms the stability of the introduced trait through traditional breeding methods. (Refer to Study 1: PHI-2009-134 for details)

The inserted DNA in GM canola line 73496 was characterized by Southern blot analysis to evaluate the integrity and stability of the inserted *gat4621* cassette. As described earlier in section A3(a), GM canola line 73496 was produced by biolistic transformation with fragment PHP28181A. Fragment PHP28181A contains the *gat4621* cassette containing the *UBQ10* promoter, *gat4621* gene, and *pin*II terminator. All probes used for the analysis are indicated on the schematic maps of PHP28181A and PHP28181 (Figures 6 and 7, respectively) and outlined in Table 4. Plasmid PHP28181 was used as a positive control for probe hybridization and to verify fragment sizes internal to PHP28181A. Individual plants of the T2, T3, T3F2, T3F3, and F1[*2] generations (refer to Figure 2 for the breeding diagram) were analysed to determine the copy number of each of the genetic elements inserted into GM canola line 73496 and to verify that the integrity of the PHP28181A fragment was maintained upon integration.

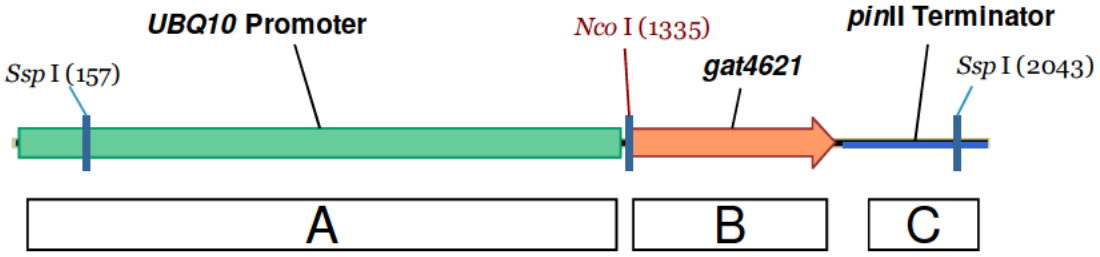

Figure 6. Restriction Enzyme Map of PHP28181A DNA Fragment.
Schematic map of fragment PHP28181A from plasmid PHP28181 used for transformation of canola. Location of genetic elements and *Ssp*I and *Nco*I restriction enzyme sites are indicated. Fragment size is 2112 bp. The locations of the probes used are shown as boxes in the lower part of the map and are identified as follows: A = *UBQ10* promoter probe, B = *gat4621* probe, and C = *pin*II terminator probe.

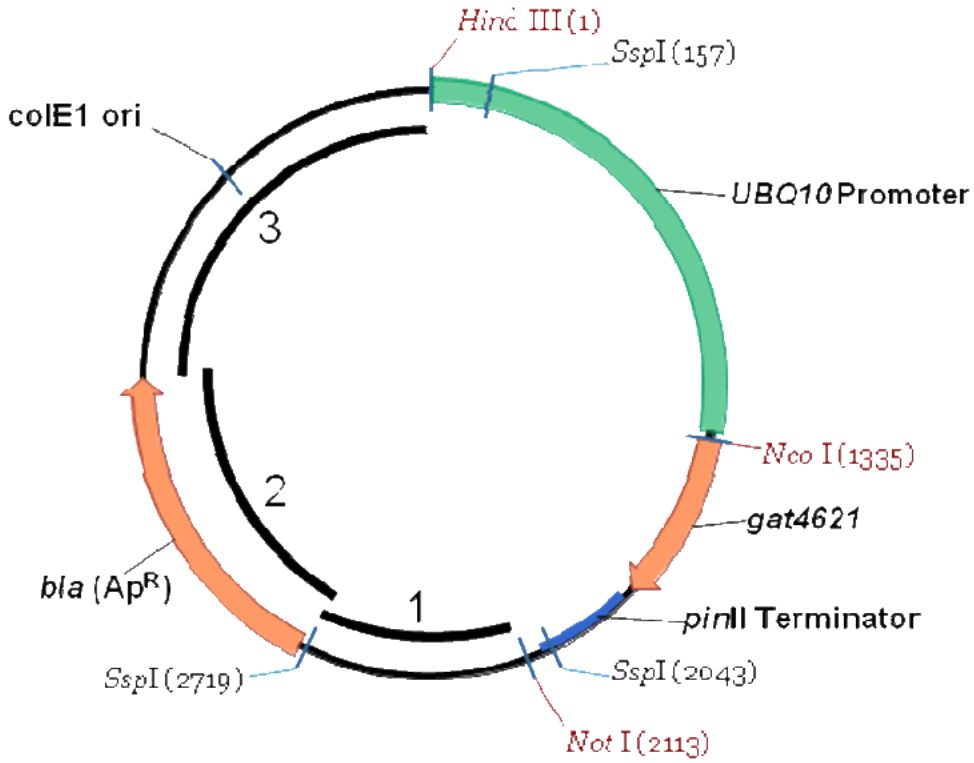

Figure 7. Restriction Enzyme Map of Plasmid PHP28181.
Schematic map of plasmid PHP28181 indicating the location of genetic elements and *Not*I, *Ssp*I, *Nco*I, and *Hind*III restriction enzyme sites. *Hind*III and *Not*I sites flank the fragment PHP28181A used for transformation of canola. The plasmid size is 4770 bp. The black arcs inside the plasmid map indicate the locations of the three probes used to confirm the absence of plasmid backbone sequences and are identified as follows: 1 = Backbone 1 probe, 2 = *bla* (Ap^R) probe, and 3 = Backbone 2 probe.

Table 4. Description of DNA Probes Used for Southern Blot Hybridization

Probe Name	Genetic Element	Figure *Probe*	Position on PHP28181A DNA Fragment (bp to bp)	Position on PHP28181 Plasmid (bp to bp)	Probe Length (bp)
UBQ10 promoter[a]	*UBQ10* promoter	Figure 6 *probe A*	26 to 699 720 to 1286	26 to 699 720 to 1286	674 567
gat4621	*gat4621* gene	Figure 6 *probe B*	1336 to 1770	1336 to 1770	435
*pin*II terminator	*pin*II terminator	Figure 6 *probe C*	1849 to 2082	1849 to 2082	234
Backbone 1[b]	Plasmid between *Not* I and *bla*	Figure 7 *probe 1*	N/A	2120 to 2762	643
bla (Ap^R)[b]	Ampicillin resistance gene	Figure 7 *probe 2*	N/A	2736 to 3566	831
Backbone 2[b]	Plasmid between *bla* and *Hind* III	Figure 7 *probe 3*	N/A	3503 to 4770	1268

N/A: Not Applicable, these are not present on the PHP28181A DNA fragment.
[a] Two non-overlapping segments were generated for this probe and were combined for hybridization.
[b] The backbond probe is comprised of three overlapping labelled fragments that are combined in the hybridization solution
The bp positions provided are the positions of each different segment.

The analyses confirmed that a single, intact PHP28181A DNA fragment was inserted into the genome of GM canola line 73496 (see Section A3(d)(ii)). In addition, these analyses also verified that the inserted DNA remained intact and stably integrated in GM canola line 73496. All five generations demonstrated identical hybridization patterns. These results confirmed the stability of the inserted DNA in GM canola line 73496 across these five breeding generations. Based on these analyses, schematic maps of the inserted DNA in GM canola line 73496 were determined and are provided in Figure 8.

All five generations were also analysed to confirm the absence of plasmid backbone sequence, i.e. the plasmid region outside of isolated fragment PHP28181A. The results verified the absence of these backbone sequences in GM canola line 73496 (see Section A3(d)(ii)(2)).

To confirm inheritance of the insertion through traditional breeding, segregation analysis using genotypic and phenotypic assays were conducted (see Section A3(f)(i)). These results indicated that the inserted DNA and the herbicide-tolerance phenotype in GM canola line 73496 segregate according to Mendel's laws of segregation and are consistent with the finding of a single locus of insertion of the *gat4621* cassette. The stability of the insertion and of the

herbicide-tolerance phenotype was demonstrated in these generations of self- and cross-pollinations.

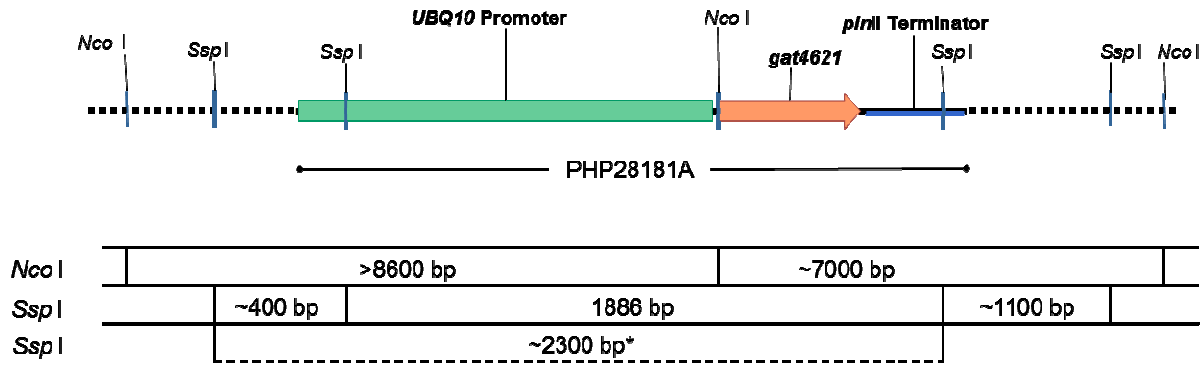

Figure 8. Physical Map of the Insertion in GM canola line 73496.
Schematic map of the insertion with Nco I, and Ssp I, sites indicated. The horizontal dotted line on the upper insertion map represents the genomic regions flanking the inserted PHP28181A fragment. The asterisk (*) and dashed line in the lower band size diagram represent the approximately 2300 bp band that resulted from incomplete digestion at the Ssp I restriction site located in the UBQ10 promoter element. Locations of restriction sites in the canola genome are not to scale.

A3(d)(ii) Determination of number of insertion sites, and copy number

The integration pattern of the insertion in GM canola line 73496 was investigated using Southern blot analysis with *Nco*I digested genomic DNA to determine copy number and with *Ssp*I digested genomic DNA to determine insertion integrity. Copy number and integrity of each genetic element were determined in five generations of GM canola line 73496 using probes specific to the *UBQ10* promoter, *gat4621* gene, and *pin*II terminator (Figure 6 and Table 4). These analyses also verified that the inserted DNA remained intact and stably integrated. In addition, probes to the plasmid backbone region of PHP28181 located outside of the PHP28181A DNA fragment (Figure 7 and Table 4) were used to show that these regions were not transferred to GM canola line 73496.

Based on the Southern blot analyses described below, it was determined that a single, intact PHP28181A DNA fragment was inserted into the genome of GM canola line 73496 as indicated in the insertion map (Figure 8) and that no region from the backbone of plasmid PHP28181 was

inserted. In addition, these results confirmed the stability of the inserted DNA in GM canola line 73496 across five breeding generations.

A3(d)(ii)(1) Insert and copy number of T-DNA in GM canola line 73496

Copy number

The *Nco*I digestion provides information about the number of copies of the PHP28181A DNA fragment that has been integrated into the genome of GM canola line 73496. The PHP28181A DNA fragment contains a single *Nco*I restriction enzyme site at bp position 1335 (Figure 6) and any additional sites would fall in the canola genome outside the fragment sequence. Therefore, hybridization with probes from the *gat4621* cassette would indicate the number of copies of each element found in GM canola line 73496 based on the number of hybridizing bands (e.g. one hybridizing band indicates one copy of the element). As the *Nco*I restriction site is located between the *UBQ10* promoter and *gat4621* gene, the promoter probe is expected to hybridize to a single band of greater than 1300 bp, and the *gat4621* and *pin*II terminator probes are expected to hybridize to a single band of greater than 800 bp (Figure 6). Predicted and observed fragment sizes for GM canola line 73496 with *Nco*I digestion are provided in Table 5.

The *UBQ10* promoter probe was hybridized to *Nco*I-digested genomic DNA from individual GM canola line 73496 plants of the T2, T3, T3F2, T3F3, and F1[*2] generations (Table 5 and Figure 9). A single fragment of greater than 8600 bp was detected in each GM canola line 73496 plant sample (Table 5 and Figure 9), indicating a single copy insertion of the promoter element. The *gat4621* and *pin*II terminator probes were hybridized to the same *Nco*I-digested genomic DNA. Both probes hybridized to the same single band of approximately 7,000 bp in each GM canola line 73496 plant sample (Table 5, Figures 10 and Figure 11).

The presence of single bands for each probe in this Southern blot analysis demonstrated the presence of a single copy of the PHP28181A DNA fragment in GM canola line 73496. The presence of identical hybridization patterns for each probe in all GM canola line 73496 plants of the five generations analysed demonstrated the stability of the DNA insertion during traditional breeding.

Table 5. Summary of Expected and Observed Hybridization Fragments on Southern Blots for GM canola line 73496

Generation	Enzyme Digestion	Expected Fragment Size in DP-Ø73496-4 Canola (bp)[1]	Expected Fragment Size in Plasmid PHP28181 (bp)[2]	Observed Fragment Size in DP-Ø73496-4 Canola (bp)[3]
UBQ10 Promoter Probe				
T2, T3, T3F2, T3F3, and F1*[2] (Figure 9)	*Nco* I	>1300 (border)	4770	>8600
T2, T3, T3F2, T3F3, and F1*[2] (Figure 12)	*Ssp* I	1886 >200 (border)	2208 1886	1886[4,5] ~400
gat4621 Probe				
T2, T3, T3F2, T3F3, and F1*[2] (Figure 10)	*Nco* I	>800 (border)	4770	~7000
T2, T3, T3F2, T3F3, and F1*[2] (Figure 13)	*Ssp* I	1886	1886	1886[4,5]
*pin*II Terminator Probe				
T2, T3, T3F2, T3F3, and F1*[2] (Figure 11)	*Nco* I	>800 (border)	4770	~7000
T2, T3, T3F2, T3F3, and F1*[2] (Figure 14)	*Ssp* I	1886 >100 (border)	1886 676	1886[4,5] ~1100

1. Predicted fragment sizes are based on the map of PHP28181A as shown in Figure 6. Border fragments are those with one restriction enzyme site within the inserted DNA and one site in the flanking maize genome.
2. Predicted fragment sizes are based on the plasmid map of PHP28181 as shown in Figure 7.
3. Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.
4. Observed size of fragment or fragments is presumed to be the same as predicted due to equivalent migration with hybridizing bands in the plasmid (PHP28181) positive control lanes.
5. A faint band is observed at about 2300 bp due to incomplete digestion of the *Ssp* I restriction enzyme site located in the *UBQ10* promoter element in the PHP28181A insertion.

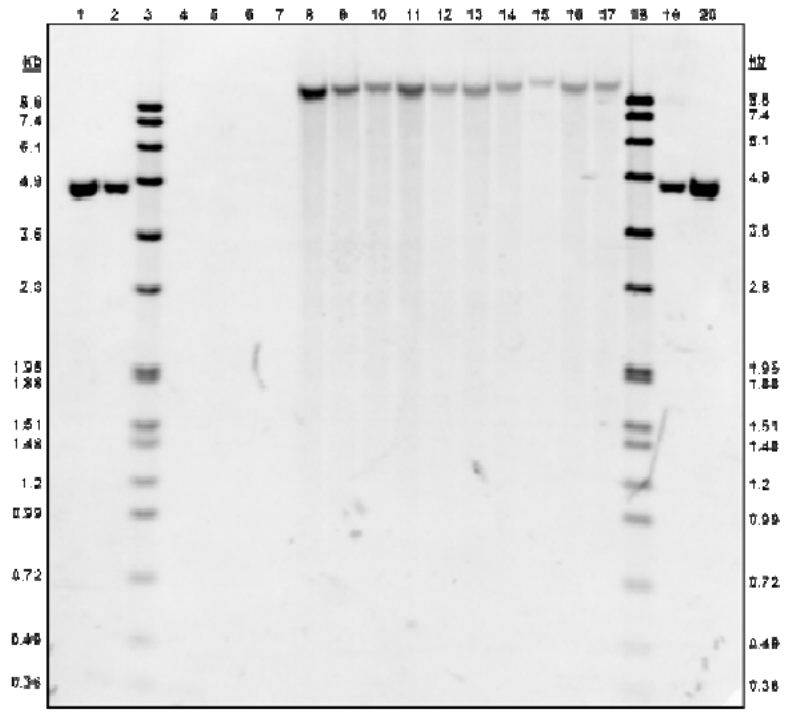

Figure 9. Southern Blot Analysis of GM canola line 73496: *Nco*I Digestion and *UBQ10* Promoter Probe.

Genomic DNA isolated from leaf tissue from individual plants from five generations of GM canola line 73496 and control canola lines was digested with *Nco*I and hybridized to the *UBQ10* promoter probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP28181 and 4 µg of control canola DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Lane	Sample
1	3 copy PHP28181 + 1822R control
2	1 copy PHP28181 + 1822R control
3	DIG VII molecular weight marker
4	1822B control
5	1822R control
6	6395B control
7	Blank
8	GM canola line 73496 / plant 1 (T2 generation)
9	GM canola line 73496 / plant 3 (T2 generation)
10	GM canola line 73496 / plant 7 (T3 generation)

Lane	Sample
11	GM canola line 73496 / plant 8 (T3 generation)
12	GM canola line 73496 / plant 9 (T3F2 generation)
13	GM canola line 73496 / plant 10 (T3F2 generation)
14	GM canola line 73496 / plant 14 (T3F3 generation)
15	GM canola line 73496 / plant 15 (T3F3 generation)
16	GM canola line 73496 / plant 19 ($F1^{*2}$ generation)
17	GM canola line 73496 / plant 20 ($F1^{*2}$ generation)
18	DIG VII molecular weight marker
19	1 copy PHP28181 + 6395B control
20	3 copy PHP28181 + 6395B control

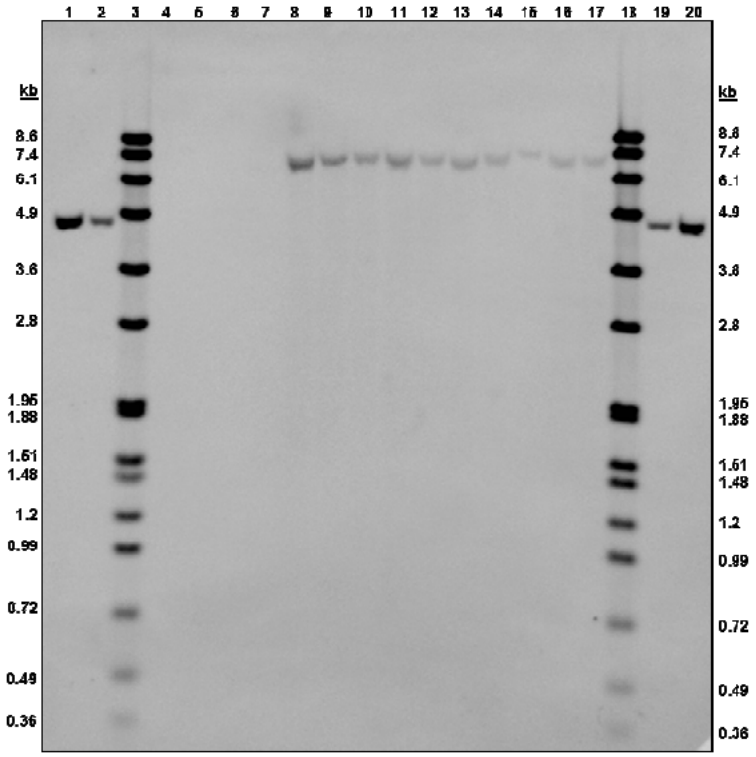

Figure 10. Southern Blot Analysis of GM canola line 73496: *Nco* I Digestion and *gat4621* Probe.

Genomic DNA isolated from leaf tissue from individual plants from five generations of GM canola line 73496 and control canola lines was digested with *Nco*I and hybridized to the *gat4621* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP28181 and 4 µg of control canola DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Lane	Sample
1	3 copy PHP28181 + 1822R control
2	1 copy PHP28181 + 1822R control
3	DIG VII molecular weight marker
4	1822B control
5	1822R control
6	6395B control
7	Blank
8	GM canola line 73496 / plant 1 (T2 generation)
9	GM canola line 73496 / plant 3 (T2 generation)
10	GM canola line 73496 / plant 7 (T3 generation)

Lane	Sample
11	GM canola line 73496 / plant 8 (T3 generation)
12	GM canola line 73496 / plant 9 (T3F2 generation)
13	GM canola line 73496 / plant 10 (T3F2 generation)
14	GM canola line 73496 / plant 14 (T3F3 generation)
15	GM canola line 73496 / plant 15 (T3F3 generation)
16	GM canola line 73496 / plant 19 ($F1^{*2}$ generation)
17	GM canola line 73496 / plant 20 ($F1^{*2}$ generation)
18	DIG VII molecular weight marker
19	1 copy PHP28181 + 6395B control
20	3 copy PHP28181 + 6395B control

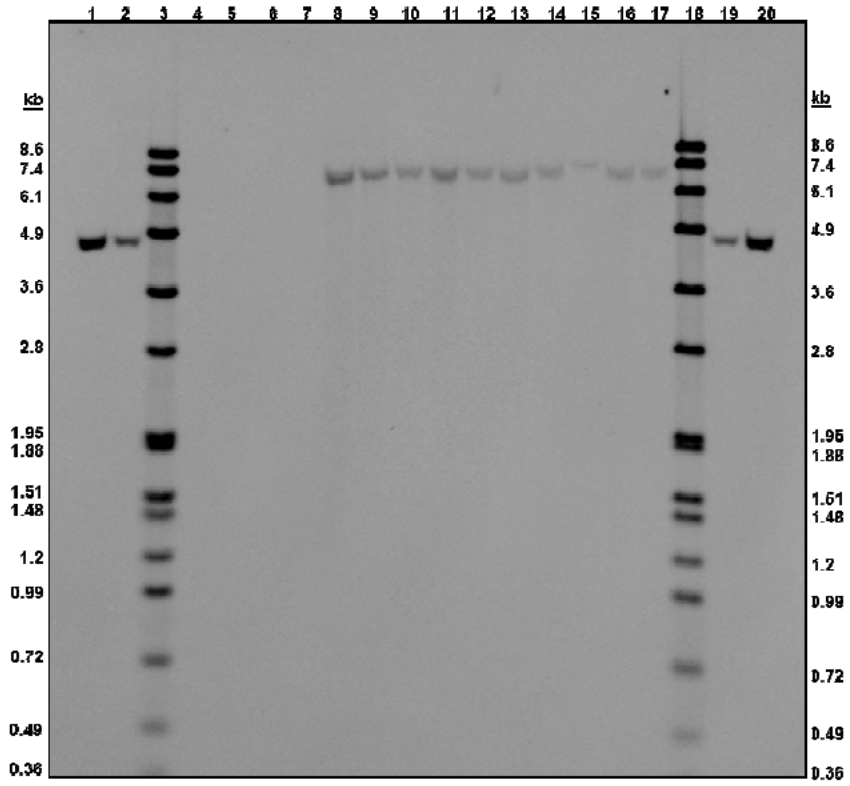

Figure 11. Southern Blot Analysis of GM canola line 73496: *Nco*I Digestion and *pin*II Terminator Probe.

Genomic DNA isolated from leaf tissue from individual plants from five generations of GM canola line 73496 and control canola lines was digested with *Nco*I and hybridized to the *pin*II terminator probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP28181 and 4 µg of control canola DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb).

Lane	Sample	Lane	Sample
1	3 copy PHP28181 + 1822R control	11	GM canola line 73496 / plant 8 (T3 generation)
2	1 copy PHP28181 + 1822R control	12	GM canola line 73496 / plant 9 (T3F2 generation)
3	DIG VII molecular weight marker	13	GM canola line 73496 / plant 10 (T3F2 generation)
4	1822B control	14	GM canola line 73496 / plant 14 (T3F3 generation)
5	1822R control	15	GM canola line 73496 / plant 15 (T3F3 generation)
6	6395B control	16	GM canola line 73496 / plant 19 ($F1^{*2}$ generation)
7	Blank	17	GM canola line 73496 / plant 20 ($F1^{*2}$ generation)
8	GM canola line 73496 / plant 1 (T2 generation)	18	DIG VII molecular weight marker
9	GM canola line 73496 / plant 3 (T2 generation)	19	1 copy PHP28181 + 6395B control
10	GM canola line 73496 / plant 7 (T3 generation)	20	3 copy PHP28181 + 6395B control

Insertion integrity

*Ssp*I digestion was used to verify that the inserted PHP28181A DNA fragment containing the *gat4621* cassette was complete and intact in GM canola line 73496. Two *Ssp*I sites are present within the PHP28181A DNA fragment (bp positions 157 and 2,043; Figure 6). Hybridization with the probes of the *gat4621* cassette confirmed that all the elements were found on the expected internal 1886 bp fragment. Expected and observed fragment sizes for GM canola line 73496 with *Ssp*I are provided in Table 5. All three probes are expected to hybridize to an internal fragment of 1,886 bp (Figure 6). Due to the locations of the *SspI* restriction sites within the *UBQ10* promoter and *pin*II terminator elements, these two probes are also expected to hybridize to additional border fragments (Table 5).

The *UBQ10* promoter, *gat4621*, and *pin*II terminator probes all hybridized to a single insert-derived band of 1886 bp that matched the plasmid control band in each GM canola line 73496 plant sample (Table 5 and Figures 12, 13, and 14). The *UBQ10* promoter probe was expected to hybridize to a border band due to the *SspI* site within the *UBQ10* promoter element. A faint border band of approximately 400 bp was detected with the *UBQ10* promoter probe (Table 5 and Figure 12). A weakly hybridizing border band of approximately 1,100 bp was also detected with the *pin*II terminator probe (Table 5 and Figure 14). The weak band resulted from a border fragment including the portion of the *pin*II terminator located 3' to the *SspI* site at bp 2,043 of PHP28181A (Figure 6) and a second *SspI* site for this fragment located in the canola genome. These hybridizing border bands with the *UBQ10* promoter and *pin*II terminator probes were observed in each GM canola line 73496 plant sample (Figures 12 and Figure 14). An additional faint band due to incomplete digestion by the *Ssp* I enzyme can also be observed at approximately 2,300 bp with all three probes. The fragment responsible for this band results from incomplete digestion at the *Ssp I* site within the *UBQ10* promoter and contains the sequences corresponding to the approximately 400 bp border band and the 1,886 bp internal band.

The presence of the 1,886 bp internal band with all probes and the border bands with the *UBQ10* promoter and *pin*II terminator probes indicate that the PHP28181A fragment was inserted intact into GM canola line 73496. Furthermore, the same hybridization pattern with each probe in the five generations confirmed the stability of the insertion in GM canola line 73496 during traditional breeding.

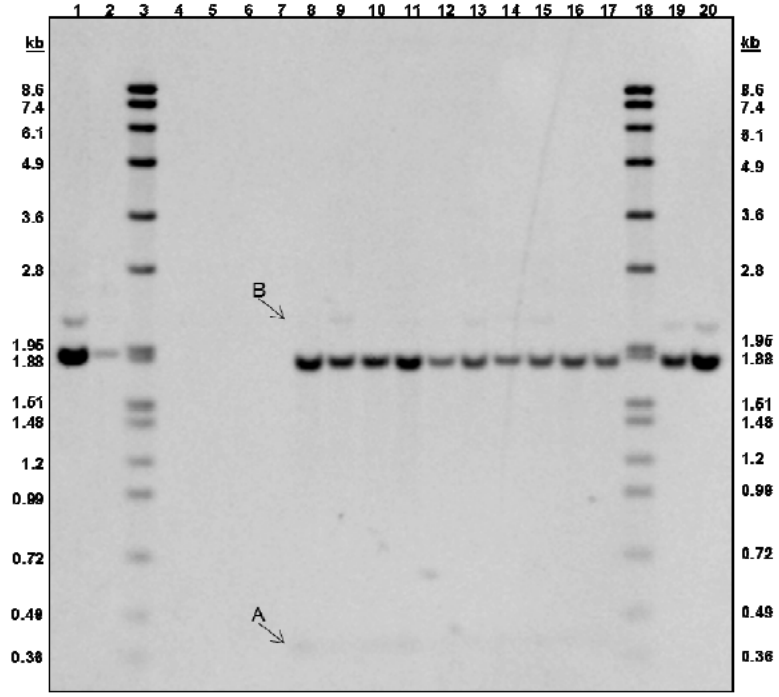

Figure 12. Southern Blot Analysis of GM canola line 73496: *Ssp*I Digestion and *UBQ10* Promoter Probe.

Genomic DNA isolated from leaf tissue from individual plants from five generations of DP-Ø73496-4 canola and control canola lines was digested with *Ssp* I and hybridized with the *UBQ10* promoter probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP28181 and 4 µg of control canola DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). **Note:** A faint band is visible on the x-ray film at about 400 bp in lanes 8 through 17 (arrow A). An additional faint band resulting from incomplete *Ssp* I digestion is visible on the x-ray film at approximately 2.3 kb in lanes 8 through 17 (arrow B).

Lane	Sample
1	3 copy PHP28181 + 1822R control
2	1 copy PHP28181 + 1822R control
3	DIG VII molecular weight marker
4	Blank
5	1822B control
6	1822R control
7	6395B control
8	GM canola line 73496 / plant 1 (T2 generation)
9	GM canola line 73496 / plant 3 (T2 generation)
10	GM canola line 73496 / plant 7 (T3 generation)

Lane	Sample
11	GM canola line 73496 / plant 8 (T3 generation)
12	GM canola line 73496 / plant 9 (T3F2 generation)
13	GM canola line 73496 / plant 10 (T3F2 generation)
14	GM canola line 73496 / plant 14 (T3F3 generation)
15	GM canola line 73496 / plant 15 (T3F3 generation)
16	GM canola line 73496 / plant 19 ($F1^{*2}$ generation)
17	GM canola line 73496 / plant 20 ($F1^{*2}$ generation)
18	DIG VII molecular weight marker
19	1 copy PHP28181 + 6395B control
20	3 copy PHP28181 + 6395B control

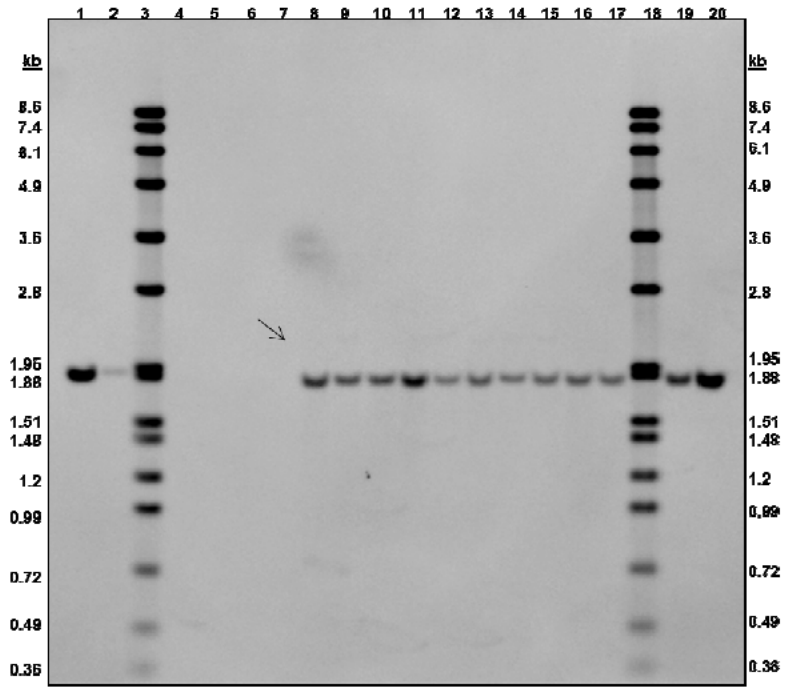

Figure 13. Southern Blot Analysis of GM canola line 73496: *Ssp*I Digestion and *gat4621* Probe.

Genomic DNA isolated from leaf tissue from individual plants from five generations of DP-Ø73496-4 canola and control canola lines was digested with *Ssp* I and hybridized with the *gat4621* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP28181 and 4 µg of control canola DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). A faint band resulting from incomplete *Ssp* I digestion is visible on the x-ray film at approximately 2.3 kb in lanes 8 through 17 (arrow).

Lane	Sample
1	3 copy PHP28181 + 1822R control
2	1 copy PHP28181 + 1822R control
3	DIG VII molecular weight marker
4	Blank
5	1822B control
6	1822R control
7	6395B control
8	GM canola line 73496 / plant 1 (T2 generation)
9	GM canola line 73496 / plant 3 (T2 generation)
10	GM canola line 73496 / plant 7 (T3 generation)

Lane	Sample
11	GM canola line 73496 / plant 8 (T3 generation)
12	GM canola line 73496 / plant 9 (T3F2 generation)
13	GM canola line 73496 / plant 10 (T3F2 generation)
14	GM canola line 73496 / plant 14 (T3F3 generation)
15	GM canola line 73496 / plant 15 (T3F3 generation)
16	GM canola line 73496 / plant 19 ($F1^{*2}$ generation)
17	GM canola line 73496 / plant 20 ($F1^{*2}$ generation)
18	DIG VII molecular weight marker
19	1 copy PHP28181 + 6395B control
20	3 copy PHP28181 + 6395B control

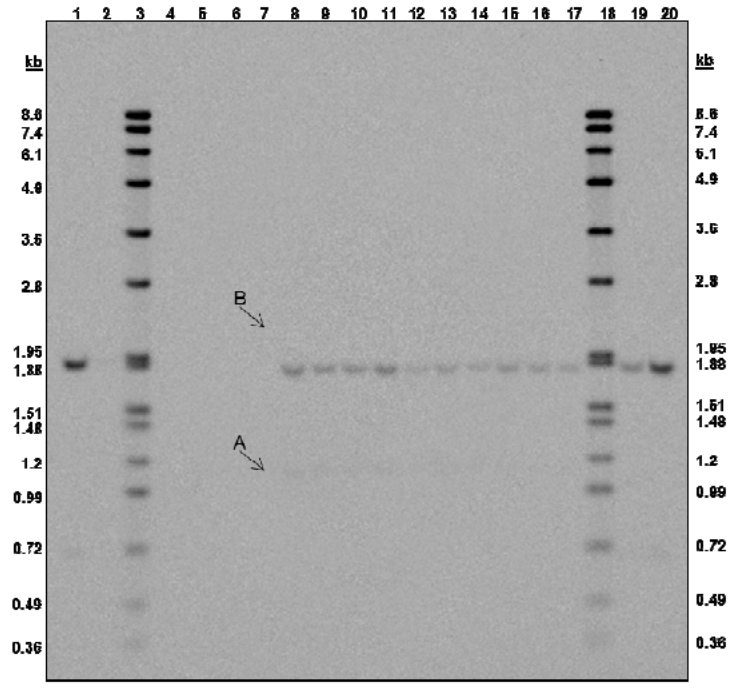

Figure 14. Southern Blot Analysis of GM canola line 73496: *Ssp*I Digestion and *pin*II Terminator Probe.

Genomic DNA isolated from leaf tissue from individual plants from five generations of DP-Ø73496-4 canola and control canola lines was digested with *Ssp I* and hybridized with the *pin*II terminator probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP28181 and 4 µg of control canola DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). Note: A faint band is visible on the x-ray film at about 1.1 kb in lanes 8 through 17 (arrow A). An additional faint band resulting from incomplete *Ssp* I digestion is visible on the x-ray film at approximately 2.3 kb in lanes 8 through 17 (arrow B).

Lane	Sample
1	3 copy PHP28181 + 1822R control
2	1 copy PHP28181 + 1822R control
3	DIG VII molecular weight marker
4	Blank
5	1822B control
6	1822R control
7	6395 control
8	GM canola line 73496 / plant 1 (T2 generation)
9	GM canola line 73496 / plant 3 (T2 generation)
10	GM canola line 73496 / plant 7 (T3 generation)

Lane	Sample
11	GM canola line 73496 / plant 8 (T3 generation)
12	GM canola line 73496 / plant 9 (T3F2 generation)
13	GM canola line 73496 / plant 10 (T3F2 generation)
14	GM canola line 73496 / plant 14 (T3F3 generation)
15	GM canola line 73496 / plant 15 (T3F3 generation)
16	GM canola line 73496 / plant 19 ($F1^{*2}$ generation)
17	GM canola line 73496 / plant 20 ($F1^{*2}$ generation)
18	DIG VII molecular weight marker
19	1 copy PHP28181 + 6395B control
20	3 copy PHP28181 + 6395B control

A3(d)(ii)(2) Southern Blot Analysis to Determine the Presence or Absence of Plasmid Vector

Five generations of GM canola line 73496 were analysed by Southern blot analysis for the presence of DNA sequences from the PHP28181 plasmid backbone. Probes for sequences from the PHP28181 plasmid backbone located outside the PHP28181A DNA fragment were used to determine if any plasmid backbone was inserted in GM canola line 73496 during transformation. Three probes covered the entire plasmid backbone region and were used in a single hybridization solution. These probes would confirm if any region of the backbone outside the PHP28181A DNA fragment was transferred into GM canola line 73496.

Genomic DNA from the T2, T3, T3F2, T3F3, and F1*2 generations were digested with *Nco*I and hybridized to the backbone probes described above. No bands were observed in the GM canola line 73496 or control canola samples, while the expected band of 4770 bp was seen in the plasmid positive control lanes (Figure 15). This confirms that no sequence from the PHP28181 plasmid backbone was inserted into GM canola line 73496 during transformation.

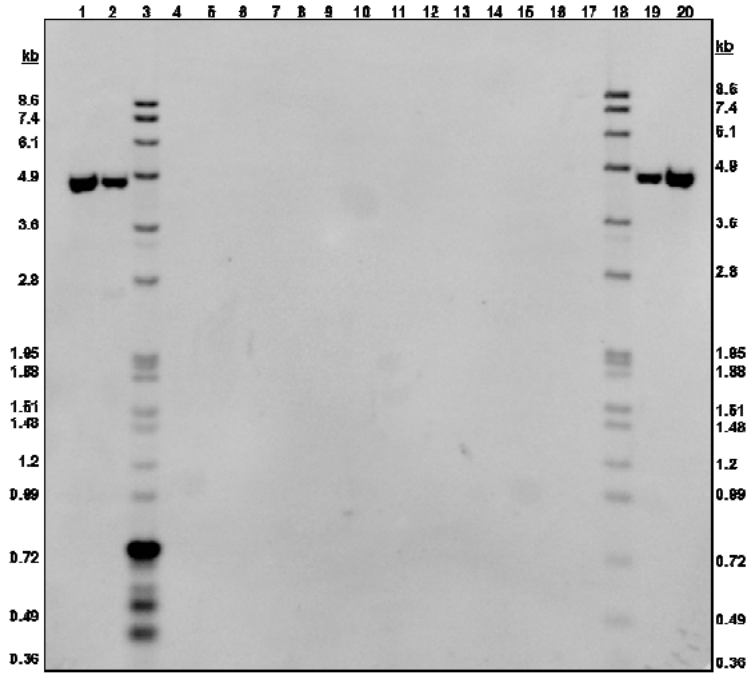

Figure 15. Southern Blot Analysis of GM canola line 73496: *Nco*I Digestion and Plasmid Backbone Probe.

Genomic DNA isolated from leaf tissue from individual plants from five generations of GM canola line 73496 and control canola lines was digested with *Nco*I and hybridized to the combined plasmid backbone probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP28181 and 4 µg of control canola DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). **Note:** Extra bands in Lane 3 near and below the 0.72 kb band are due to hybridization of the probe to the ΦX174 RF DNA/*Hae*III fragments used to monitor gel electrophoresis progress.

Lane	Sample
1	3 copy PHP28181 + 1822R control
2	1 copy PHP28181 + 1822R control
3	DIG VII molecular weight marker
4	1822B control
5	1822R control
6	6395B control
7	Blank
8	GM canola line 73496 / plant 1 (T2 generation)
9	GM canola line 73496 / plant 3 (T2 generation)
10	GM canola line 73496 / plant 7 (T3 generation)

Lane	Sample
11	GM canola line 73496 / plant 8 (T3 generation)
12	GM canola line 73496 / plant 9 (T3F2 generation)
13	GM canola line 73496 / plant 10 (T3F2 generation)
14	GM canola line 73496 / plant 14 (T3F3 generation)
15	GM canola line 73496 / plant 15 (T3F3 generation)
16	GM canola line 73496 / plant 19 ($F1^{*2}$ generation)
17	GM canola line 73496 / plant 20 ($F1^{*2}$ generation)
18	DIG VII molecular weight marker
19	1 copy PHP28181 + 6395B control
20	3 copy PHP28181 + 6395B control

A3(d)(iii) Full DNA sequence, including junction regions, or bioinformatics

Full DNA sequence of the GM canola line 73496 insertion

The sequence of the PHP28181A insert and the 5' and 3' flanking genomic regions in GM canola line 73496 was characterized from 3 overlapping PCR products (Fragments A, B and C) which are summarized in Table 6 and Figure 16. Each fragment was cloned and both sense and anti-sense strands were sequenced. In total, 6,150 bp sequences were generated (Figure 16) with 2,003 bp of the 5' flanking genomic region, 2,109 bp of the insert, and 2,038 bp of the 3' flanking genomic region (confidential sequence). A comparison of the GM canola line 73496 sequence to the PHP28181A transformation fragment revealed two sequences are identical except a three nucleotide deletion occurred at bp position 1-3 of the PHP28181A fragment in GM canola line 73496. (Refer to Study 2: PHI-2010-086/040 for details)

In order to demonstrate that the 5' and 3' flanking genomic regions of the GM canola line 73496 insertion are of canola origin, PCR was performed within these regions in both GM canola line 73496 and control canola plants. In the 5' flanking genomic region, primer pair 10-O-3568/10-O-3580 was used to amplify the canola genomic DNA (Fragment D in Table 6 and Figure 16). In the 3' flanking genomic region, primer pair 10-O-3582/10-O-3388 was used to amplify the canola genomic DNA (Fragment E in Table 6 and Figure 16). The expected PCR products from both fragments were observed (275 bp and 255 bp respectively) in the 73496 and control canola plants (Figure 17). Both fragments were sequenced from one GM canola line 73496 plant and one control plant. Sequences were found to be identical, thus confirming that the 5' and 3' flanking genomic regions in GM canola line 73496 are of canola origin.

Table 6. PCR Primers Used to Characterize the Insert and Flanking Genomic Regions in GM canola line 73496

PCR Fragment	Primer Pair	Size (bp)	Position (5' to 3')	Region
A	10-O-3386/10-O-3388	2452	1914..4365	Entire Insert with part of 5' and 3' Flanking Genomic Regions
B	10-O-3440/10-O-3369	2204	1..2204	5' Flanking Genomic Region and Insert
C	10-O-3357/11-O-4062	2154	4518..6187	Insert and 3' Flanking Genomic Region
D	10-O-3568/10-O-3580	275	1700..1974	5' Flanking Genomic Region
E	10-O-3582/10-O-3388	255	4111..4365	3' Flanking Genomic Region

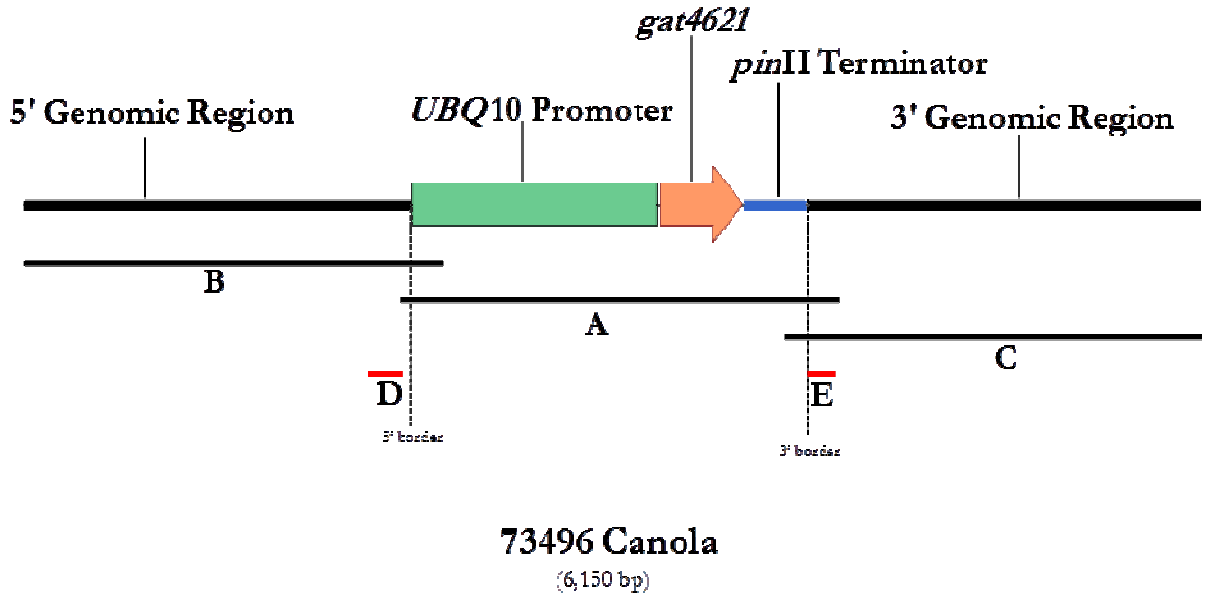

Figure 16. Schematic Representation of the Insert and the 5' and 3' Flanking Genomic Regions Sequenced in GM canola line 73496

PCR fragments shown as black horizontal bars are cloned and sequenced: Fragment A (10-O-3386/10-O-3388), Fragment B (10-O-3440/10-O-3369), and Fragment C (10-O-3357/11-O-4062). PCR fragments generated to demonstrate the canola origin of the 5' and 3' flanking genomic regions are shown as red horizontal bars (D and E). The vertical dashed lines designate the 5' and 3' insert/genomic DNA junction.

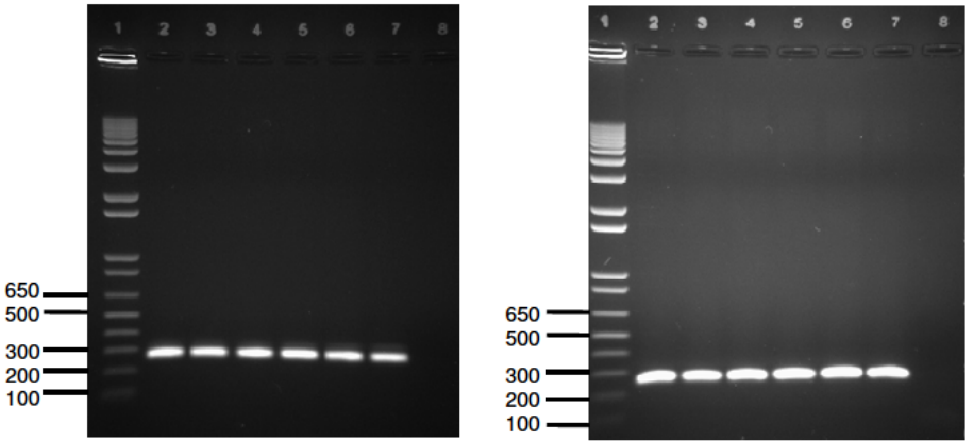

Figure 17. PCR Analysis of GM canola line 73496 5' and 3' Flanking Genomic Regions

Fragment D (10-O-3568/10-O-3580)		Fragment E (10-O-3582/10-O-3388)	
Lane	**Sample**	**Lane**	**Sample**
1	1 kb plus ladder	1	1 kb plus ladder
2	T24 (GM canola line 73496)	2	T24 (GM canola line 73496)
3	T29 (GM canola line 73496)	3	T29 (GM canola line 73496)
4	T38 (GM canola line 73496)	4	T38 (GM canola line 73496)
5	T39 (GM canola line 73496)	5	T39 (GM canola line 73496)
6	C8 (control canola)	6	C8 (control canola)
7	C19 (control canola)	7	C19 (control canola)
8	No template control	8	No template control

A3(d)(iv) Map of the organisation of the inserted DNA (each site)

Based on the Southern blot analysis, it was determined that a single, intact PHP28181A DNA fragment was inserted into the genome of GM canola line 73496. Using these data, a physical map of the inserted DNA in GM canola line 73496 showing the applicable restriction enzymes was developed and is provided in Figure 8 (section A3(d)(ii)). In addition, the sequence of the PHP28181A insert and the 5' and 3' flanking genomic regions in GM canola line 73496 was characterized by PCR and DNA sequence analysis (section A3(d)(iii)). Out of the total 6,150 bp that were sequenced, 2,003 bp were of the 5' flanking genomic region, 2,109 bp were of the PHP28181A insertion, and 2038 bp were of the 3' flanking genomic region. A comparison of the GM canola line 73496 sequence to the PHP28181A transformation fragment revealed a three nucleotide deletion occurred at bp position 1-3 of the PHP28181A fragment in GM canola line 73496. The schematic representation of the insert and the 5' and 3' flanking genomic sequence is shown in Figure 16.

A3(d)(v) Identification and characterisation of unexpected ORFs

In order to accurately assess all stop to stop open reading frames associated with the inserted region comprising the GM canola line 73496 event, the stop codons defining the longest frames extending into both the 5' and 3' genomic border regions were first identified. The external nucleotides within the most distal stop codons (corresponding to nt 1,859 and 4,185 of the 6,150 nt characterized region of the GM canola line 73496 insertion) were next used as endpoints to define the GM canola line 73496 inserted region, which encompasses all sequences capable of producing cross junction frames that lie between the genomic flanks. This 2,327 nt fragment was used to generate six-frame *in silico* start-to-stop codon translations 8 residues in length or greater using a locally installed implementation of the EMBOSS tool GETORF (http://emboss.sourceforge.net) in conjunction with a Perl script (process_ORFs.pl) that parses the data into an Excel table. A total of 40 open reading frames within the GM canola line 73496 defined insertion were identified and translated *in silico* into amino acid sequences. (Refer to Study 3: PHI-2012-283 for details)

These frames were compared to both a dataset of known and putative allergens using defined criteria (FAO/WHO, 2001; Codex Alimentarius Commission, 2003) and to publicly available protein datasets using the BLASTP algorithm in an effort to identify possible similarities to known or putative allergen or toxin proteins. Analysis of these reading frames returned no above threshold matches to known or putative allergens and no alignments to any known or putative toxin proteins, indicating no apparent safety concerns for any possible novel proteins produced as a result of the GM canola line 73496 insertion.

A3(e) Family tree or breeding map

Please refer to section A3(a), Figure 2 for the breeding diagram of GM canola line 73496 .

A3(f)(i) Pattern of inheritance of insert and no. of generations monitored

Inheritance of the inserted DNA and the herbicide-tolerance phenotype in GM canola line 73496 was evaluated to ensure stability of the trait during the plant breeding process and to confirm the trait was at a single genetic locus. Segregating generations of GM canola line 73496 (T3F2, BC1F1^{*1,*3}, BC2F1^{*1,*3}, and BC3F1^{*1,*3}) and one non-segregating generation (F1^{*1,*3}) were evaluated. The breeding history of these five generations is shown in the breeding diagram (Figure 2). Those populations with the superscript "*1" or "*3" designation were populations from two different genetic backgrounds (Figure 2). (Refer to Study 4: PHI-2010-089/010 for details)

Chi-square analysis was conducted on the four segregating generations to determine if the observed segregation ratios were consistent with the expected ratios.

The presence of the 73496 event insertion was determined by event-specific and *gat4621* gene-specific endpoint PCR analyses performed on leaf punches from seedlings of each generation. The herbicide tolerance phenotype was determined by treating the plants with herbicide and by visually evaluating each plant for the presence of herbicide injury. A positive plant exhibited no herbicidal injury and a negative plant exhibited severe herbicide injury.

Results from the segregation analysis are provided in Table 7. For one of the plants, PCR analyses were inconclusive and were unable to be repeated, and therefore were excluded from the statistical analyses. However, this excluded plant was evaluated for the herbicide-tolerance phenotype and this information was reported and analysed. For those plants where both PCR and herbicide-tolerance phenotype data were obtained, all analyses correlated (e.g., plants that were positive by PCR analyses were also tolerant to the herbicide). To confirm that the inserted DNA and the herbicide-tolerance phenotype segregate according to Mendel's laws of genetics, chi-square analysis was performed separately for the PCR and the herbicide-tolerance phenotype data. All P-values were greater than 0.05, with the exception of the BC1F1^{*1,*3} generation of GM canola line 73496, indicating that the observed segregation ratio was consistent with the expected ratio (Table 7).

In the case of the BC1F1^{*1} and BC1F1^{*3} populations, the observed segregation ratios were not consistent with the expected 1:1 (positive: negative) ratio (Table 7) and this was attributed to the hand pollinations that were performed to generate the populations. The BC1F1^{*1} and BC1F1^{*3} populations were cross-pollinated by hand with a control canola plant in order to generate a population segregating 1:1. This process required removal of pollen-producing anthers from several fertile flower buds of a hemizygous GM canola line 73496 plant. It is likely that some of the flower buds were self-pollinated inadvertently, generating a 3:1 segregating population, prior

to the cross-pollination step and resulted in a pool of seed that was a mixture of 3:1 and 1:1 segregating populations. Each of the other GM canola line 73496 generations that were analysed were either derived from the same progenitor as the BC1F1 generation (T3F2), a parent of the BC1F1 generation ($F1^{*1,*3}$), or derived directly from this BC1F1 generation ($BC2F1^{*1,*3}$ and $BC3F1^{*1,*3}$) (Figure 2). The fact that results from these other four generations were consistent with expectations indicate that the $BC1F1^{*1}$ and $BC1F1^{*3}$ populations were not representative of the segregation of the trait and that the insertion is segregating as expected.

These results indicate that the inserted DNA and the herbicide-tolerance phenotype in GM canola line 73496 segregate according to Mendel's laws of segregation and were consistent with the finding of a single locus of insertion of the *gat4621* cassette. The stability of the insertion and of the herbicide-tolerance phenotype was demonstrated in these generations of self- and cross-pollinations.

Table 7. Summary of Genotypic and Phenotypic Results for GM canola line 73496

Generation of GM canola line 73496	Expected Segregation Ratio	Seed Source	Analysis	Observed Values (n=100[a])		Statistical Analysis	
				Positive	Negative	Chi-Square[b]	P-value
T3F2	3:1	T3F2	PCR	69	30	1.4848	0.2230
			Herbicide tolerance assay	69	31	1.9200	0.1659
F1	Non-segregating	F1 *1	PCR	49	1	N/A[c]	
		F1 *3	Herbicide tolerance assay	50	0		
		F1 *1	PCR	49	1		
		F1 *3	Herbicide tolerance assay	50	0		
BC1F1	1:1	BC1F1 *1	PCR	32	18	3.9200	0.0477[d]
		BC1F1 *3	Herbicide tolerance assay	32	18	3.9200	0.0477[d]
		BC1F1 *1	PCR	32	18	3.9200	0.0477[d]
		BC1F1 *3	Herbicide tolerance assay	32	18	3.9200	0.0477[d]
BC2F1	1:1	BC2F1 *1	PCR	29	21	1.2800	0.2579
		BC2F1 *3	Herbicide tolerance assay	26	24	0.0800	0.7773
		BC2F1 *1	PCR	29	21	1.2800	0.2579
		BC2F1 *3	Herbicide tolerance assay	26	24	0.0800	0.7773
BC3F1	1:1	BC3F1 *1	PCR	24	26	0.0800	0.7773
		BC3F1 *3	Herbicide tolerance assay	27	23	0.3200	0.5716
		BC3F1 *1	PCR	24	26	0.0800	0.7773
		BC3F1 *3	Herbicide tolerance assay	27	23	0.3200	0.5716

a n = 99 for statistical analysis of T3F2 PCR results (PCR results for one T3F2 plant were inconclusive and were therefore excluded).

b Degrees of freedom = 1

c Chi-square test is not applicable (N/A) for testing a non-segregating population (i.e. a nominal proportion of 1.0).

d Statistically significant difference, P-value <0.05

A3(f)(ii) Pattern of expression of phenotype over several generations

As shown in section A3(f)(i), the inheritance of the herbicide-tolerance phenotype in GM canola line 73496 was evaluated to ensure stability of the trait during the plant breeding process and to confirm the trait was at a single genetic locus. These results (Table 7) indicate that the inserted DNA and the herbicide-tolerance phenotype in GM canola line 73496 segregate according to Mendel's laws of segregation and were consistent with the finding of a single locus of insertion of the *gat4621* cassette. The stability of the insertion and of the herbicide-tolerance phenotype was demonstrated in these generations of self- and cross-pollinations.

A4 Analytical method for detection

The DNA sequence of the insert and genomic border region in GM canola line 73496 is provided in Supporting Study 2: PHI-2010-086/040. The detection method is provided in Supporting Study 5: PHI-2010-127.

B1 Equivelancy studies

The safety of GAT4621 protein was evaluated and was assessed previously by FSANZ in the consultation of herbicide tolerant maize DP-Ø9814Ø-6 (FSANZ, 2010). That assessment considered the allergenicity and toxicity of the GAT4621 protein, which used a microbial-expressed GAT4621 protein as a means to evaluate protein safety in certain studies. The microbial-expressed GAT4621 protein was characterized for maize event DP-Ø9814Ø-6 and was used to make relevant comparisons to the GAT4621 protein in GM canola line 73496 to demonstrate equivalence and to confirm suitability of bridging to the previous GAT4621 safety assessments.

In order to verify the equivalence of the *in planta* GAT4621 to the microbial-produced protein and confirm the microbial-produced protein was appropriate for the safety assessment studies, a physicochemical characterization of the GAT4621 protein in GM canola line 73496 was conducted. This characterization of the *in planta* GAT4621 protein included the following analyses:

- Molecular weight and immunochemical cross-reactivity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblot analysis
- N-terminal amino acid sequencing to confirm protein identity
- Mass determination and protein identity using tryptic peptide mapping by matrix assisted laser desportion ionization mass spectrometry (MALDI-MS)
- Glycoprotein staining to determine potential post-translational modification (glycosylation)

A detailed description of the methods used in the equivalency studies and the resulting data is provided in section B3(b).

Utilizing the above analyses, the equivalency of GAT4621 protein expressed in *E. coli* to the protein expressed in GM canola line 73496 was demonstrated. Therefore, the GAT4621 protein derived from the microbial expression system was appropriate for utilization in safety assessment studies as a proxy for the GAT4621 protein expressed in GM canola line 73496. Microbial GAT4621 protein was used for *in vitro* digestion and acute toxicology safety assessment studies summarized in sections below. (Refer to Study 6: PHI-2010-020 for detais)

B2 Antibiotic Resistance Marker Genes

As shown in section A3(c)(i), Figure 3, the PHP28181A fragment used to transform GM canola line 73496 does not contain antibiotic resistance marker gene. In addition, Southern blot analysis with plasmid backbone probes in five generations of GM canola line 73496 confirmed that no sequence from the PHP28181 plasmid backbone was inserted into GM canola line 73496 during transformation (Figure 15).

B2(a) Clinical importance of antibiotic that GM is resistant to (if any)

Not applicable.

B2(b) Presence in food of antibiotic resistance protein (if any)

Not applicable.

B2(c) Safety of antibiotic protein

Not applicable.

B2(d) If GM organism is micro-organism, is it viable in final food?

Not applicable.

B3 Characterisation of novel proteins or other novel substances

B3(a) Biochemical function and phenotypic effects of novel protein(s)

The GM canola line 73496 has been genetically modified to express the GAT4621 (glyphosate acetyltransferase) protein and contains a glyphosate acetyltransferase gene variant *gat4621* of three *gat* genes from *Bacillus licheniformis*. The GAT4621 protein is equivalent to the protein expressed in maize event DP-Ø9814Ø-6, approved by FSANZ in May 2010, and is encoded by the *gat4621* gene that confers tolerance to glyphosate-containing herbicides by acetylating glyphosate and thereby rendering it non-phytotoxic (Figure 18).

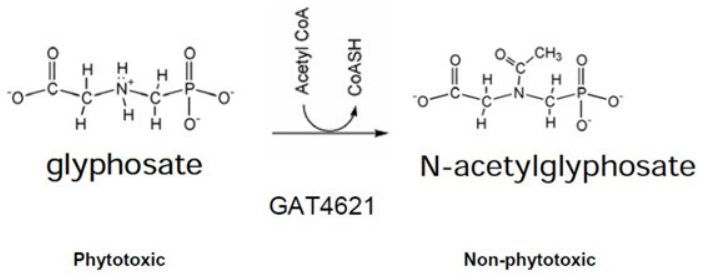

Figure 18. Enzymatic Activity of the GAT4621 Protein

The *gat4621* gene was optimized by a gene shuffling process of *Bacillus licheniformis*. The GAT4621 protein, encoded by the *gat4621* gene, confers tolerance to glyphosate-containing herbicides by acetylating glyphosate, thereby rendering it non-phytotoxic (Castle *et al*., 2004).

DNA/gene shuffling is a process that recombines genetic diversity from parental genes to create libraries of gene variants that are then screened to identify those progeny with improved properties (Stemmer, 1994; Crameri *et al*., 1998). This process of fragmentation and recombination followed by selection can be repeated using those progeny with improved properties as parents for the next round of shuffling. In the case of the *gat4621* gene this process was repeated eleven times using a combination of multi-gene shuffling and the introduction of genetic diversity via PCR.

Glyphosate acetyltransferase (GAT) proteins are members of the GCN 5-related family of *N*-acetyltransferases (also known as the GNAT superfamily). The GNAT superfamily is one of the largest enzyme superfamilies recognized to date with over 10,000 representatives from plants,

animals and microbes. Members of the GNAT superfamily all contain highly conserved GNAT motifs but have high sequence diversity (Vetting *et al*., 2005). The GNAT proteins are known to have a number of metabolic functions including detoxification (Dyda *et al*., 2000). In particular, and as described below, GAT proteins can confer herbicide tolerance by detoxification of the broad-spectrum herbicide glyphosate.

The enzyme *5-enolpyruvylshikimate-3-phosphate synthase* (EPSPS; 3-phophoshikimate 1-carboxyvinyl- transferase; EC2.5.1.19) (Steinrücken and Amrhein, 1980) is the sixth enzyme of the shikimic acid pathway (Figure 19), which is essential for the biosynthesis of aromatic amino acids (L-phenylalanine, L-tyrosine and L-tryptophan) and chorismate-derived secondary metabolites in algae, higher plants, bacteria, and fungi (Kishore and Shah, 1988). EPSPS has been identified as the primary target of glyphosate [*N*- (phosphonomethyl) glycine], which is a non-selective, broad-spectrum, foliar-applied herbicide first commercialized in 1974 and widely used for the management of annual, perennial, and biennial herbaceous species of grasses, sedges, and broadleaf weeds, as well as woody brush and tree species (Baylis, 2000; Bradshaw *et al*., 1997). Mechanisms for conferring tolerance to glyphosate herbicide in genetically engineered plants have included the introduction of microbial variants of EPSPS that are insensitive to glyphosate (e.g., CP4 EPSPS from *Agrobacterium tumefaciens*) or mutated forms of endogenous plant EPSP synthases (e.g., modified EPSPS from *Zea mays*).

GAT proteins provide an alternative mechanism of tolerance to glyphosate by detoxifying glyphosate to the non-phytotoxic form, *N*-acetylglyphosate (Figure 18). This detoxification mechanism is similar to that of the *phosphinothricin acetyltransferase* (PAT or BAR) enzymes from *Streptomyces*, which detoxify glufosinate ammonium herbicides by adding an acetyl group (De Block *et al*., 1987). GAT enzymes acetylate the secondary amine of glyphosate using acetyl coenzyme A as an acetyl donor (Figure 18; Castle *et al*., 2004). Transgenic expression of GAT proteins was shown to confer glyphosate tolerance in several plant species (Castle *et al*., 2004) and is also the basis for previously commercially approved maize (DP-Ø9814Ø-6) (FSANZ, 2010; US-FDA, 2008; Rood, 2007b; USDA-APHIS, 2009) and soybean (DP-356Ø43-5) events (FSANZ, 2009; US-FDA, 2007; Rood, 2006; USDA-APHIS, 2008).

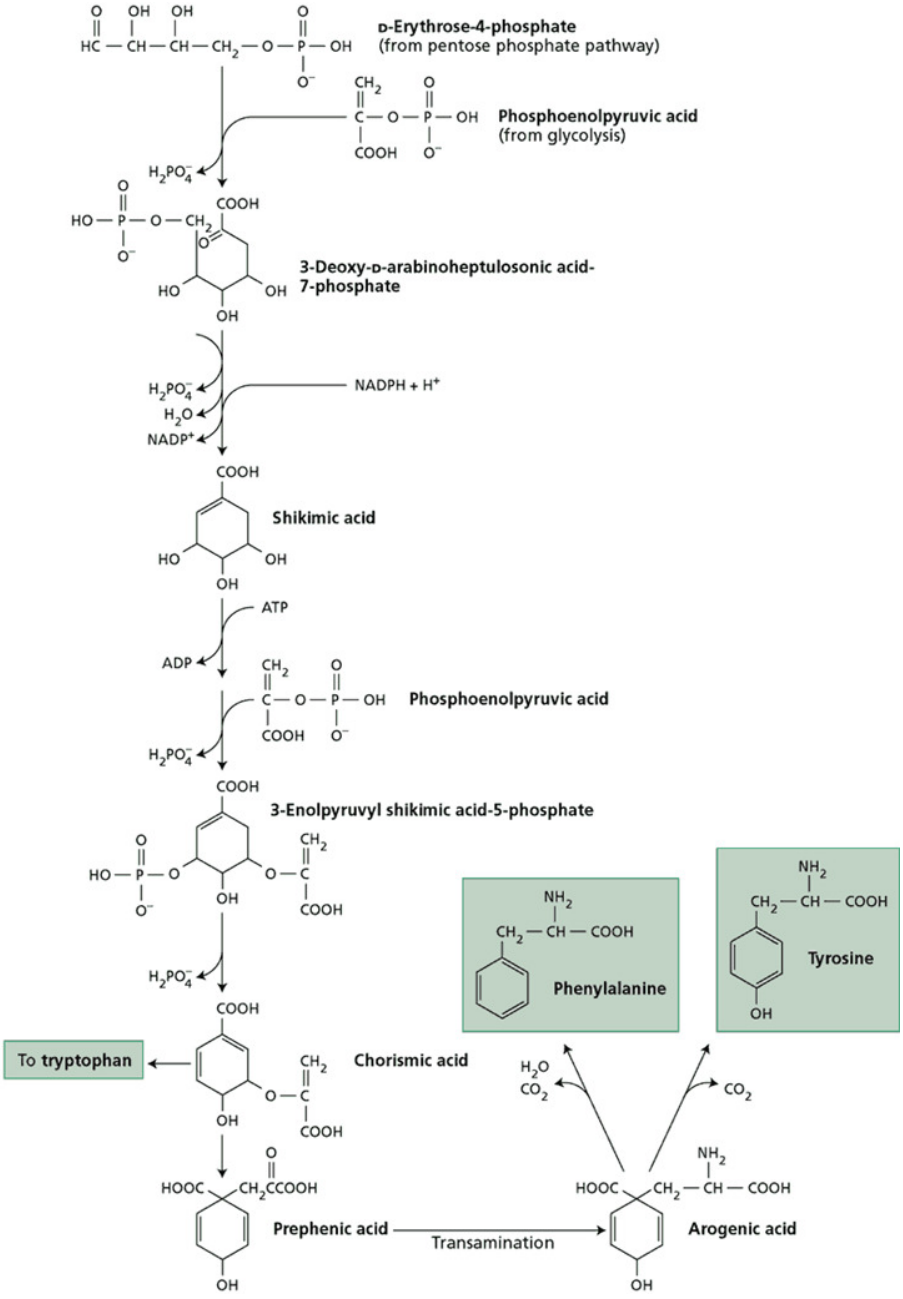

Figure 19. The Shikimic Acid Pathway (Taiz and Zeiger, 2010)

B3(b) Identification of novel substances (e.g. metabolites), levels and site

Characterization of the plant-expressed GAT4621 protein

Physicochemical characterization of the GAT4621 protein isolated from GM canola line 73496 was conducted in order to demonstrate equivalence to the microbial-expressed form of the protein used in acute toxicity testing and digestibility studies previously conducted for maize event DP-Ø9814Ø-6. This characterization of *in planta* expressed GAT4621 protein from GM canola line 73496 included: molecular weight and immunochemical cross-reactivity by SDS-PAGE and western immunoblot analysis; N-terminal amino acid sequencing; and tryptic peptide mapping by MALDI-MS. In addition, the GAT4621 protein from GM canola line 73496 was analyzed for glycosylation as part of the weight of evidence for assessing the potential allergenicity.

In the case of the N-terminal amino acid sequencing and tryptic peptide mapping by MALDI-MS, data obtained previously for the microbial-expressed GAT4621 were used for comparison to the characterization of the GAT4621 protein from GM canola line 73496.

Utilizing these analyses, the equivalency of GAT4621 protein expressed in *E. coli* to the protein expressed in GM canola line 73496 was demonstrated. Therefore, the GAT4621 protein derived from the microbial expression system was appropriate for utilization in safety assessment studies as a proxy for the GAT4621 protein expressed in GM canola line 73496. In addition, the GAT4621 protein produced in GM canola line 73496 is not glycosylated and supports the weight of evidence assessment that the GAT4621 protein is unlikely to be an allergen. (Refer to Study 6: PHI-2010-020 for details)

SDS-PAGE Analysis of the plant-expressed GAT4621 protein

Analysis of GAT4621 protein isolated from GM canola line 73496 by SDS-PAGE followed by staining revealed a prominent band with a relative mobility consistent with the GAT4621 molecular weight of 16.5 kDa (Figure 20).

Based on this analysis, the approximate size of the plant-expressed GAT4621 protein was consistent with the expected theoretical size of the GAT4621 protein.

Western immunoblot analysis of the plant-expressed and microbial-expressed GAT4621 proteins

Western blot analysis of the GAT4621 protein prepared from GM canola line 73496 revealed a predominant immunoreactive band that co-migrated with the microbial-expressed GAT4621 and corresponded to the molecular weight of 16.5 kDa for the GAT4621 protein (Figure 21).

Based on this analysis, the microbial- and the plant-expressed GAT4621 proteins were determined to be equivalent in size and immunoreactivity.

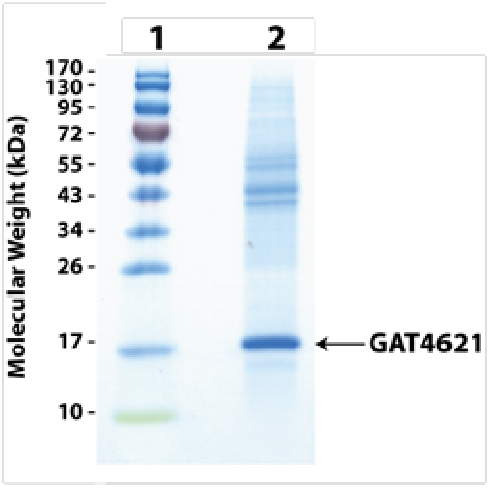

Figure 20. SDS-PAGE Analysis of the Plant-Expressed GAT4621 Protein
A sample of the purified GAT4621 protein (~1 µg total protein) from GM canola line 73496 (lane 2) was analyzed by SDS-PAGE on 10–20% gradient gels followed by staining with GelCode Coomassie Blue reagent. Molecular weight markers (PageRuler Prestained Protein Ladder) were included in lane 1. The major band corresponding to the intact size of the GAT4621 protein is indicated.

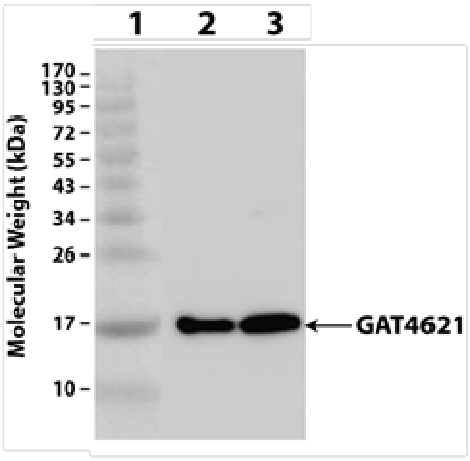

Figure 21. Western Immunoblot Analysis of Plant-Expressed and Microbial-Expressed GAT4621 Proteins
A sample of the purified GAT4621 protein (~10 ng total protein) from GM canola line 73496 (lane 2) and a sample of the microbial-expressed GAT4621 protein (Lot: PCF-0005; ~15 ng; lane 3) were analyzed by SDS-PAGE followed by electroblotting onto PVDF membrane. The blots were probed sequentially with mouse anti-GAT monoclonal antibody and horseradish peroxidase-conjugated anti-mouse IgG. Blots were then incubated in the presence of a chemiluminescent substrate and the signal was detected with Fujifilm Luminescent Image Analyzer. Molecular weight markers (PageRuler Prestained Protein Ladder) were included in lane 1.

N-Terminal amino acid sequence analysis of the plant-expressed and microbial-expressed GAT4621 proteins

The N-terminal sequence analysis of the GAT4621 protein derived from GM canola line 73496 indicated that the primary sequences matched residues 2–14 of the deduced N-terminal sequence of GAT4621 and also matched residues 2-11 of the microbial-expressed protein (Figure 22). In the plant-expressed GAT4621, the lysine signal at position 6 and the aspartic acid signal at position 12, relative to the predicted sequence, were low. The N-terminal methionine residue was not detected, which was consistent with results from MALDI-MS (Applied Biosystems/MDS SCIEX 4800 MALDI-TOF/TOF) tryptic peptide mapping (Figure 23).

Edman sequencing analysis confirmed that the N-terminal sequences of the GM canola line 73496 -derived GAT4621 protein matched both the theoretical protein sequence as well as the microbial-expressed sequences. These results provided additional evidence that the plant- and microbial-expressed GAT4621 proteins are equivalent.

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Theoretical GAT4621	M	A	I	E	V	K	P	I	N	A	E	D	T	Y
Event 73496 GAT4621		A	I	E	V	X[a]	P	I	N	A	E	(D)	T	Y
Microbial GAT4621		A	I	E	V	K	P	I	N	A	E			

Figure 22. N-Terminal Amino Acid Sequence Analysis of Plant-Expressed and Microbial-Expressed GAT4621 Proteins

A sample of purified GAT4621 protein from GM canola line 73496 was analyzed by SDS-PAGE followed by electroblotting onto PVDF membrane. The band corresponding to the GAT4621 protein was excised and subjected to Edman N-terminal sequencing. For the GM canola line 73496 -derived GAT4621 protein, the aspartic acid residue in parentheses at position 12 was assigned based on a low signal. As indicated by X^a, position 6 was not assigned due to a low signal. The theoretical and previously determined N-terminal amino acid sequence for microbial-expressed GAT4621 protein are shown for comparison.

MALDI-MS identification of tryptic peptides

MALDI-MS analysis of the tryptic peptides for GAT4621 isolated from GM canola line 73496 identified 25 unique peptides. Some of the identified peptides had overlapping sequences and several peptides had a cysteine residue modified by propionamide or a methionine residue modified by oxidation. Overall, the identified peptides accounted for 88.4% (130/147) of the deduced protein sequence for the GAT4621 protein derived from GM canola line 73496 (Figure 23). In comparison, 76% of the theoretical protein sequence was represented by uniquely identified tryptic peptides derived from the microbial-expressed GAT4621 protein. The N-terminal methionine residue was not identified in matching tryptic peptides derived from either

plant- or microbial-expressed GAT4621, consistent with the results from N-terminal amino acid sequencing.

As with the N-terminal sequencing, the analysis of tryptic peptides provides additional evidence of equivalency between the plant- and microbial-expressed GAT4621 proteins.

Figure 23. Deduced GAT4621 Amino Acid Sequence Showing Matching Peptides from MALDI-MS

Sequence shown in red corresponds to matching tryptic peptides identified for the GAT4621 protein isolated from GM canola line 73496. Sequence shown in red with shading corresponds to matching tryptic peptides identified for the microbial-expressed GAT4621 protein.

Protein glycosylation analysis of the plant-expressed GAT4621 proteins

In order to analyze glycosylation of the plant-expressed GAT4621 protein as part of the weight of evidence for protein allergenicity, the GAT4621 protein isolated from GM canola line 73496 was analyzed using a glycoprotein staining procedure. Plant-expressed GAT4621 protein did not exhibit positive staining with the glycoprotein staining reagent (Figure 24, lane 4, Glycoprotein staining panel), while the positive control horseradish peroxidase was stained and clearly visible (Figure 24, lane 3, Total protein staining panel).

Based on this analysis, the GM canola line 73496 -derived GAT4621 protein was determined not to be glycosylated and provided support to the weight of evidence assessment that the GAT4621 protein is unlikely to be an allergen.

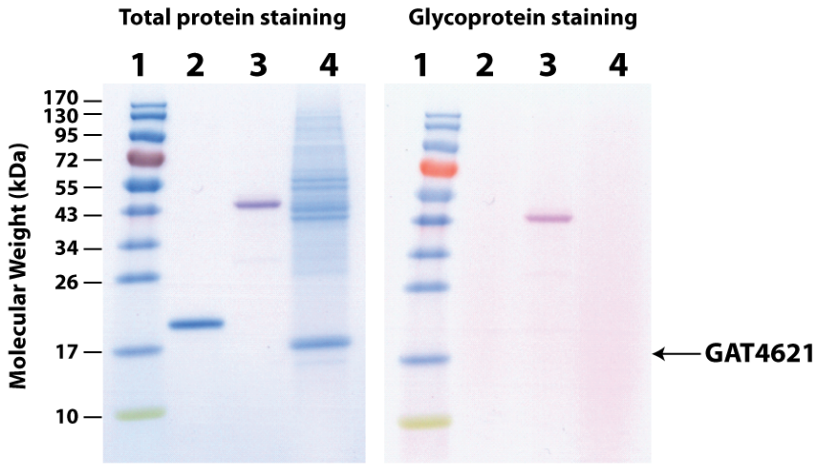

Figure 24. Glycosylation Analysis of Plant-Expressed GAT4621 Protein
Samples containing soybean trypsin inhibitor (~1 µg) as a negative control (lane 2), horseradish peroxidase (~1 µg) as a positive control (lane 3), and GAT4621 protein (~1 µg) isolated from GM canola line 73496 (lane 4) were subjected to SDS-PAGE and analyzed for the presence of carbohydrates using a periodate-acid-Schiff glycoprotein staining reagent (Glycoprotein staining panel). Following image capture, the same gel was stained with Coomassie Blue to visual all proteins (Total protein staining panel). Molecular weight markers (PageRuler Prestained Protein Ladder) were included in lane 1.

B3(c) Site of expression of all novel substances and levels

In order to understand the level of expression of the GAT4621 protein in GM canola line 73496 and the potential exposure, the concentration of the protein was measured in relevant plant tissues. Expression of the GAT4621 protein is driven by the constitutive *UBQ10* promoter from *Arabidopsis thaliana* and would be expected to be measurable in all tissues tested from GM canola line 73496. (Refer to Study 7: PHI-2009-039/010 for details)

The range of expression of the GAT4621 protein in GM canola line 73496 was determined by quantitative enzyme linked immunosorbent assay (ELISA) of samples of seed, roots, and whole plants (i.e., the entire above-ground portion of the plant) obtained from up to six field trial locations in Canada and the United States in 2009. Near-isoline canola plants were grown and sampled as controls.

As indicated below, tissue samples were collected at various developmental stages described by Lancashire *et al*. (1991):

- **BBCH15** – five true leaves unfolded (whole plant sample)
- **BBCH33** – three visibly extended internodes (whole plant sample)
- **BBCH65** – full flowering; 50% of flowers open on main raceme, older petals falling (whole plant and root samples)

- **BBCH90** – senescence (seed sample)

As expected, quantifiable amounts of GAT4621 protein were detected in each plant tissue tested from GM canola line 73496 (Table 8). The range of mean concentrations in whole plant samples across growth stages was 5.2-6.9 ng/mg dry weight. The mean concentration of the GAT4621 protein measured in root was 6.6 ng/mg dry weight. The mean concentration in seed was 6.2 ng/mg dry weight.

Table 8. Concentration of GAT4621 Protein in GM canola line 73496 Plant Tissues

Tissue	GAT4621 Concentration [ng/mg dry weight ± SD (range)][a]			
	Plant Growth Stage[b]			
	BBCH15	BBCH33	BBCH65	BBCH90
Whole Plant (n=24)	6.9 ± 1.3 (3.9-10)	5.3 ± 1.2 (3.1-8.4)	5.2 ± 0.88 (3.9-7.6)	NC
Root (n=24)	NC[c]	NC	6.6 ± 2.4 (3.9-13)	NC
Seed (n=20)	NC	NC	NC	6.2 ± 0.94 (4.8-8.4)

[a] Values are expressed as the mean of four replicate tissue samples collected from each of six locations except seed samples, which were only collected from five locations. SD = standard deviation; range, in parentheses, denotes the lowest and highest individual value across sites.

[b] Plant growth stages: BBCH15 – five true leaves unfolded; BBCH33 – three visibly extended internodes; BBCH65 – full flowering; BBCH90 – senescence (Lancashire *et al.*, 1991).

[c] NC = Not collected.

B3(d) Post-translational modifications to the novel protein(s)

Please refer to section B3(b).

B3(e) Evidence of silencing, if silencing is the method of modification

Not applicable.

B3(f) History of human consumption of novel substances or similarity to substances previously consumed in food

Food and feed safety assessments have been conducted to assess the allergenicity and toxicity potential of the GAT4621 protein. A detailed assessment of the food and feed safety of the GAT4621 protein was submitted to FDA on January 31, 2007 as part of New Protein Consultation (NPC) 005 and was completed on October 7, 2009 (US-FDA, 2009; Rood, 2007a). A summary of the information in NPC 005, along with recently updated bioinformatic analyses on *GAT4621* are described below. Further, a Safety Assessment for the *gat4621* gene and GAT4621 protein has been conducted by FSANZ in association with assessment of the dual herbicide-tolerant maize event DP-Ø9814Ø-6 that contains the *gat4621* gene concluding that no potential public health and safety concerns were identified and that on the basis of the data provided, and other available information, food derived from event DP-Ø9814Ø-6 containing the *gat4621* gene is considered as safe for human consumption as food derived from conventional maize varieties (FSANZ, 2010).Canola seed is not commonly consumed without processing and its fractions (i.e. oil and meal) have different uses for human and livestock consumption, together with a range of industrial uses. The primary use of the refined oil from the processed canola seed is used for:

- Cooking oil
- Spreads and shortening
- Prepared foods
- Cosmetics, and
- Lubricants, fuels and other industrial applications

B4 Assessment of Potential Toxicity

B4(a) Bioinformatic comparison (aa) of novel protein(s) to toxins

An updated sequence similarity search of the GAT4621 protein sequence against the National Center for Biotechnology Information (NCBI) Entrez Protein dataset was conducted using the BLASTP 2.2.25 algorithm against the current NCBI Entrez Protein dataset (Release 188.0, 2/15/2012). A cut off E score of 1.0 was used to generate biologically meaningful similarity between the GAT4621 protein and proteins in the NCBI database, which allowed for identification of proteins with limited similarity in the search. Low complexity filtering was turned off and the maximum number of alignments returned was set to 2000. None of the similar proteins returned by the search were identified as toxins, demonstrating that the GAT4621 protein is unlikely to share relevant sequence similarities with known protein toxins and is therefore unlikely to be a toxin itself. (Refer to Study 8: PHI-2007-009/073 for detais)

B4(b) Stability to heat or processing and/or degradation in gastric model

Heat stability of GAT4621

Evaluation of the stability of a novel protein during heating is considered in an effort to mimic cooking/processing effects and exposure conditions. The GAT4621 enzyme was subjected to heat treatment over a temperature incubation range of 36-60°C and then analyzed using a continuous absorbance spectrophotometric enzyme activity assay. The results of the study showed that the GAT4621 enzyme lost half of its activity when incubated in the range of 49 – 50°C for 15 minutes and was essentially inactivated following incubation for 15 minutes at temperatures above 53°C (Figure 25). (Refer to Study 9: PHI-2006-184/018 for details)

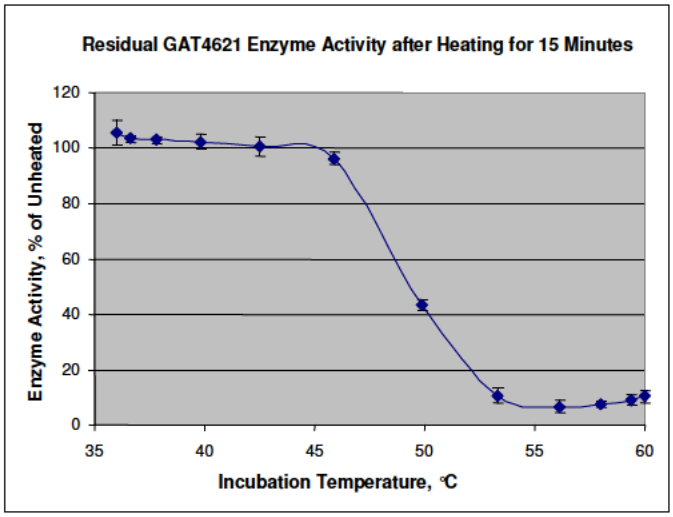

Figure 25. Graph Illustrating the Residual Enzyme Activity versus the Incubation Temperature

Degradation of GAT4621 in simulated gastric fluid (SGF) model

SGF was used to assess the susceptibility of microbially expressed and purified GAT4621 proteins to proteolytic digestion by pepsin *in vitro*. The International Life Sciences Institute (ILSI) has standardized the pepsin digestibility assay protocol in a multi-laboratory evaluation (Thomas *et al*., 2004). The SGF formulation, time course, and experimental parameters followed in the evaluation of GAT4621 were similar to conditions used in the ILSI multi-laboratory evaluation. Bovine serum albumin (BSA) and β-lactoglobulin were used as positive and negative controls, respectively (data not shown). GAT4621 along with BSA and β-lactoglobulin were incubated in SGF containing pepsin at pH 1.2 for specific time intervals and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The molar ratio of pepsin to GAT4621 protein in the study was ~0.02 mM pepsin to ~0.015 mM GAT4621, or ~1.3:1. This is equivalent to ~ 3:1 pepsin to GAT4621 protein ratio on a weight basis (Thomas *et al*., 2004). (Refer to Study 10: PHI-2006-120 for details)

The GAT4621 protein was not detectable at 30 seconds in SGF (Figure 26, lane 4). Two faint low molecular weight bands were visible in lanes 4-6 near the dye front, and the lower of the two bands persisted through 60 minutes (lane 11). These bands are likely a mix of breakdown products from the GAT4621 protein. Results of the SGF study demonstrate that the GAT4621 protein is rapidly (< 30 seconds) hydrolyzed in SGF containing pepsin at pH 1.2, as shown by SDS-PAGE analysis (Figure 26).

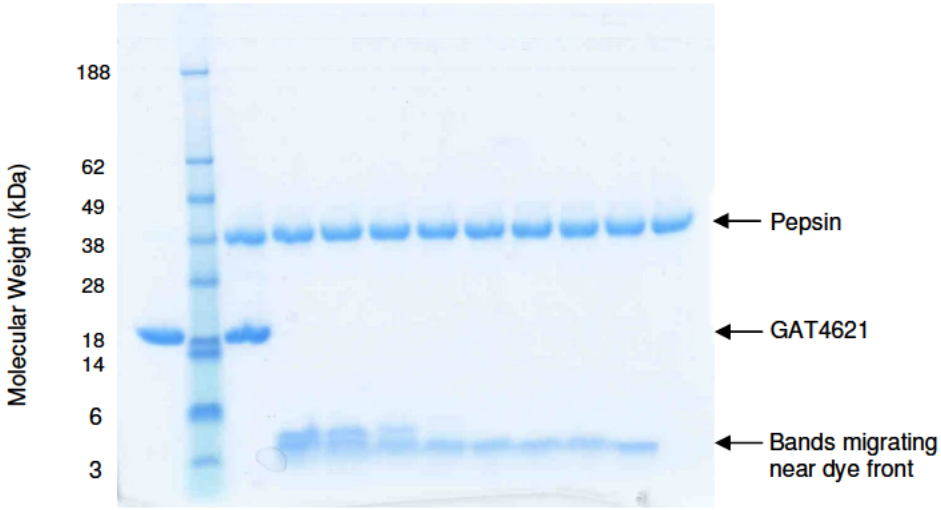

Figure 26. Scanned Image of SDS-PAGE Gel Showing Pepsin Resistance of GAT4621 Protein

Lane	Load Volume µL	Sample ID
1	20	GAT4621 (~2.3 µg) in water "Time 0"
2	13	SeeBlue[a] molecular weight marker
3	20	GAT4621 (~2.3 µg) in SGF "Time 0"
4	20	GAT4621 in SGF for 0.5 minutes
5	20	GAT4621 in SGF for 1 minute
6	20	GAT4621 in SGF for 2 minutes
7	20	GAT4621 in SGF for 5 minutes
8	20	GAT4621 in SGF for 10 minutes
9	20	GAT4621 in SGF for 20 minutes
10	20	GAT4621 in SGF for 30 minutes
11	20	GAT4621 in SGF for 60 minutes
12	20	SGF control ~60 minutes

[a] Registered trademark of Invitrogen Corporation.

B4(c) Acute or short-term oral toxicity on novel protein(s)

Oral exposure to most proteins does not cause adverse effects. Those that do cause toxicity are believed to act through acute mechanisms of action (Sjoblad *et al.,* 1992; Hammond and Fuchs, 1998; Pariza and Johnson, 2001). An acute oral mouse toxicity study of the GAT4621 protein was conducted. The oral route of exposure was selected because it is the most likely route of exposure for humans.

A single dose of GAT4621 protein preparation (containing approximately 82% microbially expressed, purified GAT4621 protein) was administered by oral gavage to groups of five fasted male and five fasted female Crl:CD®-1(ICR)BR mice at a target dose of 2000 mg/kg body weight (OECD, 2001b). The actual dose of purified GAT4621 protein was 1640 mg/kg. A control group of five fasted male and five fasted female mice was administered bovine serum albumin at a dose of 2000 mg/kg or water alone at an equivalent dose volume to the GAT4621 treated mice. (Refer to Study 11: PHI-2005-110 for details)

The GAT4621 protein used for this study was produced in and purified from *E. coli* BL21 (DE3) by cation exchange chromatography, anion exchange chromatography, hydrophobic interaction chromatography and diafiltration. The GAT4621 protein was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine molecular weight, western blotting to determine immunoreactivity, N-terminal amino acid sequencing and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) to determine peptide mass and, indirectly, protein sequence, and glycoprotein staining to demonstrate lack of glycosylation.

The mice were observed for mortality, body weight gain, and clinical signs for 14 days post dosing, after which they were euthanized and subjected to gross necropsy to detect observable evidence of organ or tissue damage.

All mice survived until the scheduled euthanization on Day 14. No clinical signs of toxicity or test substance-related body weight losses were observed in any mice. No gross lesions were observed in the mice at necropsy.

Under the conditions of this study, administration of GAT4621 protein to male and female mice at a dose of 1640 mg/kg produced no test substance-related clinical signs of toxicity, body weight losses, gross lesions, or mortality. From this study, it was concluded that the GAT4621 protein is not acutely toxic.

B5 Assessment of Potential Allergenicity

The GAT4621 protein is unlikely to cause an allergic reaction in humans and therefore is safe for human exposure and animal consumption. The following is supporting evidence to these claims:

1. The donor organism, *B. licheniformis*, is a common soil bacterium widely distributed in the environment. It is widely known as a contaminant of food but not associated with any adverse effects (section B5(a)).
2. *B. licheniformis* has a history of safe use for the production of food enzymes in the United States, Canada, and Europe (e.g., alpha-amylase, cyclodextrin glycosyltransferase, hemicellulase, proteases, pullulanase), biocontrol agents (European Commission, 2000; US-FDA, 2001) and as a probiotic (Alexopoulos *et al*., 2004a; Alexopoulos *et al*., 2004b; Kritas *et al*., 2006). *B. licheniformis* was determined by the EPA to present low risk of adverse effects to human health and the environment and was subsequently granted an exemption for use in certain industrial fermentation processes (US-EPA, 1997).
3. The GAT4621 protein is not glycosylated (section B3(b)).
4. The GAT4621 protein is rapidly hydrolysed in both simulated gastric (section B4(b)) and intestinal fluids (section B5(c)) (less than 30 seconds in simulated gastric fluid containing pepsin at pH 1.2 as demonstrated by SDS-PAGE analysis, and less than 5 minutes in simulated intestinal fluid containing pancreatin at pH 7.5 as demonstrated by SDS-PAGE analysis).
5. The amino acid sequence of the GAT4621 protein was compared to a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (FARRP, 2012), which contains the amino acid sequences of 1603 known and putative allergenic proteins. The results indicate the lack of both amino acid identity and immunologically relevant similarities between the GAT4621 protein and known protein allergens (section B5(b)).

B5(a) Source of introduced protein

The donor organism, *B. licheniformis*, is a common soil bacterium widely distributed in the environment. Due to its ubiquitous presence as spores in soil and dust, *B. licheniformis* is widely known as a contaminant of food but is not associated with any adverse effects. Although derived from *B. licheniformis*, the *gat4621* gene is inserted in isolation from other *B. licheniformis* genes. Further, a Safety Assessment for the *gat4621* gene has been conducted by FSANZ in association with assessment of the dual herbicide-tolerant maize event DP-Ø9814Ø-6 that contains the *gat4621* gene concluding that no potential public health and safety concerns were identified and that on the basis of the data provided, and other available information, food

derived from maize event DP-Ø9814Ø-6 containing the *gat4621* gene is considered as safe for human consumption as food derived from conventional maize varieties.

B5(b) Bioinformatic comparison (aa) of novel protein(s) to allergens

An updated bioinformatic analyses were conducted to evaluate the potential allergenicity of the GAT4621 protein. The amino acid sequence of the GAT4621 protein was compared to a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (FARRP, 2012), which contains the amino acid sequences of 1603 known and putative allergenic proteins. Potential identities between the GAT4621 protein and proteins in the allergen database were evaluated using the FASTA35 sequence alignment program (Pearson and Lipman, 1988) set to the default parameters (ktup = 2, scoring matrix = BLOSUM50, gap creation penalty = -10, gap extension penalty = -2, E score cut off = 10). The top 20 high scoring alignments were reviewed for sequence identities greater than or equal to 35% over 80 or greater amino acid residues. None of the alignments met or exceeded the 35% over 80 or greater amino acid threshold. The GAT4621 amino acid sequence was also evaluated for any eight or greater contiguous identical amino acid matches to the same database of allergens noted above. There were no eight or greater contiguous identical amino acid matches observed with the GAT4621 amino acid sequence. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the GAT4621 protein and known protein allergens. (Refer to Study 12: PHI-2007-008/073 for details)

B5(c) Structural properties, including digestion by pepsin, heat treatment

B5(c)(i) Digestive fate of the GAT4621 protein

Degradation of GAT4621 in simulated gastric fluid (SGF) model

The GAT4621 protein is rapidly digested in simulated gastric fluids containing pepsin at pH 1.2 in less than 30 seconds (section B4(b)).

Degradation of GAT4621 in simulated intestinal fluid (SIF) model

In order to assess lability of the GAT4621 protein in the intestinal tract, microbially expressed and purified GAT4621 protein were incubated in SIF containing pancreatin prepared as described in the United States Pharmacopoeia (USP-NF, 2000) for specific time intervals and analyzed by SDS-PAGE. SIF contained 0.25 mg/ml of GAT4621 protein in 50 mM KH_2PO_4, 1% w/v pancreatin, pH 7.5. This is equivalent to a ~ 40:1 pancreatin to GAT4621 protein ratio on a weight basis. ß-lactoglobulin and BSA were used as controls (data not shown). (Refer to Study 13: PHI-2006-122 for detais)

Porcine pancreatin (from Sigma; meets United States Pharmacopoeia standards) contains many enzymes, including amylase, lipase and protease. In Figure 27, the mixture of proteins in pancreatin can be seen as multiple stained protein bands in lane 12.

Results of the SIF study for GAT 4621 are shown in Figure 27. The GAT4621 protein was not detectable at 5 minutes in SIF (Figure 27, lane 7). The low molecular weight bands that were seen in SGF were not seen in SIF, indicating complete lability of the GAT4621 protein.

Results of the SIF study demonstrate that the GAT4621 protein is rapidly (< 5 minutes) hydrolyzed in SIF containing pancreatin at pH 7.5, as shown by SDS-PAGE analysis.

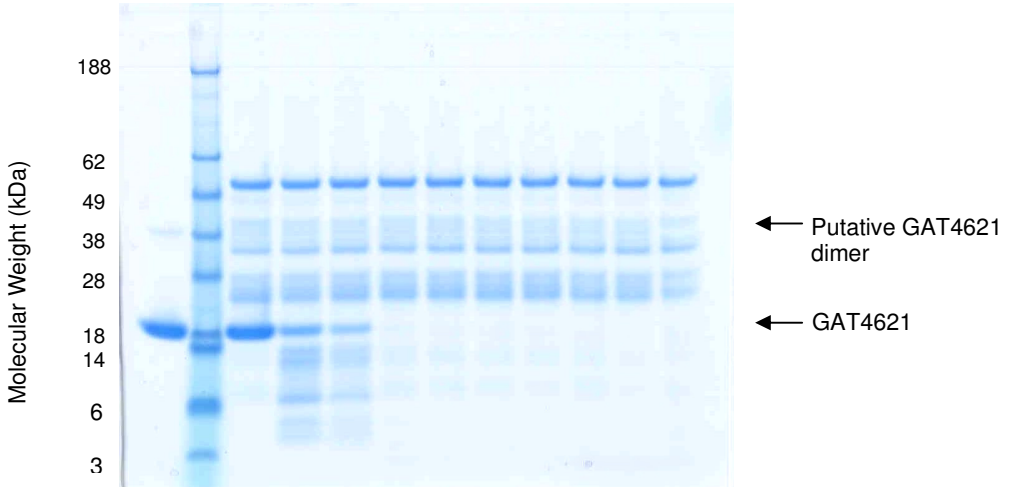

Figure 27. Image of SDS-PAGE Gel Showing Pancreatin Resistance of the GAT4621 Protein

Lane	Load Volume µL	Sample ID
1	20	GAT4621 (~3.3 µg) in water "Time 0"
2	13	SeeBlue[a] molecular weight marker
3	20	GAT4621 in SIF (~3.3 µg) "Time 0"
4	20	GAT4621 in SIF 0.5 minutes
5	20	GAT4621 in SIF 1 minute
6	20	GAT4621 in SIF 2 minutes
7	20	GAT4621 in SIF 5 minutes
8	20	GAT4621 in SIF 10 minutes
9	20	GAT4621 in SIF 20 minutes
10	20	GAT4621 in SIF 30 minutes
11	20	GAT4621 in SIF 60 minutes
12	20	SIF control ~60 minutes

[a] Registered trademark of Invitrogen Corporation

B5(c)(ii) Heat Stability of GAT4621Protein

The GAT4621 enzyme lost half of its activity when incubated in the range of 49 – 50℃ for 15 minutes and was essentially inactivated following incubation for 15 minutes at temperatures above 53℃ (section B4(b)).

B5(d) Specific serum screening if protein from allergenic source

The donor organism, *B. licheniformis*, is a common soil bacterium widely distributed in the environment. It is widely known as a contaminant of food but not associated with any adverse effects or considered as an allergenic source.

B5(e) GAT4621 protein as a proportion of total protein

The GAT4621 protein was measured in relevant plant tissues of GM canola line 73496 at different developmental stages (Table 8). Since the majority of harvested canola seeds are processed into canola oil, which represents the major exposure route of GM canola line 73496 in human diet (section A2(b)(iii)), it would be the most relevant to assess the allergenicity of GM canola line 73496 with the percentage of GAT4621 protien as a proportion of total protein in seeds. The mean concentration of GAT4621 protein in GM canola line 73496 seeds is 6.2 ng/mg (Table 8), and the average percentage of crude protein in GM canola line 73496 seeds is 25.9% dry weight (Table 13), therefore the percentage of GAT4621 protein as a proportion of total protein is calculated as following:

6.2ng / (1 mg x 1,000,000) x 25.9% ≈ 0.002% of total protein in GM canola line 73496 seed

As a consequence, the proportion of GAT4621 protein would be relatively low in harvested GM canola line 73496 seeds. Additionally, the residual protein in oil extracted from canola seed can be diminished to as low as 0.00002% or 0.2 ppm (Martín-Hernández *et al.*, 2008), that would further limit the exposure of GAT4621 protein in human diet. As the canola oil represents the major exposure route of GAT4621 protein in human diet, the exposed amount of GAT4621 protein in human diet is almost neglectable (0.002% in 0.2ppm of residual protein in canola oil).

B6 Toxicity of novel herbicide metabolites in GM herbicide-tolerant plants

The Residue Level of Novel Metabolites

The metabolism of glyphosate in conventional crops and glyphosate-tolerant crops with *cp4 epsps* gene has been identified the same with the aminomethylphosphonic acid (AMPA) as the major degrade from glyphosate (FAO, 2005). AMPA can be further conjugated with natural plant constituents to give trace level metabolites, or degraded to one carbon fragments that are incorporated into natural products (FAO, 2005). The GM canola line 73496 has been genetically modified to express the GAT4621 (glyphosate acetyltransferase) protein and contains a glyphosate acetyltransferase gene variant *gat4621* of three *gat* genes from *Bacillus licheniformis*. The GAT4621 protein is equivalent to the protein expressed in both maize event DP-Ø9814Ø-6, approved by FSANZ in May 2010, and soybean event, DP-356Ø43-5, approved by FSANZ in December 2009, and is encoded by the *gat4621* gene that confers tolerance to glyphosate-containing herbicides by acetylating glyphosate and thereby rendering it non-phytotoxic (Figure 18). Metabolism studies in genetically modified corn and soybean with *gat4621* gene demonstrated that *N*-acetylglyphosate was formed as novel metabolite, whereas glyphosate, *N*-acetyl AMPA and AMPA were found in low concentrations in the edible parts of the crops (FAO, 2005). Comprehensive toxicological studies have revealed that no public health and safety concern is related to *N*-acetylglyphosate and *N*-acetyl-AMPA as both metabolites are less toxic than glyphosate itself (FSANZ 2009; FSANZ 2010).

The following glyphosate residue study was conducted to determine the redisdues for glyphosate and its plant metabolites *N*-acetyl-*N*-(phosphonomethyl) glycine (*N*-acetylglyphosate), aminomethylphosphonic acid (AMPA) and *N*-acetylaminomethylphosphonic acid (*N*-acetyl AMPA) in seed and whole plant (forage) after the treatment of Touchdown Total® (TT) Herbicide on GM canola line 73496 grown at 16 sites in the Canada during 2009. All sites contain one untreated (control) plot and two TT-treated plots. The Touchdown Total® Herbicide label for Canada allows for one pre-emergent application and two post-emergent applications in-crop. The spectifications related to application method of glyphosate is summarized in Table 9. The first application of Touchdown Total® Herbicide was at preemergent stage with the application rate of 675 g a.e./hectare. The second application of Touchdown Total® Herbicide was at 6-leaf stage with the application rate of 675 g a.e./hectare. The last application was at 7 days before harvest with the rate of 900 g a.e./hectare. (Please refer to Study 14: PHI-2009-057 for details)

Residues of glyphosate and plant metabolites found in replicated treated forage and seed samples following Canada treatment parameter of glyphosate are summarized in Table 10 and 11 respectively. The average residues of glyphosate + *N*-acetylglyphosate + AMPA + *N*-acetyl AMPA in glyphosate equivalents are presented in Table 12. Forage sampling occurred after the 2nd application and has the average residues of 0.49 ppm, which is lower than the average

residues of 3.9 ppm detected in seed. Comparing with the residue study conducted in the US sites[b], a higher residue in seed samples was detected which was likely resulted from the late (3rd) application at 7 days before harvest. While the United States EPA (U.S. EPA, 2008) and international agency, CODEX (Codex Alimentarius,2012), have established the Maximum Residue Limit (MRL) of 20 ppm for glyphosate in conventional and glyphosate-tolerant canola, Pioneer Hi-Bred Australia Pty Ltd. is seeking the consideration from FSANZ on amending **Standard 1.4.2** – *Maximum Residue Limits* to harmonise the MRL tolerance for glyphosate on canola food imports with the extend established in the Code of Federal Regulations of the United States of America and the Codex Alimentarius. Nevertheless, local residue trials will also be conducted and the results will be submitted to Australian Pesticides and Veterinary Medicines Authority by Pioneer Hi-Bred Australia Pty Ltd. before the commercialization of GM canola line 73496 to ensure the MRL cultivation tolerance complies with the practice in Australia.

Table 9. The Summary of Specification of Application Rates, Test Substance, and Application Timing.

Appl. No.	Test substance	Rate (g a.e./hectare)	Appl. Method	Appl. Timing (± 1 DAY)	Spray Volume	Adjuvant
A1	Touchdown Total ®	675	Broadcast	Preemergent	50-100 L/ha	None
A2	Touchdown Total ®	675	Broadcast	At but no later than 6 leaf stage	50-100 L/ha	None
A3	Touchdown Total ®	900	Broadcast	7 days before harvest	50-100 L/ha	None

[b] Study conducted at 8 sites in the US with US label of Touchdown Total® Herbicide has two applications, which are at preemergent stage and 6-leaf stage, respectively. The average residues in forage and seed samples are 0.4 ppm and 0.7 ppm, respectively. Please refer to Study 14: PHI-2009-057 for details.

Table 10. Magnitude of Residues in Forage.

Forage Residues in ppm (mg/kg)[a]				
Statistic[b]	**Glyphosate**	***N*-acetylglyphosate**	**AMPA**	***N*-acetyl-AMPA**
mean	0.02	0.45	ND	ND
median	ND	0.39	ND	ND
min	ND	ND	ND	ND
max	0.26	1.4	0.072	0.03

a. The designation "ND" is used for treated specimens for which no peak was observed with a signal-to-noise ratio greater than 3:1. All residues reported as glyphosate equivalents.

b. Each statistic (mean, median, minimum, and maximum) is calculated from the individual residue values across all test sites. For calculations, one-half LOD was used for specimens when the residue value was reported as ND.

Table 11. Magnitude of Residues in Seed.

Seed Residues in ppm (mg/kg)[a]				
Statistic[b]	**Glyphosate**	***N*-acetylglyphosate**	**AMPA**	***N*-acetyl-AMPA**
mean	2.5	1.3	0.03	0.04
median	1.9	0.41	0.02	0.03
min	0.29	0.21	ND	ND
max	9.9	17	0.089	0.035

a. The designation "ND" is used for treated specimens for which no peak was observed with a signal-to-noise ratio greater than 3:1. All residues reported as glyphosate equivalents.

b. Each statistic (mean, median, minimum, and maximum) is calculated from the individual residue values across all test sites. For calculations, one-half LOD was used for specimens when the residue value was reported as ND.

Table 12. The Average Residues in Seed and Forage.

Commodity	Residues in ppm (mg/kg)
Seed	3.9
Forage	0.49

The Safety of Novel Metabolites

The GM canola line 73496 has been genetically modified to express the GAT4621 (glyphosate acetyltransferase) protein and contains a glyphosate acetyltransferase gene variant *gat4621* of three *gat* genes from *Bacillus licheniformis*. The GAT4621 protein is equivalent to the protein expressed in both maize event DP-Ø9814Ø-6, approved by FSANZ in May 2010, and soybean event, DP-356Ø43-5, approved by FSANZ in December 2009, and is encoded by the *gat4621* gene that confers tolerance to glyphosate-containing herbicides by acetylating glyphosate and thereby rendering it non-phytotoxic (Figure 18). Metabolism studies in genetically modified maize and soybean with *gat4621* gene demonstrated that *N*-acetylglyphosate was formed as novel metabolite, whereas glyphosate, *N*-acetyl AMPA and AMPA were found in low concentrations in the edible parts of the crops (FAO, 2005). Comprehensive toxicological studies have revealed that no public health and safety concern is related to *N*-acetylglyphosate and *N*-acetyl-AMPA as both metabolites are less toxic than glyphosate itself (FSANZ 2009; FSANZ 2010).

B7 Compositional Assessment

Compositional comparisons between transgenic crops and conventional varieties are a key part of a nutritional and safety assessment and provide assurance of the food safety of transgenic crops. Pioneer has conducted compositional assessments performed in accordance with the principles outlined in the OECD consensus document on compositional considerations for canola (OECD, 2001a). This document emphasises quantitative measurements of essential nutrients, and known anti-nutrients and toxicants. These analyses effectively highlight any compositional changes that may indicate potential safety and anti-nutritional concerns.

For the GM canola line 73496, the following analytes were measured for the comparative assessment: proximates, fibre, fatty acids, amino acids, vitamins, minerals, glucosinolates, secondary metabolites, phytosterols, and anti-nutrients. Levels of the analytes were measured in the seed of the F1[*4] generation (Figure 2) of GM canola line 73496 and were compared to corresponding levels in the near-isoline control and were also compared to statistical tolerance intervals generated from non-modified conventional commercial varieties. These comparisons formed the basis for determining compositional comparability of GM canola line 73496 to conventional canola. Canola seed was chosen as the test material for the compositional analysis of GM canola line 73496 because oil fractions are derived from seed. Compositional evaluation of seed would be representative of these derived materials.

Seed samples were collected from five separate sites in Canada and the United States during the 2009 growing season (three sites in Manitoba, Canada: Elm Creek, Minto, and Portage la Prairie; two sites in the United States: Velva, ND and Ephrata, WA). Each site utilised a randomized complete block design with four blocks and each block containing GM canola line 73496 and the control canola. Each plot of GM canola line 73496 was treated with glyphosate at a rate of 0.367-0.414 pounds acid equivalent per acre (411-464 grams acid equivalent per hectare) consistent with typical agronomic practices, and each plot of the control canola was left untreated. Seed samples collected from GM canola line 73496 and the control canola were analysed for key nutritional components in accordance with the OECD consensus document on compositional considerations for new varieties of canola (OECD, 2001a), which included analysis of proximates, fiber, fatty acids, amino acids, vitamins, minerals, glucosinolates, secondary metabolites, phytosterols, and anti-nutrients. (Refer to Study 15: PHI-2009-039/020 for details)

In a separate experiment for the statistical tolerance intervals, seed was also collected from five conventional (i.e., non-modified) commercial canola lines grown at five field locations in canola-growing areas of U.S. and Canada in 2008 and 2009. Planting, harvesting, processing, and compositional analysis procedures for the reference hybrid trials were similar to those employed for the trials containing near-isoline control and GM canola line 73496. Compositional analyses

of the reference lines were used to determine the statistical tolerance intervals, which established the normal variation for the measured analytes in canola.

Statistical analysis of nutrient composition data was conducted to test for differences in the analyte mean values between GM canola line 73496 and the near-isoline control. When numerous analytes are being evaluated on the same samples, controlling false positive outcomes is important. A false positive outcome occurs when an analyte mean of the transgenic line is deemed significantly different from the analyte mean of the control line, when in fact the two means are not different. If one uses a 5% type I error rate for each analyte, then the number of false positives increases as the number of analytes increase. In order to help manage the false positive rate, the false discovery rate (FDR) method of Benjamini and Hochberg was applied to account for making multiple comparisons (Benjamini and Hochberg, 1995; Westfall *et al.*, 1999). P-values were adjusted accordingly, resulting in the false positive rate being held to 5%. Both adjusted and non-adjusted P-values are provided in this submission. In the discussion of these data, a significant difference between the mean of GM canola line 73496 and that of the near isoline was established with an FDR-adjusted P- value <0.05.

Using the data obtained from the commercial lines, a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial canola. Any negative limits were set to zero. This statistical tolerance interval provided further context for interpretation of the composition results for GM canola line 73496.

B7(a) Levels of key nutrients, toxicants and anti-nutrients

Proximate and fiber analysis

Analysis of the major constituents of canola seed, or proximates, was used to determine the nutritional properties of seed from GM canola line 73496 and control canola. No statistically significant differences were observed between GM canola line 73496 and the control canola mean values for any of the proximate and fibre analytes (Table 13).

In conclusion, proximate and fibre analysis of canola seed demonstrates that GM canola line 73496 is comparable to conventional canola.

Table 13. Summary Analysis of Proximate and Fibre Results in Canola Seed

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval[a]
Proximates and Fiber Composition (% Dry Weight)				
Crude Protein	Mean[b]	26.6	25.9	11.3 - 54.4
	Range[c]	23.4 - 31.9	22.4 - 31.6	
	CI[d]	23.4 - 30.3	22.8 - 29.5	
	P-Value[e]		0.0275	
	Adjusted P-Value[f]		0.0974	
Crude Fat	Mean	43.6	43.9	30.6 - 65.8
	Range	37.4 - 51.5	37.0 - 49.6	
	CI	40.1 - 47.3	40.3 - 47.7	
	P-Value		0.608	
	Adjusted P-Value		0.737	
ADF[g]	Mean	32.4	32.3	14.5 - 63.4
	Range	27.1 - 38.6	26.6 - 39.5	
	CI	28.8 - 36.5	28.7 - 36.4	
	P-Value		0.929	
	Adjusted P-Value		0.957	
Crude Fiber	Mean	28.4	28.5	12.9 - 55.5
	Range	24.3 - 35.1	22.8 - 35.5	
	CI	25.1 - 32.1	25.2 - 32.2	
	P-Value		0.889	
	Adjusted P-Value		0.935	
NDF[f]	Mean	33.4	33.3	15.5 - 67.7
	Range	28.8 - 40.3	28.0 - 38.9	
	CI	30.4 - 36.8	30.2 - 36.7	
	P-Value		0.844	
	Adjusted P-Value		0.911	
Ash	Mean	4.08	3.92	2.01 - 7.63
	Range	2.98 - 5.10	3.00 - 5.06	
	CI	3.29 - 5.07	3.16 - 4.87	
	P-Value		0.0549	
	Adjusted P-Value		0.141	
Carbohydrates	Mean	25.3	26.0	19.6 - 33.5
	Range	16.9 - 29.1	23.7 - 28.8	
	CI	24.0 - 26.7	24.6 - 27.4	
	P-Value		0.320	
	Adjusted P-Value		0.468	

[a] The statistical tolerance interval was calculated from commercial canola varieties and calculated to contain with 95 percent confidence, 99 percent of the population of canola, negative limits set to zero

[b] Least squares mean were generated from a mixed model analysis

[c] Range denotes the lowest and highest individual value across sites

[d] Confidence Interval

[e] Non-adjusted P-value

[f] False Discovery Rate (FDR) adjusted P-value

[g] Acid Detergent Fiber

[h] Neutral Detergent Fiber

Fatty acid analysis

Five fatty acids account for more than 96% of the total fatty acids in canola seed, with the most abundant being oleic (C18:1 Δ9; ~ 60%) and linoleic (C18:2 Δ9,12; ~ 20%) acids. Less abundant, but occurring at measurable concentrations are palmitic (C16:0), stearic (C18:0) and α-linolenic (C18:3 Δ9,12,15) acids. The desaturation of oleic acid to form linoleic acid, and its subsequent desaturation to form α-linolenic acid, occurs only in plants, hence both linoleic and α-linolenic acids are essential fatty acids for mammals. For this reason, it was important to analyse any unintended changes in the concentrations of linoleic and α-linolenic acids, and their key precursors palmitic, stearic, and oleic acids, in seed from GM canola line 73496.

Other polyunsaturated fatty acids, such as γ-linolenic (C18:3 Δ6,9,12), eicosatrienoic (C20:3 Δ8,11,14) and arachidonic (C20:4 Δ5,8,11,14) acids can all be synthesized by mammals from dietary sources of α-linolenic and linoleic acid. Hence, small changes in the concentrations of these trace fatty acids in GM canola line 73496 seed would have little or no biological significance to either humans or animals consuming derived products. Similarly, the synthesis of palmitoleic (C16:1 Δ9) and saturated fatty acids with chain lengths greater than 18 (e.g., C20:0, C22:0, C24:0), can be accomplished in mammals through *de novo* fatty acid synthesis without dietary requirements for palmitic and stearic acids, respectively.

The fatty acids reported in Table 14 were identified by OECD as those that should be measured for compositional comparisons in canola (OECD, 2001a). Based on consultation with the Codex Alimentarius Commission definition of canola oil (Codex, 2005), six additional fatty acids were reported in this analysis. Non-detectable fatty acids are defined as <0.05% of total fatty acids (Codex, 2005). Heptadecanoic acid (C17:0), γ-linolenic acid (C18:3), and erucic acid (C22:1) were below this threshold and were not included in Table 14. As expected per the canola definition, erucic acid (C22:1) was not detected in the analysis.

Statistically significant differences were observed using the adjusted P-value in concentrations of oleic and linoleic acid between GM canola line 73496 and control canola (Table 14). However, these fatty acid concentrations were within the respective tolerance intervals derived from commercial canola varieties. Therefore, the statistically significant differences observed for certain fatty acids are unlikely to be biologically meaningful.

In conclusion, fatty acid analysis of canola seed demonstrates that GM canola line 73496 is comparable to conventional canola.

Table 14. Summary Analysis of Fatty Acid Composition in Canola Seed

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Fatty Acids Composition (% Total Fatty Acids)				
Lauric Acid (C12:0)	Mean	0.0816	0.0811	0 - 0.216
	Range	0.0568 - 0.105	0.0563 - 0.113	
	CI	0.0639 - 0.104	0.0634 - 0.104	
	P-Value		0.863	
	Adjusted P-Value		0.919	
Myristic Acid (C14:0)	Mean	0.0530	0.0546	0.0239 - 0.112
	Range	0.0470 - 0.0749	0.0484 - 0.0670	
	CI	0.0466 - 0.0603	0.0480 - 0.0621	
	P-Value		0.212	
	Adjusted P-Value		0.355	
Palmitic Acid (C16:0)	Mean	4.08	4.19	3.10 - 5.60
	Range	3.72 - 4.40	3.85 - 4.45	
	CI	3.87 - 4.31	3.97 - 4.42	
	P-Value		0.0111	
	Adjusted P-Value		0.0605	
Palmitoleic Acid (C16:1)	Mean	0.262	0.272	0.154 - 0.378
	Range	0.225 - 0.306	0.224 - 0.317	
	CI	0.232 - 0.295	0.241 - 0.307	
	P-Value		0.0173	
	Adjusted P-Value		0.0790	
Heptadecenoic Acid (C17:1)	Mean	0.108	0.105	0 - 0.264
	Range	0.0675 - 0.140	0.0666 - 0.137	
	CI	0.0802 - 0.146	0.0776 - 0.141	
	P-Value		0.0580	
	Adjusted P-Value		0.144	
Stearic Acid (C18:0)	Mean	1.71	1.61	1.17 - 3.47
	Range	1.47 - 1.89	1.44 - 1.93	
	CI	1.57 - 1.86	1.48 - 1.76	
	P-Value		0.00577	
	Adjusted P-Value		0.0507	
Oleic Acid (C18:1)	Mean	61.6	60.1	53.1 - 74.4
	Range	58.6 - 64.1	56.8 - 63.3	
	CI	58.9 - 64.4	57.5 - 62.9	
	P-Value		<0.0001	
	Adjusted P-Value		0.00216[d]	
Linoleic Acid (C18:2)	Mean	20.1	21.3	13.1 - 27.1
	Range	18.5 - 22.5	19.5 - 23.4	
	CI	18.5 - 21.8	19.5 - 23.1	
	P-Value		0.000107	
	Adjusted P-Value		0.00220[d]	

Table 14. Summary Analysis of Fatty Acid Composition in Canola Seed (continued)

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
(9,15) Isomer of Linoleic Acid (C18:2)	Mean	0.0986	0.0924	0 - 0.524[c]
	Range	0.0626 - 1.45	0.0611 - 0.496	
	CI	0.0697 - 0.139	0.0653 - 0.131	
	P-Value		0.764	
	Adjusted P-Value		0.868	
Linolenic Acid (C18:3)	Mean	8.92	9.29	4.23 - 16.8
	Range	7.46 - 10.0	8.08 - 10.5	
	CI	8.04 - 9.89	8.38 - 10.3	
	P-Value		0.00884	
	Adjusted P-Value		0.0525	
Arachidic Acid (C20:0)	Mean	0.615	0.586	0.496 - 1.01
	Range	0.585 - 0.645	0.552 - 0.640	
	CI	0.598 - 0.632	0.570 - 0.602	
	P-Value		0.00618	
	Adjusted P-Value		0.0507	
Eicosenoic Acid (C20:1)	Mean	1.38	1.36	0.999 - 1.88
	Range	1.19 - 1.54	1.24 - 1.45	
	CI	1.27 - 1.51	1.25 - 1.48	
	P-Value		0.128	
	Adjusted P-Value		0.269	
Eicosadienoic Acid (C20:2)	Mean	0.0690	0.0713	0.0308 - 0.107
	Range	0.0565 - 0.0805	0.0563 - 0.0831	
	CI	0.0583 - 0.0817	0.0602 - 0.0844	
	P-Value		0.0256	
	Adjusted P-Value		0.0974	
Behenic Acid (C22:0)	Mean	0.328	0.319	0.241 - 0.465
	Range	0.290 - 0.365	0.286 - 0.359	
	CI	0.300 - 0.358	0.292 - 0.348	
	P-Value		0.0182	
	Adjusted P-Value		0.0790	
Lignoceric Acid (C24:0)	Mean	0.178	0.170	0.0601 - 0.657
	Range	0.160 - 0.208	0.151 - 0.206	
	CI	0.160 - 0.198	0.153 - 0.189	
	P-Value		0.0183	
	Adjusted P-Value		0.0790	
Nervonic Acid (C24:1)	Mean	0.191	0.194	0.0295 - 0.542
	Range	0.121 - 0.257	0.120 - 0.254	
	CI	0.141 - 0.259	0.143 - 0.263	
	P-Value		0.303	
	Adjusted P-Value		0.458	

[a] Statistical analysis was not available (NA).
[b] Tolerance interval could not be calculated (NC) due to all sample values below the assay LLOQ.
[c] Tolerance interval was not available due to insufficient sample values being detected above the assay LLOQ. Minimum and maximum values are used in place of a tolerance interval.
[d] Statistically significant difference, FDR adjusted P-Value <0.05.

Amino acid analysis

Canola seed is a good source of essential and non-essential amino acids for most domestic animal species. Total levels of 18 amino acids were measured in GM canola line 73496 and control seed.

Results are shown in Table 15 for the 18 total amino acids. No statistically significant differences between the GM canola line 73496 and control were noted for any amino acids measured (adjusted P-value).

In conclusion, the total amino acid analysis of canola seed demonstrates that GM canola line 73496 is comparable to conventional canola.

Table 15. Summary Analysis of Amino Acid Composition in Canola Seed

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Amino Acids Composition (% Dry Weight)				
Alanine	Mean	1.14	1.12	0.588 - 1.90
	Range	1.01 - 1.37	0.970 - 1.32	
	CI	1.02 - 1.28	0.996 - 1.25	
	P-Value		0.0446	
	Adjusted P-Value		0.133	
Arginine	Mean	1.62	1.57	0.741 - 3.07
	Range	1.30 - 1.98	1.32 - 1.92	
	CI	1.42 - 1.85	1.37 - 1.79	
	P-Value		0.0502	
	Adjusted P-Value		0.133	
Aspartic Acid	Mean	2.00	2.06	0.980 - 3.52
	Range	1.26 - 2.54	1.77 - 2.50	
	CI	1.71 - 2.33	1.77 - 2.40	
	P-Value		0.152	
	Adjusted P-Value		0.312	
Cystine	Mean	0.606	0.618	0.311 - 1.24
	Range	0.505 - 0.751	0.523 - 0.722	
	CI	0.516 - 0.712	0.526 - 0.726	
	P-Value		0.592	
	Adjusted P-Value		0.737	
Glycine	Mean	1.38	1.35	0.688 - 2.52
	Range	1.09 - 1.61	1.17 - 1.54	
	CI	1.25 - 1.52	1.22 - 1.49	
	P-Value		0.162	
	Adjusted P-Value		0.317	
Glutamic Acid	Mean	5.05	5.01	1.99 - 11.9
	Range	2.48 - 6.68	4.30 - 6.40	
	CI	4.23 - 6.02	4.20 - 5.97	
	P-Value		0.783	
	Adjusted P-Value		0.868	
Histidine	Mean	0.800	0.801	0.342 - 1.72
	Range	0.644 - 0.966	0.667 - 0.939	
	CI	0.712 - 0.899	0.713 - 0.900	
	P-Value		0.945	
	Adjusted P-Value		0.957	
Isoleucine	Mean	1.08	1.06	0.533 - 1.95
	Range	0.869 - 1.30	0.922 - 1.25	
	CI	0.958 - 1.22	0.935 - 1.19	
	P-Value		0.113	
	Adjusted P-Value		0.250	
Leucine	Mean	1.88	1.83	0.910 - 3.42
	Range	1.52 - 2.26	1.59 - 2.19	
	CI	1.66 - 2.12	1.62 - 2.07	
	P-Value		0.0498	
	Adjusted P-Value		0.133	

Table 15. Summary Analysis of Amino Acid Composition in Canola Seed (continued)

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Lysine	Mean	1.65	1.64	0.729 - 3.16
	Range	1.25 - 2.02	1.48 - 1.97	
	CI	1.45 - 1.87	1.45 - 1.86	
	P-Value		0.669	
	Adjusted P-Value		0.793	
Methionine	Mean	0.464	0.472	0.258 - 0.857
	Range	0.383 - 0.546	0.402 - 0.546	
	CI	0.408 - 0.529	0.415 - 0.538	
	P-Value		0.596	
	Adjusted P-Value		0.737	
Phenylalanine	Mean	1.12	1.10	0.545 - 2.12
	Range	0.901 - 1.35	0.927 - 1.28	
	CI	1.01 - 1.26	0.985 - 1.23	
	P-Value		0.167	
	Adjusted P-Value		0.319	
Proline	Mean	1.63	1.59	0.717 - 3.36
	Range	1.46 - 1.98	1.38 - 1.95	
	CI	1.43 - 1.87	1.39 - 1.83	
	P-Value		0.123	
	Adjusted P-Value		0.265	
Serine	Mean	1.12	1.12	0.576 - 2.08
	Range	0.719 - 1.34	0.985 - 1.31	
	CI	1.01 - 1.25	1.00 - 1.25	
	P-Value		0.971	
	Adjusted P-Value		0.971	
Threonine	Mean	1.11	1.11	0.619 - 1.95
	Range	0.847 - 1.29	0.997 - 1.26	
	CI	1.02 - 1.22	1.01 - 1.21	
	P-Value		0.687	
	Adjusted P-Value		0.793	
Tryptophan	Mean	0.325	0.312	0.153 - 0.537
	Range	0.242 - 0.429	0.236 - 0.427	
	CI	0.256 - 0.382	0.239 - 0.371	
	P-Value		0.212	
	Adjusted P-Value		0.355	
Tyrosine	Mean	0.635	0.620	0.337 - 1.20
	Range	0.552 - 0.757	0.508 - 0.737	
	CI	0.573 - 0.704	0.560 - 0.688	
	P-Value		0.205	
	Adjusted P-Value		0.355	
Valine	Mean	1.39	1.36	0.682 - 2.49
	Range	1.04 - 1.66	1.20 - 1.59	
	CI	1.23 - 1.57	1.20 - 1.54	
	P-Value		0.159	
	Adjusted P-Value		0.317	

Mineral analysis

Several mineral ions are recognized as essential plant nutrients and are required by the plant in significant quantities. These macronutrients include calcium, phosphorus, magnesium, potassium and sodium. The micronutrient minerals, iron, copper, manganese and zinc are incorporated in plant tissues in only trace amounts. Both macro- and micro-nutrient minerals were analysed in seed samples from GM canola line 73496 and compared with corresponding values from samples of near-isoline control canola seed.

A statistically significant difference was observed in magnesium concentration between GM canola line 73496 and control canola using the adjusted P-value (Table 16). The magnitude of the difference was small and the range of individual values was within the tolerance interval determined for seed magnesium concentrations using commercial canola varieties.

In conclusion, mineral analysis of canola seed demonstrates that GM canola line 73496 is comparable to conventional canola.

Table 16. Summary Analysis of Mineral Composition in Canola Seed

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Minerals Composition (% Dry Weight)				
Calcium	Mean	0.465	0.474	0.172 - 0.939
	Range	0.365 - 0.576	0.397 - 0.572	
	CI	0.406 - 0.532	0.414 - 0.542	
	P-Value		0.457	
	Adjusted P-Value		0.625	
Phosphorus	Mean	0.788	0.756	0.299 - 1.58
	Range	0.543 - 1.13	0.567 - 1.14	
	CI	0.608 - 1.02	0.583 - 0.980	
	P-Value		0.0285	
	Adjusted P-Value		0.0974	
Magnesium	Mean	0.357	0.325	0.219 - 0.538
	Range	0.308 - 0.410	0.277 - 0.385	
	CI	0.327 - 0.390	0.298 - 0.356	
	P-Value		<0.0001	
	Adjusted P-Value		0.00216[a]	
Manganese	Mean	0.00381	0.00351	0.00174 - 0.00769
	Range	0.00275 - 0.00463	0.00278 - 0.00418	
	CI	0.00318 - 0.00458	0.00292 - 0.00422	
	P-Value		0.0334	
	Adjusted P-Value		0.110	

Table 16. Summary Analysis of Mineral Composition in Canola Seed (continued)

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Copper	Mean	0.000175	0.000185	0 - 0.000772
	Range	<0.000125[b] - 0.000251	<0.000125[b] - 0.000276	
	CI	0.000129 - 0.000236	0.000137 - 0.000250	
	P-Value		0.0972	
	Adjusted P-Value		0.221	
Iron	Mean	0.00677	0.00634	0.00163 - 0.0259
	Range	0.00452 - 0.0200	0.00412 - 0.0116	
	CI	0.00427 - 0.0107	0.00400 - 0.0100	
	P-Value		0.288	
	Adjusted P-Value		0.454	
Potassium	Mean	0.649	0.659	0.284 - 1.79
	Range	0.489 - 0.866	0.487 - 0.898	
	CI	0.525 - 0.803	0.533 - 0.816	
	P-Value		0.401	
	Adjusted P-Value		0.567	
Sodium	Mean	0.00168	0.00179	0.0000886 - 0.0270
	Range	0.000568 - 0.00599	0.000849 - 0.00505	
	CI	0.000790 - 0.00358	0.000840 - 0.00381	
	P-Value		0.507	
	Adjusted P-Value		0.671	
Zinc	Mean	0.00398	0.00384	0.00163 - 0.0101
	Range	0.00277 - 0.00548	0.00233 - 0.00594	
	CI	0.00317 - 0.00498	0.00306 - 0.00481	
	P-Value		0.288	
	Adjusted P-Value		0.454	

[a] Statistically significant difference, FDR adjusted P-Value <0.05.
[b] Analyte values were below the assay LLOQ. Sample results below the LLOQ were assigned a value equal to the LLOQ.

Vitamin analysis

Although not specifically mentioned in the OECD consensus document, a standard B vitamin analysis was conducted. No statistically significant differences were observed between GM canola line 73496 and the control canola mean values for vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B3 (niacin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), and vitamin B9 (folic acid; Table 13). These results indicate GM canola line 73496 is comparable to conventional canola with respect to key vitamins.

Tocopherols are listed in the OECD consensus document as additional important components of canola oil as natural antioxidants (OECD, 2001a). Concentrations of δ-tocopherol and total tocopherols were statistically significantly different between GM canola line 73496 and control canola seed samples using the adjusted P-value (Table 13). However, the differences were small in magnitude and in every case the range of values was within the respective tolerance interval determined using commercial canola varieties. The statistically significant differences observed for certain tocopherols are unlikely to be biologically meaningful.

In conclusion, vitamin analysis of canola seed demonstrates that GM canola line 73496 is comparable to conventional canola.

Table 17. Summary Analysis of Vitamin Composition in Canola Seed

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Vitamins Composition (mg/kg Dry Weight)				
Vitamin B1 (Thiamine)	Mean	14.2	14.8	4.92 - 33.1
	Range	9.65 - 21.1	10.6 - 25.8	
	CI	11.8 - 17.2	12.2 - 17.8	
	P-Value		0.611	
	Adjusted P-Value		0.737	
Vitamin B2 (Riboflavin)	Mean	2.70	3.14	1.45 - 7.33
	Range	2.04 - 3.98	2.27 - 5.88	
	CI	2.29 - 3.19	2.66 - 3.71	
	P-Value		0.00531	
	Adjusted P-Value		0.0507	
Vitamin B3 (Niacin)	Mean	188	180	61.0 - 444
	Range	156 - 245	154 – 208	
	CI	179 - 197	171 – 189	
	P-Value		0.188	
	Adjusted P-Value		0.338	
Vitamin B5 (Pantothenic Acid)	Mean	4.74	4.56	3.52 - 9.88
	Range	4.08 - 5.77	3.94 - 5.05	
	CI	4.31 - 5.21	4.15 - 5.01	
	P-Value		0.0273	
	Adjusted P-Value		0.0974	

Table 17. Summary Analysis of Vitamin Composition in Canola Seed (continued)

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Vitamin B6 (Pyridoxine)	Mean	3.69	3.39	1.17 - 20.9
	Range	2.58 - 6.01	2.54 - 5.25	
	CI	2.94 - 4.64	2.70 - 4.26	
	P-Value		0.0670	
	Adjusted P-Value		0.162	
Vitamin B9 (Folic Acid)	Mean	2.89	2.63	0.769 - 5.27
	Range	1.57 - 7.88	1.12 - 5.68	
	CI	2.05 - 4.06	1.87 - 3.69	
	P-Value		0.307	
	Adjusted P-Value		0.458	
α-Tocopherol	Mean	109	114	54.7 - 189
	Range	96.5 - 130	101 – 129	
	CI	98.8 - 120	103 – 125	
	P-Value		0.0179	
	Adjusted P-Value		0.0790	
β-Tocopherol	Mean	<1.25[a]	<1.25[a]	0 - 1.67[b]
	Range	<1.25[a]	<1.25[a]	
	CI	NA	NA	
	P-Value		NA	
	Adjusted P-Value		NA	
δ-Tocopherol	Mean	3.23	3.65	1.93 - 11.9
	Range	2.80 - 4.04	3.25 - 3.99	
	CI	3.11 - 3.35	3.51 - 3.78	
	P-Value		<0.0001	
	Adjusted P-Value		0.00216[c]	
γ-Tocopherol	Mean	170	177	106 - 378
	Range	143 - 196	146 – 205	
	CI	155 - 187	161 – 195	
	P-Value		0.00770	
	Adjusted P-Value		0.0525	
Total Tocopherols	Mean	283	295	191 - 499
	Range	243 - 313	250 – 332	
	CI	262 - 305	273 – 318	
	P-Value		0.00397	
	Adjusted P-Value		0.0465[c]	

[a] Analyte values were below the assay LLOQ. Sample results below the LLOQ were assigned a value equal to the LLOQ.

[b] Tolerance interval was not available due to insufficient sample values being detected above the assay LLOQ. Minimum and maximum values are used in place of a tolerance interval.

[c] Statistically significant difference, FDR adjusted P-Value <0.05.

Glucosinolate analysis

Glucosinolates are considered key toxicants of canola (OECD, 2001a). The major glucosinolates in canola are 3-butenyl glucosinolate (gluconapin), 4-pentenyl glucosinolate (glucobrassicanapin), 2- hydroxy-3- butenyl glucosinolate (progoitrin) and 2-hydroxy-4-pentenyl glucosinolate (napoleiferin).

Glucosinolates themselves are generally considered to be innocuous; however the hydrolysis products have negative effects on animal production. The low palatability and the adverse effects of glucosinolates due to their antithyroid activity led to the development of varieties of rapeseed which have combined low concentrations of both glucosinolates and erucic acid (also known as "double low" varieties). The total glucosinolate concentration for GM canola line 73496 was 5.66 µmoles/g dry weight, which is within the acceptable definition for canola (maximum 30 µmoles/g dry weight; OECD, 2001a).

A statistically significant difference was observed in progoitrin concentration between GM canola line 73496 and control canola samples based on the adjusted P-value. The range of concentrations of progoitrin in GM canola line 73496 was within the tolerance intervals established using commercial canola varieties (Table 18).

In conclusion, glucosinolate analysis of canola seed demonstrates that GM canola line 73496 is comparable to conventional canola.

Table 18. Summary Analysis of Glucosinolate Composition in Canola Seed

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Glucosinolates Composition (µmol/g Dry Weight)				
Glucoiberin	Mean	<0.176[a]	<0.176[a]	NC
	Range	<0.176[a]	<0.176[a]	
	CI	NA	NA	
	P-Value		NA	
	Adjusted P-Value		NA	
Progoitrin	Mean	0.524	0.412	0.130 - 11.6
	Range	0.224 - 1.29	0.181 - 1.37	
	CI	0.233 - 1.18	0.183 - 0.928	
	P-Value		0.00174	
	Adjusted P-Value		0.0238[b]	
Epi-Progoitrin	Mean	0.0717	<0.0708[a]	0 - 0.159[c]
	Range	<0.0708[a]- 0.0883	<0.0708[a]	
	CI	NA	NA	
	P-Value		NA	
	Adjusted P-Value		NA	
Glucoraphanin	Mean	0.0847	0.0789	0 - 0.565
	Range	<0.0488[g] - 0.217	<0.0488[g] - 0.143	
	CI	0.0655 - 0.110	0.0610 - 0.102	
	P-Value		0.401	
	Adjusted P-Value		0.567	

Table 18. Summary Analysis of Glucosinolate Composition in Canola Seed (continued)

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Gluconapoleiferin	Mean	0.0339	0.0330	0 - 0.372
	Range	<0.0299[a] - 0.0618	<0.0299[a] - 0.0544	
	CI	0.0280 - 0.0411	0.0272 - 0.0400	
	P-Value		0.272	
	Adjusted P-Value		0.446	
Gluconapin	Mean	0.759	0.773	0.197 - 10.0
	Range	0.286 - 2.01	0.304 - 2.66	
	CI	0.328 - 1.75	0.335 - 1.79	
	P-Value		0.777	
	Adjusted P-Value		0.868	
Glucoalyssin	Mean	0.0753	0.0897	0 - 0.663
	Range	<0.0676[a] - 0.125	<0.0676[a] - 0.223	
	CI	0.0508 - 0.111	0.0606 - 0.133	
	P-Value		0.0496	
	Adjusted P-Value		0.133	
4-Hydroxyglucobrassicin	Mean	3.36	3.41	1.28 - 11.3
	Range	1.53 - 6.06	1.78 - 8.33	
	CI	2.40 - 4.71	2.44 - 4.76	
	P-Value		0.836	
	Adjusted P-Value		0.911	
Glucobrassicin	Mean	0.404	0.383	0.0376 - 5.25
	Range	0.215 - 0.787	0.206 - 1.14	
	CI	0.271 - 0.600	0.257 - 0.569	
	P-Value		0.189	
	Adjusted P-Value		0.338	
Glucobrassicinapin	Mean	0.401	0.395	0 - 1.80
	Range	<0.390[a] - 0.475	<0.390[a] - 0.441	
	CI	NA	NA	
	P-Value		NA	
	Adjusted P-Value		NA	
Gluconasturtiin	Mean	0.255	0.286	0 - 5.39
	Range	0.133 - 0.991	0.139 - 0.610	
	CI	0.166 - 0.391	0.186 - 0.439	
	P-Value		0.302	
	Adjusted P-Value		0.458	
4-Methoxyglucobrassicin	Mean	<0.0774[a]	<0.0774[a]	NC
	Range	<0.0774[a]	<0.0774[a]	
	CI	NA	NA	
	P-Value		NA	
	Adjusted P-Value		NA	
Neoglucobrassicin	Mean	0.0466	0.0641	0 - 0.192
	Range	<0.0198[a] - 0.0903	0.0230 - 0.162	
	CI	0.0266 - 0.0815	0.0366 - 0.112	
	P-Value		0.00897	
	Adjusted P-Value		0.0525	

Table 18. Summary Analysis of Glucosinolate Composition in Canola Seed (continued)

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Total Glucosinolates	Mean	5.77	5.66	2.17 - 30.0
	Range	2.61 - 10.5	2.77 - 13.5	
	CI	3.72 - 8.94	3.65 - 8.77	
	P-Value		0.686	
	Adjusted P-Value		0.793	

[a] Theoretical LLOQ values were calculated by mathematically proportioning a known or measured canola seed glucosinolate concentration per measured signal-to-noise (S:N) ratio to a theoretical glucosinolate concentration per S:N of 10. Sample results below the theoretical LLOQ were designated < [LLOQ].
[b] Statistically significant difference, FDR adjusted P-Value <0.05.
[c] Tolerance interval was not available due to insufficient sample values being detected above the assay LLOQ. Minimum and maximum values are used in place of a tolerance interval.

Secondary metabolite and anti-nutrient analysis

Tannins, sinapine, and phytic acid are considered to be anti-nutrients in canola meal (OECD, 2001a). A major phenolic compound in canola, sinapine imparts a bitter taste to canola meal (OECD, 2001a). Phytic acid is the major form of phosphorus in plants; however, it is unavailable as a nutrient source for animals (OECD, 2001a).

Phytosterols are cholesterol-like molecules found in all plant foods, with the highest concentrations occurring in vegetable oils. They are absorbed only in trace amounts but have the beneficial effect of inhibiting the absorption of dietary cholesterol (Ostlund, 2002). Phytosterols are not endogenously synthesized in the body but are derived solely from the diet (Rao and Koratkar, 1997).

The only statistically significant difference found between GM canola line 73496 compared with control canola was for cholesterol based on the adjusted P-value. However, the range of values for cholesterol was within the established tolerance interval and is unlikely to be biologically meaningful (Table 19).

In conclusion, secondary metabolite and anti-nutrient analysis of canola seed demonstrates that GM canola line 73496 is comparable to conventional canola.

Table 19. Summary Analysis of Secondary Metabolite and Anti-Nutrient Composition in Canola Seed

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
Secondary Metabolites and Anti-Nutrients Composition (% Dry Weight)				
Tannins-Soluble	Mean	0.113	0.109	0.0521 - 0.297
	Range	0.0768 - 0.180	0.0777 - 0.176	
	CI	0.0840 - 0.152	0.0812 - 0.147	
	P-Value		0.415	
	Adjusted P-Value		0.577	
Tannins-Insoluble	Mean	0.330	0.237	0.0731 - 2.32
	Range	0.155 - 0.824	0.142 - 0.492	
	CI	0.218 - 0.499	0.157 - 0.359	
	P-Value		0.00874	
	Adjusted P-Value		0.0525	
Phytic Acid	Mean	1.84	1.73	0.684 - 6.06
	Range	1.04 - 2.86	1.01 - 3.00	
	CI	1.27 - 2.68	1.19 - 2.52	
	P-Value		0.0401	
	Adjusted P-Value		0.126	
Sinapine	Mean	0.928	0.937	0.538 - 1.33
	Range	0.763 - 1.12	0.804 - 1.09	
	CI	0.852 - 1.01	0.861 - 1.02	
	P-Value		0.558	
	Adjusted P-Value		0.727	
Cholesterol	Mean	0.000866	0.00110	0 - 0.00238
	Range	0.000685 - 0.00121	0.000850 - 0.00135	
	CI	0.000801 - 0.000938	0.00101 - 0.00119	
	P-Value		0.000281	
	Adjusted P-Value		0.00461[a]	
Brassicasterol	Mean	0.0147	0.0160	0.00830 - 0.108
	Range	0.0106 - 0.0190	0.0124 - 0.0200	
	CI	0.0129 - 0.0168	0.0141 - 0.0183	
	P-Value		0.0230	
	Adjusted P-Value		0.0945	
Campesterol	Mean	0.0921	0.0990	0.0249 - 0.257
	Range	0.0738 - 0.111	0.0707 - 0.125	
	CI	0.0775 - 0.109	0.0833 - 0.118	
	P-Value		0.0492	
	Adjusted P-Value		0.133	
Stigmasterol	Mean	0.00269	0.00268	0.000832 - 0.0113
	Range	0.00234 - 0.00316	0.00228 - 0.00335	
	CI	0.00248 - 0.00292	0.00247 - 0.00291	
	P-Value		0.942	
	Adjusted P-Value		0.957	

Table 19. Summary Analysis of Secondary Metabolite and Anti-Nutrient Composition in Canola Seed (continued)

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval
β-Sitosterol	Mean	0.132	0.137	0.0428 - 0.387
	Range	0.0967 - 0.158	0.103 - 0.174	
	CI	0.115 - 0.151	0.120 - 0.157	
	P-Value		0.176	
	Adjusted P-Value		0.328	
Total Sterols	Mean	0.242	0.257	0.0801 - 0.741
	Range	0.189 - 0.287	0.206 - 0.323	
	CI	0.215 - 0.274	0.228 - 0.290	
	P-Value		0.0846	
	Adjusted P-Value		0.198	

[a] Statistically significant difference, FDR adjusted P-Value <0.05.

B7(b) Levels of other GM-influenced constituents

Acetylated amino acids

The GAT4621 enzyme exhibits measureable activity with five amino acid substrates (L-aspartate, L-glutamate, L-serine, glycine and L-threonine) in addition to glyphosate. In studies using microbial-produced GAT4621, the level of catalytic efficiency of GAT4621 on aspartate, glutamate, serine, and threonine was 1%, 0.8%, 0.05%, and 0.06%, respectively, of that observed for glyphosate (Pioneer data not shown). The affinity of the GAT4621 enzyme for glycine was too low to estimate the level of catalytic efficiency. Levels of activity with other tested substrates, including a wide range of other amino acids and antibiotics, were below the limit of quantification (Pioneer data not shown).

Based on the activity of the GAT4621 enzyme, the concentrations of *N*-acetylaspartate (NAA), *N*-acetylglutamate (NAG), *N*-acetylserine (NAS), *N*-acetylthreonine (NAT), and *N*-acetylglycine (NAGly) were measured in samples of seed, whole plant, and processed fractions derived from GM canola line 73496 and control canola. (Refer to Study 16: PHI-2010-018/020, Study 17: 2010-019-020 for details, Study 18: PHI-2010-145/020, Study 19: 2010-155/020, Study 20: PHI-2010-107/020 and Study 21: PHI-2010-108/020 for details)

Acetylated amino acids in seed and whole plant samples

Concentrations of the five acetylated amino acids were measured in seed samples of GM canola line 73496 and control canola. Results are provided in Table 20. As expected based on catalytic efficiency, NAA and NAG were the two most abundant in GM canola line 73496. Mean concentrations of NAA and NAG were 1480 µg/g dry weight and 32.8 µg/g dry weight, respectively, for GM canola line 73496 seed samples (Table 20). Although the mean concentrations of NAT and NAS were elevated (statistically significant) in GM canola line 73496 seed, relative to the near-isoline control line, the range of individual values for both these acetylated amino acids were within the tolerance interval established using commercial canola varieties (Table 20). There was no statistically significant difference in NAGly concentration measured in GM canola line 73496 seed samples relative to the control line.

Concentrations of the five acetylated amino acids were measured in whole plant samples derived from greenhouse-grown GM canola line 73496 and control canola. Concentrations of all measured acetylated amino acids except NAS were elevated in whole plant samples of GM canola line 73496 plants compared to control (Table 21). Consistent with results obtained for seed samples obtained from GM canola line 73496, NAA and NAG were the two most abundant with mean concentrations of 4560 µg/g and 26 µg/g, in whole plant samples, respectively. These acetylated amino acids are normal components of food and feed, have a safe history of food and feed use, and are not novel substances (Appendix 1). The safety of these substances has also been evaluated in published studies as described in Appendix 1; therefore, it is not expected that the increase in these acetylated amino acids would adversely affect the safety of processed products from GM canola line 73496. (Refer to Study 18: PHI-2010-145/020 and Study 19: 2010-155/020 for details)

Table 20. Concentrations of Acetylated Amino Acids in Canola Seed

Analyte		Control Canola n=20	Herbicide-Treated 73496 Canola n=20	Tolerance Interval[a]
µg/g Dry Weight				
NAA	Mean[b]	1.24	1480	0.00861 - 4.43
	Range[c]	0.377 - 5.39	1200 – 1770	
	CI[d]	0[g] - 9.38	1340 – 1640	
	P-Value[e]		<0.0001	
	Adjusted P-Value[f]		<0.0001[g]	
NAG	Mean	0.628	32.8	0.0968 - 5.37
	Range	0.428 - 1.46	20.3 - 61.1	
	CI	0.00000752 - 2.50	24.4 - 42.5	
	P-Value		<0.0001	
	Adjusted P-Value		<0.0001[h]	
NAGly	Mean	0.0751	0.0825	0.0240 - 0.338
	Range	0.0481 - 0.125	0.0424 - 0.182	
	CI	0.0540 - 0.105	0.0592 - 0.115	
	P-Value		0.454	
	Adjusted P-Value		0.454	
NAS	Mean	0.843	1.04	0.0524 - 27.2
	Range	0.389 - 3.05	0.491 - 3.55	
	CI	0.437 - 1.63	0.542 - 2.01	
	P-Value		0.0035	
	Adjusted P-Value		0.00528[h]	
NAT	Mean	0.110	0.546	0.0140 - 1.74
	Range	0.0531 - 0.212	0.260 - 1.64	
	CI	0.0665 - 0.181	0.331 - 0.902	
	P-Value		<0.0001	
	Adjusted P-Value		<0.0001[h]	

[a] The statistical tolerance interval was calculated from commercial canola varieties and calculated to contain with 95 percent confidence, 99 percent of the population of canola, negative limits set to zero
[b] Least squares mean were generated from a mixed model analysis
[c] Range denotes the lowest and highest individual value across sites
[d] Confidence Interval
[e] Non-adjusted P-value
[f] False Discovery Rate (FDR) adjusted P-value
[g] The lower limit of the confidence interval was negative on the transformed scale and was set to zero.prior to back-transformation
[h] Statistically significant difference, FDR adjusted P-Value <0.05.

Table 21. Concentrations of Acetylated Amino Acids in Canola Whole Plant Samples

Analyte		Control Canola n=15	Herbicide-Treated 73496 Canola n=15
		µg/g[a]	
NAA	Mean[b]	0.705	4560
	Range[c]	0.404 - 1.23	3730 - 5340
	CI[d]	0.649 - 0.766	4190 - 4950
	P-value[e]		<0.0001
	Adjusted P-value[f]		<0.0001[g]
NAG	Mean	2.04	26.0
	Range	1.45 - 3.27	21.0 - 35.9
	CI	1.87 - 2.22	23.8 - 28.3
	P-value		<0.0001
	Adjusted P-value		<0.0001[g]
NAGly	Mean	0.152	0.344
	Range	0.122 - 0.193	0.247 - 0.445
	CI	0.139 - 0.166	0.316 - 0.376
	P-value		<0.0001
	Adjusted P-value		<0.0001[g]
NAS	Mean	14.0	13.0
	Range	10.2 - 21.9	9.17 - 22.0
	CI	12.3 - 15.9	11.5 - 14.7
	P-value		0.406
	Adjusted P-value		0.406
NAT	Mean	1.92	7.67
	Range	1.60 - 2.50	5.43 - 11.8
	CI	1.76 - 2.11	7.00 - 8.39
	P-value		<0.0001
	Adjusted P-value		<0.0001[g]

[a] Results reported as-is after lyophilization without adjusting for moisture
[b] Least squares mean were generated from a mixed model analysis
[c] Range denotes the lowest and highest individual value across sites.
[d] Confidence Interval
[e] Non-adjusted P-value
[f] False Discovery Rate (FDR) adjusted P-value
[g] Statistically significant difference, FDR adjusted P-Value <0.05.

Acetylated amino acids in processed fractions

Whole canola seed is not commonly consumed. Because the typical processed canola products consumed by animals and humans are meal and oil, respectively, the concentrations of the five acetylated amino acids were measured in processed fractions of GM canola line 73496 and control canola.

As expected, based on data from whole seed samples, NAA and NAG were the two most abundant acetylated amino acids in GM canola line 73496 meal. Mean concentrations of NAA and NAG were 2872 µg/g and 62.3 µg/g, respectively, for GM canola line 73496 toasted meal from seed with hulls (i.e., seed coats) (Table 22). The corresponding concentrations from toasted meal produced from canola seed without hulls of each event were similar at 3013 and 66.0 µg/g, respectively for NAA and NAG. The concentrations of each of the remaining acetylated amino acids, NAT, NAS and NAGly, were approximately 30–100 fold less than the concentration of NAG measured in toasted meal produced from GM canola line 73496 seed either with or without hulls. (Refer to Study 20: PHI-2010-107/020 and Study 21: PHI-2010-108/020 for details)

On an equivalent weight basis, the elevated concentrations of acetylated amino acids in toasted meal, relative to concentrations measured in whole seed samples, reflect removal of the oil component, which is not expected to contain these compounds. This was confirmed through analysis of oil samples, where the concentrations of each acetylated amino acid were either not detectable or below the limit of quantification in all samples of refined, bleached, deodorized oil (RBD oil, Table 22).). Moreover, these acetylated amino acids are normal components of food and feed, have a safe history of food and feed use, and are not novel substances (Appendix 1). The safety of these substances has also been evaluated in published studies as described in Appendix 1; therefore, it is not expected that the increase in these acetylated amino acids would adversely affect the safety of processed products from GM canola line 73496.

Table 22. Concentrations of Acetylated Amino Acids in Processed Fractions Produced from GM Canola Line 73496 and Control Canola

Fraction	Canola Line	Concentration (Range) n=2[a]				
		NAA[b]	NAG	NAT	NAS	NAGly
		µg/g[c]				
Toasted Meal (with hulls)	73496	2872 (2822 - 2921)	62.3 (57.7 - 66.8)	0.585 (0.558 - 0.612)	1.20 (1.11 - 1.29)	0.151 (0.145 - 0.157)
	Control	2.31 (2.15 - 2.47)	1.36 (1.31 - 1.41)	0.161 (0.146 - 0.175)	1.46 (1.39 - 1.53)	0.129 (0.114 - 0.144)
Toasted Meal (without hulls)	73496	3013 (3002 - 3024)	66.0 (62.6 - 69.3)	0.788 (0.535 - 1.04)	2.27 (1.98 - 2.55)	0.224 (0.184 - 0.265)
	Control	2.58 (2.00 - 3.16)	1.71 (1.63 - 1.80)	0.226 (0.195 - 0.258)	1.76 (1.51 - 2.01)	0.180 0.163 - 0.197)
RBD Oil (with hulls)	73496	ND[d]	ND - <LLOQ	ND - <LLOQ	ND	ND - <LLOQ
	Control	ND	ND	<LLOQ	ND	ND - <LLOQ
RBD Oil (without hulls)	73496	ND - <LLOQ[e]	<LLOQ	ND	ND	ND -<LLOQ
	Control	ND	ND	ND	ND	ND

[a] Statistics are not provided due to small sample size.
[b] NAA = N-acetylaspartate; NAG = N-acetylglutamate; NAT = N-acetylthreonine; NAS = N-acetylserine; NAGly = N-acetylglycine
[c] Values are not corrected for moisture content.
[d] ND = Not detected
[e] LLOQ = Lower limit of quantification, which was 0.01 µg/g for a 100 mg sample

Conclusions on the compositional analysis of GM canola line 73496

The analytes for compositional assessment were selected considering the OECD consensus document on compositional considerations for new varieties of canola (OECD, 2001a). Among the numerous compositional analyses that were carried out, concentrations of most analytes were not significantly different between GM canola line 73496 and control canola. Statistically significant differences were noted for concentrations of oleic and linoleic fatty acids; delta- and total tocopherols; magnesium; the glucosinolate progoitrin; and cholesterol. However, the magnitudes of the differences were small and in every case the ranges of values was all within the respective tolerance interval established using commercial canola varieties. Overall, no consistent patterns emerged to suggest that biologically significant changes in composition or nutritive value of the seed had occurred as an unexpected result of the transformation process.

In addition to the compositional analytes per OECD guidelines, the concentrations of NAA, NAG, NAS, NAT, and NAGly were measured in samples of seed, whole plant, and processed fractions derived from GM canola line 73496. As described earlier, these analyses were conducted because the GAT4621 protein is known to acetylate certain free amino acids (L-

aspartate, L-glutamate, glycine, L-serine, and L-threonine) resulting in the production of NAA, NAG, NAGly, NAS, and NAT, respectively. As expected, NAA and NAG were the most abundant in seed, whole plant, and processed meal fractions. Refined, bleached, deodorized oil fractions did not contain measurable levels of these five acetylated amino acids. These acetylated amino acids are normal components of food and feed, have a safe history of food and feed use, and are not novel substances (Appendix 1). The safety of these substances has also been evaluated in published studies as described in Appendix 1; therefore, it is not expected that the increase in these acetylated amino acids would adversely affect the safety of processed products from GM canola line 73496.

Based on the OECD guidelines for compositional equivalence, we have concluded that GM canola line 73496 was compositionally comparable to conventional canola. The increases in certain acetylated amino acids do not negatively impact the safety of GM canola line 73496 and will not have an adverse impact on processed commodity products from GM canola line 73496.

B7(c) Levels of naturally-occurring allergenic proteins

Not applicable.

C Nutritional Impact

C1 Data on nutritional impact of compositional changes

Nutritional impact of GM canola line 73496 on human diet

Extensive nutritional compositional analyses of seed were conducted to evaluate the composition of GM canola line 73496 as compared to control canola. Compositional analysis of GM canola line 73496 was used to evaluate any changes in the levels of key nutrients, anti-nutrients or secondary metabolites (section B7(a)). Based on the compositional evaluation, the seed of GM canola line 73496 is considered to be comparable to conventional canola with respect to nutrient composition.

Although findings demonstrated elevated concentrations of NAA and NAG, and in some instances NAGly, NAS, and NAT when compared with the corresponding concentrations of these substances in unmodified canola, the levels of each *N*-acetylated amino acid in refined, bleached, deodorized (RBD) oil (major fraction that is consumed by humans) were either not detectable or below the limit of quantification (section B7(b), Table 22). And, these five acetylated amino acids are not novel substances as they are present in conventional canola as well as in other plants. Therefore, the increased concentration of these acetylated amino acids in GM canola line 73496 will have no nutritional inpact on humandiet.

C2 Data from an animal feeding study, if available

Based on the food safety assessment of GM canola line 73496, Pioneer concludes that there will be no adverse effects to human health resulting from the consumption of GM canola line 73496. Therefore, animal feeding study would not be warranted or add further value to the food safety assessment of GM canola line 73496.

STATUTORY DECLARATION – AUSTRALIA

Statutory Declarations Act 1959

I, [redacted] Managing Director for Pioneer Hi-Bred Australia, LMB 9001 204 Wyreema Road, Toowoomba, QLD 4350 AU, make the following declaration under the *Statutory Declarations Act 1959*:

1. the information provided in this application fully sets out the matters required
2. the information provided in this application is true to the best of my knowledge and belief
3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

…….....
[redacted]

Declared at……..on ……..of…….

[place] [day] [month] [year]

Before me,

…… ……
Signature of person before whom this declaration is made

…….....
[Full name, qualification and address of person before whom this declaration is made]

Supporting studies

1. PHI-2009-134: Characterization of DP-Ø73496-4 Canola: Insertion Integrity, Stability, Copy Number, and Backbone Analysis
2. PHI-2010-086/040: Sequence Characterization of Insert and Flanking Genomic Regions of Canola Event DP-Ø73496-4 **(CCI)**
3. PHI-2012-283: ORF Analysis at the Insertion Site of Canola Event DP-Ø73496-4
4. PHI-2010-089/010: Endpoint PCR Analysis of Five Generations of Canola Containing Event DP Ø73496-4
5. PHI-2010-127: Development and Validation of Event-Specific qPCR Detection Method for DP-Ø73496-4 **(CCI)**
6. PHI-2010-020: Characterization of GAT4621 Protein Derived from Canola Containing Event DP-Ø73496-4 and Equivalency Assessment with the GAT4621 Protein Derived from a Microbial Expression System
7. PHI-2009-039/010: Quantification of GAT4621 Protein in Tissues of Herbicide-Treated Canola Lines Containing Event DP-Ø73496-4: U.S. and Canada Test Sites
8. PHI-2007-009/073: Evaluation of the Amino Acid Sequence Similarity of the GAT4621 Protein to the NCBI Protein Sequence Datasets
9. PHI-2006-184/018: Characterization of the Thermal Stability of Glyphosate Acetyltransferase Enzyme Activity: GAT4621
10. PHI-2006-120: Characterization of the *In Vitro* Pepsin Resistance of Glyphosate *N*-acetyltransferase 4621 Protein
11. PHI-2005-110: GAT4621: Acute Oral Toxicity Study in Mice
12. PHI-2007-008/073: Comparison of the Amino Acid Sequence Identity Between the GAT4621 Protein and Known Protein Allergens
13. PHI-2006-122: Characterization of the *In Vitro* Pancreatin Resistance of Glyphosate *N*-acetyltransferase 4621
14. PHI-2009-057: Magnitude and Decline of Glyphosate Related Residues in Forage and Seed of Genetically Modified Canola Event DP-Ø73496-4 and Magnitude of Glyphosate Related Residues in Canola Event DP-Ø73496-4 Seed Process Fractions Following Applications of Touchdown Total® Herbicide- Locations in the United States and Canada, Season 2009
15. PHI-2009-039/020: Nutrient Composition of an Herbicide-Treated Canola Line Containing Event DP-Ø73496-4: U.S. and Canada Test Sites

16. PHI-2010-018/020: *N*-Acetylaspartate and *N*-Acetylglutamate Concentrations in Seed of an Herbicide-Treated Canola Line Containing Event DP Ø73496 4: U.S. and Canada Test Sites
17. PHI-2010-019/020: *N*-Acetylglycine, *N*-Acetylserine, and *N*-Acetylthreonine Concentrations in Seed of an Herbicide Treated Canola Line Containing Event DP Ø73496 4: U.S. and Canada Test Sites
18. PHI-2010-145/020: Concentration of *N*-Acetylaspartate and *N*-Acetylglutamate in Whole Plant Tissues Derived from a Canola Line Containing Event DP-Ø73496-4
19. 2010-155/020: Concentration of *N*-Acetylglycine, *N*-Acetylserine, and *N*-Acetylthreonine in Whole Plant Tissues Derived from a Canola Line Containing Event DP-Ø73496-4
20. PHI-2010-107/020: Concentration of *N*-Acetylaspartate and *N*-Acetylglutamate in Processed Products from Seed of a Canola Line Containing Event DP-Ø73496-4: U.S. and Canada Test Sites
21. PHI-2010-108/020: Concentration of *N*-Acetylglycine, *N*-Acetylserine, and *N*-Acetylthreonine in Processed Products from Seed of a Canola Line Containing Event DP-Ø73496-4: U.S. and Canada Test Sites

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Appendices

Appendix 1. Safety and History of Consumption of Acetylated Amino Acids

A1.1 Summary

In addition to glyphosate, the GAT4621 enzyme is known to acetylate five amino acids: aspartate, glutamate, threonine, serine, and glycine. Acetylated amino acids are ubiquitous in nature, and are part of many biological systems in plants and animals. In addition, acetylated amino acids can be used in animal feed applications and industrial applications. A large amount of data has been developed on the safety of consumption of NAA, NAG, NAT, NAS, and NAGly. Collectively, these data indicate that acetylated amino acids have a history of safe use and are as safe for consumption as table salt.

A1.2 Function and Use of Acetylated Amino Acids

Acetylated amino acids are naturally occurring substances that have been identified in many biological systems. Acetylation of *N*-terminal amino acids is the most commonly observed posttranslational modification of cytosolic proteins (Persson *et al.,* 1985; Polevoda and Sherman, 2002). It has been estimated that up to 80% of all cytosolic proteins in mammalian systems are *N*-acetylated (Brown and Roberts, 1976; Driessen *et al*., 1985). Enzymatic acetylation of amino acids using acetyl-CoA as the acetyl donor group can occur either co-translationally or post-translationally depending on the biological system (Polevoda and Sherman, 2000). Enzymes responsible for intracellular acetylation of amino acids (*N*-acetyltransferases) have been identified in rat, yeast, and other eukaryotic organisms (Lee *et al*., 1988; Lee *et al*., 1989; Mullen *et al*., 1989; Yamada and Bradshaw, 1991a; Yamada and Bradshaw, 1991b). The biological role of acetylation of *N*-terminal amino acids of cytosolic proteins has been investigated and evidence indicates that this modification protects proteins from proteolysis by intracellular aminopeptidases (Berger *et al*., 1981; Brown, 1979; Jörnvall, 1975). A variety of additional roles for *N*-acetylation of amino acids in biological systems have been established (Polevoda and Sherman, 2002).

Taking into account the wide distribution and biological roles of acetylation, it is not surprising that a number of enzymes responsible for deacetylation of acetylated amino acids (*i.e.*, acylases) have also been described. It has long been speculated that enzymatic deacetylation of amino acids is a “general” phenomenon in mammals because this reaction has been observed in numerous organs (Neuberger and Sanger, 1943). It was later hypothesized that this enzymatic reaction plays a role in the salvage of acetylated amino acids formed during the metabolic degradation of N-terminal acetylated proteins (Endo, 1980; Gade and Brown, 1981). To date, four classes of acylases (Types I – IV) that mediate deacetylation of acetylated amino

acids have been described in mammalian systems that differ with regard to distribution and specificity.

A1.3 History of Safe Use of Acetylated Amino Acids

Acetylation of proteins is commonly employed in the food industry to alter the solubility, water absorption capacity and emulsifying properties of protein concentrates (*e.g.*, El-Adawy, 2000; Ramos and Bora, 2004). Another well-characterized use of acetylated amino acids is in the livestock industry in cases when it is unsuitable to use free amino acids in feed. For example, the quality of soy protein fractions can be limited by the concentrations of the essential amino acid L-methionine. This deficiency can be overcome by supplementation of diets with free L-methionine; however this can lead to development of objectionable odours and flavours from conversion of L-methionine to methional by Strecker degradation (Ballance, 1961). Therefore, feed may be supplemented with *N*-acetyl-L-methionine. In rats fed diets produced with soy protein isolates, growth and weight gains were similar regardless of whether they were supplemented with L-methionine or *N*-acetyl-L-methionine (Amos *et al*., 1975; Boggs *et al*., 1975).

Metabolism studies have demonstrated that *N*-acetyl-^{14}C-L-methionine is readily metabolized toL-methionine in rats and in human infants (Boggs, 1978; Stegink *et al.,* 1980; Stegink *et al.,* 1982). Similarly, metabolic deacetylation of N-acetyl-L-methionine has been reported in *in vitro* studies using rabbit intestinal epithelial cells (Brachet *et al*., 1991). While these reports demonstrate that this enzymatic deacetylation occurs within the digestive system, there is also evidence that *N*-acetyl amino acids are deacetylated in other tissues (Yoshida and Lin, 1972).

Nutritional and metabolic studies with the *N*-acetyl forms of some amino acids have been conducted in humans, rats, and pigs. In most cases, these studies have reported that the *N*-acetyl form of amino acids substitute for the constituent amino acid via metabolic deacetylation. Such results have been reported for glutamate (Arnaud *et al*., 2004; Magnusson *et al*., 1989; Neuhäuser and Bässler, 1986), phenylalanine and tryptophan (du Vigneaud *et al.,* 1934) and threonine (Boggs, 1978). Although specific information is not available for aspartate, threonine, serine, or glycine, there is no reason to believe these amino acids would not also be biologically available when acetylated.

A1.4 Presence of NAA, NAG, NAT, NAS, and NAGly in Biological Systems

Plants expressing the GAT4621 enzyme are known to contain increased concentrations of some or all of the following five acetylated amino acids: NAA, NAG, NAT, NAS, and NAGly. As described earlier, acetylated amino acids are known to be widely present in biological systems and some additional information about the cellular location and possible function of these five acetylated amino acids are described further below.

A1.4.1 NAA

NAA is a component of the mammalian central nervous system (CNS). It was first isolated from the brains of cats in 1956 (Tallan *et al*., 1956). Since that report NAA has been found to be a common constituent of the CNS of all mammals (reviewed in Moffett *et al*., 2007) where it is located almost entirely within the neurons and produced at high concentrations. It has been reported that NAA is the second most abundant free amino acid in the mammalian CNS – second only to the concentrations of free glutamate (Miyake *et al*., 1981; Simmons *et al*., 1991; Tsai and Coyle, 1995; Urenjak *et al*., 1993).

The metabolic pathway by which NAA is synthesized in the mammalian CNS has also been determined. It is produced enzymatically by acetylation of L-aspartate using acetyl-CoA as a cofactor. The enzyme responsible for this reaction is aspartate-*N*-acetyltransferase (E.C. 2.3.1.17). It is expressed exclusively in neuron mitochondria (Demougeot *et al*., 2004; Truckenmiller *et al*., 1985).

Within the mammalian CNS, NAA is readily metabolized to L-aspartate and free acetate by the enzyme *N*-acetyl-L-aspartate amidohydrolase (EC 3.5.1.15; D'Adamo *et al*., 1973; Goldstein, 1976). This enzyme has also been called acylase II and aspartoacylase in published literature and will hereafter be referred to as aspartoacylase. Recent studies have demonstrated that aspartoacylase is expressed by oligodendrocytes (the cells responsible for synthesis of myelin in the CNS) but not by neurons or astrocytes (Baslow *et al*., 1999; Madhavarao *et al*., 2004).

NAA is the primary source of acetate required for lipid synthesis used for axonal myelination during development of the mammalian CNS (D'Adamo *et al*., 1968; Patel and Clark, 1979). Aspartoacylase liberates acetate from NAA which is then used in the biosynthesis of lipids used for myelination of the axons of the developing CNS. In fact, it has been reported that the activity of aspartoacylase in the brains of developing rats correlates with the time course of myelination of the brain (Bhakoo *et al*., 2001; Kirmani *et al*., 2002; Kirmani *et al*., 2003); therefore NAA is critical for proper development of the mammalian CNS.

A1.4.2 NAG

NAG has been identified as a component of a number of organs (liver, small intestine, brain, kidneys, spleen and heart) in numerous animal species (rats, mice and other animals) using different analytical methods (Alonso *et al*., 1991; Reichelt and Kvamme, 1967; Shigesada and Tatibana, 1971a), and serves as the first intermediate in the biosynthesis of arginine in prokaryotes, lower eukaryotes and plants (Caldovic and Tuchman, 2003). Glutamate is the most abundant amino acid in the mammalian brain and it is a principal neurotransmitter in the central nervous system (CNS; Al-Sarraf and Philip, 2003; reviewed by Meldrum, 2000).

Biochemical studies have demonstrated that NAG in mammals is produced enzymatically by N-acetylglutamate synthase (NAGS; E.C. 2.3.1.1), which acetylates glutamate using acetyl-CoA as a cofactor (Caldovic and Tuchman, 2003). This occurs in mitochondria of parenchymal cells from most tissues, as high levels of NAGS activity and NAGS specific mRNA have been

identified in liver and small intestinal mucosal cells of rats and humans though neither NAGS protein nor mRNA have been detected in brain tissue (Caldovic *et al*., 2002a; Caldovic *et al*., 2002b; Uchiyama *et al*., 1981).

Mammalian metabolism of NAG is also well characterized. NAG is hydrolyzed enzymatically by aminoacylase I (EC 3.5.1.14) to glutamate and acetate within the cytosol of kidney and liver parenchymal cells (Reglero *et al*., 1977; Shigesada and Tatibana, 1971b). The primary biological activity of NAG is the allosteric regulation of carbamyl phosphate synthetase I (CPSI; E.C. 6.3.4.16) activity; the first enzymatic step in the urea cycle which is responsible for elimination of excess ammonia from metabolic processes (Caldovic and Tuchman, 2003). CPSI is an intramitochondrial enzyme that converts ammonia and bicarbonate into carbamyl phosphate which is subsequently combined with ornithine via ornithine transcarbamylase (EC 2.1.3.3) to form citrulline which is then exported from the mitochondria. The activity of CPSI is so dependent on NAG that the activity of this enzyme is virtually undetectable in the absence of NAG (Caldovic and Tuchman, 2003; Hall *et al*., 1958). This dependence has clinical relevance for humans and other mammals because deficiency of NAG can arrest urea cycle metabolism and lead to hyperammonemia (Caldovic *et al*., 2002a).

A1.4.3 NAT

NAT is a derivative of the amino acid L-threonine bearing an acetyl group covalently linked to the amine nitrogen. It has been identified as the N-terminal amino acid of a number of dietary proteins though the biological function is unknown (Polevoda and Sherman, 2003).

Acetylation of proteins has been reported to protect proteins from degradation (Persson *et al*., 1985), assist in the export of soluble proteins from the cell (Chang *et al*., 2008), and block activation of signalling pathways by antagonizing phosphorylation of threonine residues (Mukherjee *et al*., 2007).

A1.4.4 NAS

NAS is an acetylated derivative of the amino acid L-serine and has been identified on the N-terminus of proteins as a commonly occurring post-translational modification of proteins in eukaryotes (Brown and Roberts, 1976; Polevoda and Sherman, 2003). Acetylation of proteins has been reported to protect proteins from degradation (Persson *et al*., 1985), assist in the export of soluble proteins from the cell (Chang *et al*., 2008), and block activation of signalling pathways by antagonizing phosphorylation of serine residues (Mukherjee *et al*., 2007).

Within nature, NAS is one of the most commonly acetylated amino acids of proteins. In fact, it has been estimated that approximately 90% of proteins with N-terminal serine residues are acetylated (Driessen *et al*., 1985). Accordingly, proteins containing NAS are likely to be routinely consumed in the diet from a wide variety of plant and animal sources (Brown and Roberts, 1976; Persson *et al*., 1985).

Plants and microorganisms are able to reduce inorganic sulphur resulting in L-cysteine biosynthesis. A pathway for this has been described in microorganisms involving free NAS. Through a feedback inhibition mechanism, L-cysteine inhibits the biosynthesis of O-acetyl-L-serine, which is the precursor for NAS, an inducer of the cysteine regulon. (Kredich, 1996).

A1.4.5 NAGly

A number of naturally occurring proteins from eukaryotic cells contain NAGly, including; cytochrome c, hemoglobin and ovalbumin (Brown and Roberts, 1976). Specific activity for the hydrolysis of NAGly has been observed in liver, kidney and brain tissue (Bray *et al*., 1949; Bray *et al*., 1950; Giardina *et al*., 2000; Goldstein, 1976; Mounter *et al*., 1958; Reglero *et al*., 1977).

Aminoacylase I (ACY1; EC 3.5.1.14) is an enzyme involved in the cytoplasmic degradation of N-acetylated derivatives of serine, glutamic acid, alanine, methionine, glycine, leucine, and valine. When aminoacylase activity is compromised, as in the case of ACY1 deficiency, there is marked increase in urinary excretion of acetylated amino acids including NAGly (Gerlo *et al*., 2006; Sass *et al*., 2006).

A1.5 History of Safe Consumption of NAA, NAG, NAS, NAT, and NAGly

NAA, NAG, NAS, NAT, and NAGly are normal components of food and feedstuffs. As demonstrated by the compositional analysis described in Section VIII and more extensively in submissions for previously approved 98140 maize (US-FDA, 2008; USDA-APHIS, 2009), these compounds are found in plant species including canola, are not novel, and are normal components of animal diets.

A1.6 Toxicology Studies Conducted with NAA, NAG, NAS, NAT, and NAGly

A substantial number of toxicology studies have been conducted with NAA, NAG, NAS, NAT, and NAGly. There was no evidence of mutagenicity in individual *in vitro* and *in vivo* studies with NAA, NAG, NAS, NAT and NAGly (Harper *et al.,* 2009; Harper *et al.,* 2010; Karaman *et al*., 2009; van de Mortel *et al.,* 2010a; van de Mortel *et al.,* 2010b). In addition, no adverse effects were observed in rats following acute oral exposure to NAA, NAG, NAS, NAT and NAGly individually at 2000 mg/kg of body weight or following repeated dose dietary exposure to approximately 1000 mg/kg of body weight (Delaney *et al*., 2008; Harper *et al.,* 2009; Harper *et al.,* 2010; van de Mortel *et al.,* 2010a; van de Mortel *et al.,* 2010b). Mortalities and clinical signs of toxicity were observed in rats that were orally dosed with NAA at 5000 mg/kg of body weight in an acute toxicity study, indicating that the acute toxicity of this particular substance is similar to that reported for table salt (Delaney, 2010). In addition, there was no evidence of adverse effects in longer term (i.e., 90 day) NAA feeding studies at doses of approximately 500 mg/kg of body weight in which brain myelination was evaluated (Karaman *et al*., 2011). A two generation reproductive toxicity study with NAA that included evaluation of brain lipid myelination and tissue

concentrations of NAA in which no evidence of adverse effects were observed was also conducted, however, this study has not yet been published. The full list of toxicology studies conducted by Pioneer is listed in Table 23.

Table 23. Full List of Studies Conducted with NAA, NAG, NAS, NAT, and NAGly

Acetylated Amino Acid	Publication	Study
NAA	Karaman et al., 2009	Ames *in vitro* mutagenicity. Bone marrow micronucleus *in vivo* mutagenicity
	Delaney et al., 2008 and Delaney, 2010	Acute oral toxicity
	Delaney et al., 2008	28-Day repeated dose oral toxicity
	Karaman et al., 2011	90-Day repeated dose oral toxicity
	Not yet published	2-Generation reproduction toxicity
NAG	Harper et al., 2009	Ames *in vitro* mutagenicity. Bone marrow micronucleus *in vivo* mutagenicity Acute oral toxicity 28-Day repeated dose oral toxicity.
NAT	van de Mortel et al., 2010a	Ames *in vitro* mutagenicity. Bone marrow micronucleus *in vivo* mutagenicity. Acute oral toxicity 28-Day repeated dose oral toxicity.
NAS	van de Mortel et al., 2010b	Ames *in vitro* mutagenicity. Bone marrow micronucleus *in vivo* mutagenicity. Acute oral toxicity 28-Day repeated dose oral toxicity.
NAGly	Harper et al., 2010	Ames *in vitro* mutagenicity. Bone marrow micronucleus *in vivo* mutagenicity. Acute oral toxicity 28-Day repeated dose oral toxicity.

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