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Petition for the Determination of Nonregulated Status for Herbicide-Tolerant 73496 Canola

We submit this petition under 7 CFR §340.6 to request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article should no longer be regulated under 7 CFR §340.

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TABLE OF CONTENTS

LIST OF TABLES 5

LIST OF FIGURES 7

RELEASE OF INFORMATION 9

CERTIFICATION 10

SUMMARY 11

ABBREVIATIONS, ACRONYMS, AND DEFINITIONS 13

I. RATIONALE FOR THE DEVELOPMENT OF 73496 CANOLA 15

I-A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR §340.6 15
I-B. Benefits of 73496 Canola 15
I-C. Submissions to Other Regulatory Agencies 15

II. THE BIOLOGY OF CANOLA 16

II-A. Canola as a Crop 16
II-B. Description of the Non-Transformed Recipient Canola Line 16

III. METHOD OF DEVELOPMENT OF 73496 CANOLA 16

III-A. Description of Transformation, Selection, and Breeding 16
III-B. Selection of Comparators for 73496 Canola 18

IV. DONOR GENES AND REGULATORY SEQUENCES 18

IV-A. Fragment PHP28181A Used in Transformation 18
IV-B. Identity and Source of the *gat4621* Gene Cassette on Fragment PHP28181A 20

V. GENETIC CHARACTERIZATION OF 73496 CANOLA 21

V-A. Molecular Analysis Overview 21
V-B. Results of Molecular Characterization of 73496 Canola by Southern Blot Analysis 21
V-C. Inheritance and Genetic Stability of the Introduced Trait in 73496 Canola 34
V-D. Conclusions on Molecular Characterization and Genetic Stability of 73496 Canola 35

VI. CHARACTERIZATION OF THE INTRODUCED GAT4621 PROTEIN 36

VI-A. The GAT4621 Protein 36
VI-B. Physicochemical Characterization of the GAT4621 Protein 38
VI-C. Concentrations of GAT4621 in 73496 Canola 38
VI-D. Summary of the Food and Feed Safety Assessment for the GAT4621 Protein 40

VII. AGRONOMIC PERFORMANCE AND ECOLOGICAL OBSERVATIONS 42

VII-A. Germination Evaluations 42
VII-B. Field Trial Evaluations 43
VII-C. Ecological Observations 52
VII-D. Conclusions on Agronomic Performance and Ecological Observations 53

TABLE OF CONTENTS (CONTINUED)

VIII. COMPOSITIONAL ASSESSMENT 54

VIII-A. Proximate and Fiber Analysis 55
VIII-B. Fatty Acid Analysis 57
VIII-C. Amino Acid Analysis 60
VIII-D. Vitamin Analysis 63
VIII-E. Mineral Analysis 65
VIII-F. Glucosinolate Analysis 67
VIII-G. Secondary Metabolite and Anti-Nutrient Analysis 70
VIII-H. Acetylated Amino Acids 72
VIII-I. Conclusions on the Compositional Analysis of 73496 Canola 75

IX. ASSESSMENT OF PLANT PEST RISK FOR 73496 CANOLA 77

IX-A. Environmental Assessment of the Transgenic Protein 77
IX-B. Fate of Transgenic DNA in Humans and Animals 77
IX-C. Weediness Potential of 73496 Canola 77
IX-D. Gene Flow Assessment 81

APPENDIX 1. MATERIALS AND METHODS FOR GENETIC CHARACTERIZATION OF 73496 CANOLA 92

1.1. Southern Blot Characterization of 73496 Canola 92
1.2. Mendelian Inheritance and Genetic Stability of the Trait in 73496 Canola 95

APPENDIX 2. DESCRIPTION OF STATISTICAL ANALYSES PERFORMED FOR AGRONOMIC AND NUTRIENT COMPOSITION DATA 97

2.1. Statistical Analysis of Germination Data 97
2.2. Statistical Analyses of Agronomic Characteristic, Yield, and Nutrient Composition Data 97
2.3. Special Considerations 100
2.4. SAS Procedures 101

APPENDIX 3. MATERIALS AND METHODS FOR DETERMINATION OF GAT4621 PROTEIN CONCENTRATIONS 102

3.1. Plant Material 102
3.2. Storage and Processing of ELISA Samples 102
3.3. Protein Extraction from Ground Canola Tissues 102
3.4. Determination of GAT4621 Protein Concentrations 102

APPENDIX 4. CHARACTERIZATION OF THE PLANT-EXPRESSED AND MICROBIAL-EXPRESSED GAT4621 PROTEINS 104

APPENDIX 5. ECOLOGICAL OBSERVATIONS FOR 73496 CANOLA 111

APPENDIX 6. USDA AND CANADIAN FIELD TRIALS OF 73496 CANOLA 115

APPENDIX 7. SURVEY OF POTENTIAL SUBSTRATES FOR GAT4621 116

TABLE OF CONTENTS (CONTINUED)

APPENDIX 8. SAFETY AND HISTORY OF CONSUMPTION OF ACETYLATED AMINO ACIDS 118

8.1. Summary 118
8.2. Function and Use of Acetylated Amino Acids 118
8.3. History of Safe Use of Acetylated Amino Acids 118
8.4. Presence of NAA, NAG, NAT, NAS, and NAGly in Biological Systems 119
8.5. History of Safe Consumption of NAA, NAG, NAS, NAT, and NAGly 121
8.6 Toxicology Studies Conducted with NAA, NAG, NAS, NAT, and NAGly 121

APPENDIX 9. HERBICIDE RESISTANT WEEDS 122

9.1. Evolution of Herbicide Resistant Weeds 122
9.2. Characteristics of Glyphosate 124
9.3. Evolution of Resistance to Glyphosate 124
9.4. Stewardship of Herbicide Tolerant 73496 Canola 125

APPENDIX 10. ENVIRONMENTAL REPORT ON THE DEREGULATION OF 73496 CANOLA 130

10.1. Overview of U.S. Canola Production, Market, and Trade 130
10.2. Potential Impact of the Introduction of 73496 Canola on U.S. and Canada Production, Market, and Trade 130
10.3. Current Agronomic Practices for Canola in the U.S. 130
10.4. Potential Impact of the Introduction of 73496 Canola on Agronomic Practices 130
10.5. Additional Potential Impacts of the Introduction of 73496 Canola 130
10.6. Cumulative Impacts 131

APPENDIX 11. GAT4621 NEW PROTEIN CONSULTATION 132

REFERENCES 150

LIST OF TABLES

Table 1. Generations and Comparators Used for Analysis of 73496 Canola 18

Table 2. Description of the Genetic Elements in Fragment PHP28181A 20

Table 3. Description of DNA Probes Used for Southern Blot Hybridization 24

Table 4. Summary of Expected and Observed Hybridization Fragments on Southern Blots for 73496 Canola 25

Table 5. Summary of Genotypic and Phenotypic Results for 73496 Canola 35

Table 6. Comparison of Amino Acid Sequence Similarity Between Parental GAT Enzymes and GAT4621 38

Table 7. Concentrations of GAT4621 Protein in 73496 Canola Plant Tissues 39

Table 8. Description of Seed Germination Conditions 42

Table 9. Summary of Germination Results for 73496 Canola 43

Table 10. Site Locations for 2008 and 2009 Field Trial Experiments 45

Table 11. Experiment A: 2008 Field Trial Agronomic Characteristics Measured 47

Table 12. Experiment A: Summary of Agronomic Characteristics of 73496 Canola Across Seven Locations 48

Table 13. Experiment B: 2009 Field Trial Agronomic Characteristics Measured 49

Table 14. Experiment B: Summary of Agronomic Characteristics of 73496 Canola Across Three Locations 51

Table 15. Summary Analysis of Proximate and Fiber Results in Canola Seed 56

Table 16. Summary Analysis of Fatty Acid Composition in Canola Seed 58

Table 17. Summary Analysis of Amino Acid Composition in Canola Seed 61

Table 18. Summary Analysis of Vitamin Composition in Canola Seed 64

Table 19. Summary Analysis of Mineral Composition in Canola Seed 66

Table 20. Summary Analysis of Glucosinolate Composition in Canola Seed 68

Table 21. Summary Analysis of Secondary Metabolite and Anti-Nutrient Composition in Canola Seed 71

Table 22. Concentrations of Acetylated Amino Acids in Canola Seed 73

Table 23. Concentrations of Acetylated Amino Acids in Canola Whole Plant Samples 74

LIST OF TABLES (CONTINUED)

Table 24. Concentrations of Acetylated Amino Acids in Processed Fractions Produced from 73496 and Control Canola 75

Table 25. Common Characteristics of Weeds 78

Table 26. List of Species with Reports of Hand-Pollinated or Spontaneous Hybridization with *B. napus* 84

Table 5.1. United States: Insect Stressor Comparison between 73496 Canola and Controls 111

Table 5.2. United States: Disease Stressor Comparison between 73496 Canola and Controls 112

Table 5.3. Canada: Insect Stressor Comparison between 73496 Canola and Controls 113

Table 5.4. Canada: Disease Stressor Comparison between 73496 Canola and Controls 114

Table 6.1. USDA Field Trials of 73496 Canola 115

Table 6.2. Canada Field Trials of 73496 Canola 115

Table 7.1. k_{cat}/K_M for the GAT4621 Enzyme on Glyphosate and Selected Amino Acids 116

Table 8.1. Full list of Studies Conducted with NAA, NAG, NAS, NAT, and NAGly 121

Table 9.1. Herbicide Resistant Weeds Summary Table (Heap, 2010b) 123

LIST OF FIGURES

Figure 1. Schematic Diagram of the Development of 73496 Canola 17

Figure 2. Breeding Diagram for 73496 Canola and Generations Used for Analyses 17

Figure 3. Schematic Diagram of Fragment PHP28181A 19

Figure 4. Schematic Diagram of Plasmid PHP28181 19

Figure 5. Restriction Enzyme Map of PHP28181A DNA Fragment 25

Figure 6. Restriction Enzyme Map of Plasmid PHP28181 26

Figure 7. Physical Map of the Insertion in 73496 Canola 26

Figure 8. Southern Blot Analysis of 73496 Canola: *Nco* I Digestion and *UBQ10* Promoter Probe 27

Figure 9. Southern Blot Analysis of 73496 Canola: *Nco* I Digestion and *gat4621* Probe 28

Figure 10. Southern Blot Analysis of 73496 Canola: *Nco* I Digestion and *pin*II Terminator Probe 29

Figure 11. Southern Blot Analysis of 73496 Canola: *Ssp* I Digestion and *UBQ10* Promoter Probe 30

Figure 12. Southern Blot Analysis of 73496 Canola: *Ssp* I Digestion and *gat4621* Probe 31

Figure 13. Southern Blot Analysis of 73496 Canola: *Ssp* I Digestion and *pin*II Terminator Probe 32

Figure 14. Southern Blot Analysis of 73496 Canola: *Nco* I Digestion and Plasmid Backbone Probe 33

Figure 15. Deduced Amino Acid Sequence of the GAT4621 Protein 36

Figure 16. Enzymatic Activity of the GAT4621 Protein 37

Figure 17. Map of Locations for Agronomic Data Collection for 73496 Canola 45

Figure 18. Map Indicating Zones with Similar Growing Conditions for Field Trials 46

Figure 19. Genome Relationships Among Cultivated Brassicaceae 82

Figure 4.1. SDS-PAGE Analysis of the Plant-Expressed GAT4621 Protein 104

Figure 4.2. Western Immunoblot Analysis of the Plant-Expressed and Microbial-Expressed GAT4621 Proteins 105

Figure 4.3. N-Terminal Amino Acid Sequence Analysis of the Plant-Expressed and Microbial-Expressed GAT4621 Proteins 106

LIST OF FIGURES (CONTINUED)

Figure 4.4. Deduced GAT4621 Amino Acid Sequence Showing Matching Peptides from MALDI-MS 106

Figure 4.5. Glycosylation Analysis of the Plant-Expressed GAT4621 Protein..... 107

Figure 9.1. Number of Resistant Biotypes of Weeds by Herbicide Class and Year (Heap, 2010d)..... 124

Figure 9.2. Resistant Weed Management Label..... 127

Release of Information

Pioneer Hi-Bred International, Inc. (Pioneer) is submitting the information in this assessment for review by USDA as part of the regulatory process. By submitting this information, Pioneer does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., Section 552; USDA complies with the provisions of FOIA and USDA's implementation regulations (7 CFR Part 1.4); and this informaiton is responsive to the specific request. Except in accordance with the Freedom of Information Act, Pioneer does not authorize the release, publication or other distribution of this information (including website posting) without Pioneer's prior notice or consent.

Certification

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioners, which are unfavorable to the petition.

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Summary

Pioneer Hi-Bred International, Inc. (Pioneer) is submitting this Petition for Determination of Nonregulated Status for herbicide-tolerant canola event DP-Ø73496-4, hereafter referred to as 73496 canola. Canola line 73496 was developed by Pioneer Hi-Bred, a DuPont Business. The data in this petition support the conclusion that 73496 canola is not likely to pose an increased plant pest potential. Therefore, Pioneer requests a determination from USDA - Animal and Plant Health Inspection Service (APHIS) that 73496 canola and any crosses of this line with other nonregulated *Brassica napus* no longer be considered regulated articles under 7 CFR §340.

73496 canola is a transgenic plant line that is tolerant to the herbicidal active ingredient glyphosate. The availability of 73496 canola will provide an alternative to currently available glyphosate-tolerant canola lines. Herbicide-tolerant 73496 canola will provide the same benefits as currently available glyphosate-tolerant canola lines, in that growers will be able to proactively manage weed populations and delay the development of adverse populations of troublesome weeds.

73496 canola 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 98140 maize deregulated by USDA in 2009 and reviewed by FDA in 2008, and is encoded by the *gat4621* gene which confers tolerance to glyphosate-containing herbicides by acetylating glyphosate and thereby rendering it non-phytotoxic. In 73496 canola, the expression of the *gat4621* gene is driven by the *Arabidopsis* polyubiquitin constitutive promoter.

73496 canola was generated using biolistic transformation of canola microspores with a gel-purified DNA fragment isolated from plasmid PHP28181 containing the *gat4621* gene cassette. Molecular characterization of 73496 canola by Southern blot analysis confirmed that a single, intact PHP28181A DNA fragment has been inserted into the genome with no plasmid backbone DNA. Segregation analysis of 73496 canola confirmed Mendelian inheritance of the *gat4621* gene.

The potential for allergenicity and toxicity of 73496 canola 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 73496 canola 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 not glycosylated and is rapidly digested (within 30 seconds) in simulated gastric fluid. In simulated intestinal fluid, the GAT4621 protein hydrolyzed within 5 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 or animals and therefore support the food and feed safety of GAT4621. A New Protein Consultation for the GAT4621 protein was submitted to FDA on January 31, 2007 and completed on October 7, 2009.

73496 canola has been field tested since 2007 in the major canola-growing regions of Canada and the United States. All field tests that have occurred in the United States were under field permits and notifications granted by USDA - APHIS. Comprehensive agronomic performance for 73496 canola were conducted in replicated field studies at a total of 10 locations across Canada and the United States. Characteristics such as early growth, days to flower, days to maturity, plant height, yield, seedling vigor, flowering duration, lodging, shattering, disease incidence and insect damage were assessed. Seed germination and dormancy data were also collected in laboratory experiments. All field trials of 73496 canola were observed for naturally occurring disease or insect biotic stressors. Analysis of agronomic and ecological data showed no biologically meaningful differences between 73496 canola and control canola lines, indicating the similarity of 73496 canola to conventional canola and indicating no plant pest

characteristics or selective advantage in natural habitats. These data support the conclusion of agronomic comparability of 73496 canola to commercially available canola with respect to the lack of increased weediness and plant pest potential.

Extensive nutrient composition analysis of seed was conducted to compare the composition of 73496 canola to that of a control line and five commercial canola varieties. Compositional analysis of 73496 canola was used to evaluate any changes in the levels of key nutrients, anti-nutrients, and secondary metabolites in accordance with the OECD consensus document for new varieties of canola. Based on the results of the compositional evaluation, the seed of 73496 canola are comparable to commercially available canola. Along with the agronomic data included in this petition, compositional comparability is a general indicator that 73496 canola will not exhibit unintended effects due to the inserted DNA.

In addition to glyphosate, 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 *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. Because of the potential for these five acetylated amino acids to be increased in 73496 canola, concentrations were measured in seed, whole plant, and processed product samples. An increase in concentrations of NAA, NAG, and, in some instances, NAGly, NAS, and NAT, was confirmed through the analysis of seed, whole plant, and processed product samples. Low but quantifiable concentrations 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.

In conclusion, based on the data contained herein that support the conclusion that 73496 canola is not likely to pose an increased plant pest potential, Pioneer requests that APHIS grant the request for a determination of nonregulated status for 73496 canola and any crosses of this line with other nonregulated *Brassica napus*.

Abbreviations, Acronyms, and Definitions

~	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
73496 canola	canola lines containing the DP-Ø73496-4 event
ACY1	Aminoacylase I
ADF	acid detergent fiber
ALS	Acetylactate synthase
AOSA	Association of Official Seed Analysis
APHIS	Animal and Plant Health Inspection Service of USDA
ATCC	American Type Culture Collection
*Bam*H I	Restriction enzyme from *Bacillus amyloliquefaciens*
BAR	phosphinothricin acetyltransferase
BBCH	Bundesanstalt, Bundessortenamt and Chemical industry
bla (Ap^R)	Ampicillin resistance gene
B. licheniformis	*Bacillus licheniformis*
B. napus	*Brassica napus* L.
bp	base pair
CAC	Codex Alimentarius Commission
CCA	Certified Crop Adviser
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
CNS	Central nervous system
CPSI	Carbamyl phosphate synthetase I
C_T	threshold cycle
Da	Dalton
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
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
E score	expectation score
ELISA	enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
EPSPS	enolpyruvylshikimate-3-phosphate synthase
EU	European Union
FARRP	Food Allergy Research and Resource Program
FDA	Food and Drug Administration
FDR	false discovery rate
FOIA	Freedom of Information Act
GAT	glyphosate acetyltransferase
GAT4621	specific GAT protein
gat4621	specific *gat* gene
GLMM	Generalized Linear Mixed Model
GNAT	GCN 5-related *N*-acetyltransferases
*Hae*III	Restriction enzyme from *Haemophilus aegyptius*
*Hin*d III	Restriction enzyme from *Haemophilus influenzae*

Abbreviations, Acronyms, and Definitions (continued)

HRAC	Herbicide Resistance Action Committee
HRP	horseradish peroxidase
IgG	immunoglobulin G
ISAAA	International Service for the Acquisition of Agri-biotech Applications
kb	kilobase pair
kDa	kilodalton
LLOQ	lower limit of quantitation
LS-Mean	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
Not I	Restriction enzyme from *Nocardia otitidis-caviarum*
NRCS	Natural Resources Conservation Service
OD	optical density
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
*pin*II	proteinase inhibitor II
PMRA	Pest Management Regulatory Agency (Canada)
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*
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
WSSA	Weed Science Society of America

*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/).

I. Rationale for the Development of 73496 Canola

I-A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR §340.6

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. 7701-7772) and the Plant Quarantine Act (7 U.S.C. 151-167), to prevent the introduction or dissemination of plant pests into or within the United States. Part 340 regulates introduction of organisms altered or produced through genetic engineering which are plant pests or for which there is a reason to believe are plant pests. The APHIS regulations at 7 CFR §430.6 provide that an applicant may petition APHIS to evaluate submitted data on the genetically engineered crop to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

Pioneer Hi-Bred International, Inc. is submitting data for genetically engineered herbicide tolerant 73496 canola and requests a determination from USDA - Animal and Plant Health Inspection Service (APHIS) that event DP-Ø73496-4 and any crosses with other nonregulated *Brassica napus* no longer be considered regulated articles under 7 CFR §340.

I-B. Benefits of 73496 Canola

Herbicide-tolerant canola varieties have provided growers with a very useful tool to help manage their canola crops. Herbicide-tolerant canola varieties have helped to increase the adoption of low- and no-till practices which are important in the minimization of soil erosion and also have helped to reduce the volume of herbicides applied to canola crops to control weeds (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 competing for soil nutrients and moisture.

In particular, glyphosate has become a popular post-emergence herbicide for canola to control weeds that reduce yields. As a broad-spectrum herbicide, it is effective in controlling both grasses and annual broadleaf weeds, while possessing an excellent environmental profile and low mammalian toxicity.

73496 canola was developed with glyphosate-tolerance to provide an alternative to existing herbicide-tolerant canola products on the market. The commercialization of 73496 canola is not expected to have an impact on existing weed control practices and could replace the use of other glyphosate-tolerant canola products already commercialized. The availability of 73496 canola will provide growers with an additional market choice for herbicide-tolerant canola.

I-C. Submissions to Other Regulatory Agencies

A New Protein Consultation for the GAT4621 protein was submitted to FDA on January 31, 2007 and completed on October 7, 2009. A safety and nutritional assessment for feed and food derived from 73496 canola will be submitted to FDA in the first quarter of 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 on February 18, 2011.

Submissions for food, feed, and environmental approval were made to Canada in August 2010. Submissions will also be made in Mexico, Japan, South Korea, China, EU, and other countries as appropriate. Pioneer is committed to robust product stewardship prior to and continuing after all relevant authorizations are granted. A full commercial launch of 73496 canola will not occur until import regulatory approvals have been obtained in key canola import markets with functioning regulatory systems.

II. The Biology of Canola

II-A. Canola as a Crop

Biology documents on the unmodified plant species, canola (*Brassica napus* L.), have been published by the 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
- centers of origin and diversity
- crosses, including intra- and inter-specific/genus crosses and gene flow
- agro-ecology, including information about cultivation, volunteers and weediness, soil ecology, and canola-insect interactions

II-B. Description of the Non-Transformed Recipient Canola Line

A doubled-haploid form of the Pioneer proprietary line, 1822B, was used as the recipient line for the production of 73496 canola. Line 1822B was chosen because it is receptive to transformation and is also an elite line (*i.e.*, Pioneer proprietary line used for commercial products). The use of this doubled-haploid line ensured that the recipient line was homozygous at all genetic loci, which aided in the breeding and testing of the transformed events.

III. Method of Development of 73496 Canola

III-A. Description of Transformation, Selection, and Breeding

A gel-purified DNA fragment isolated from plasmid PHP28181 (containing the *gat4621* gene cassette) was used to generate 73496 canola. Refer to Section 3.1 for a detailed description of the gel-purified *Hin*d III/*Not* I DNA fragment, PHP28181A, used for transformation.

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 biolistic transformation 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°C and then for four weeks at 25°C 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 73496 canola.

The subsequent breeding of 73496 canola 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.

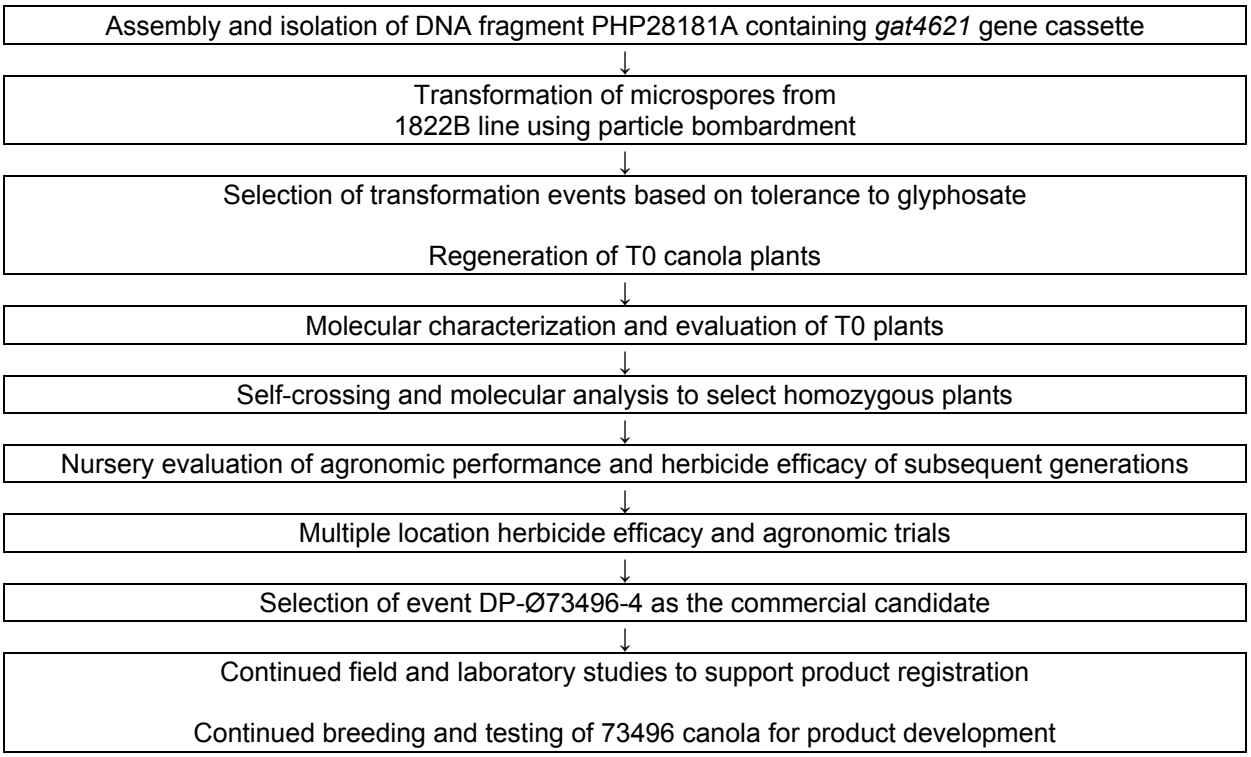

Figure 1. Schematic Diagram of the Development of 73496 Canola

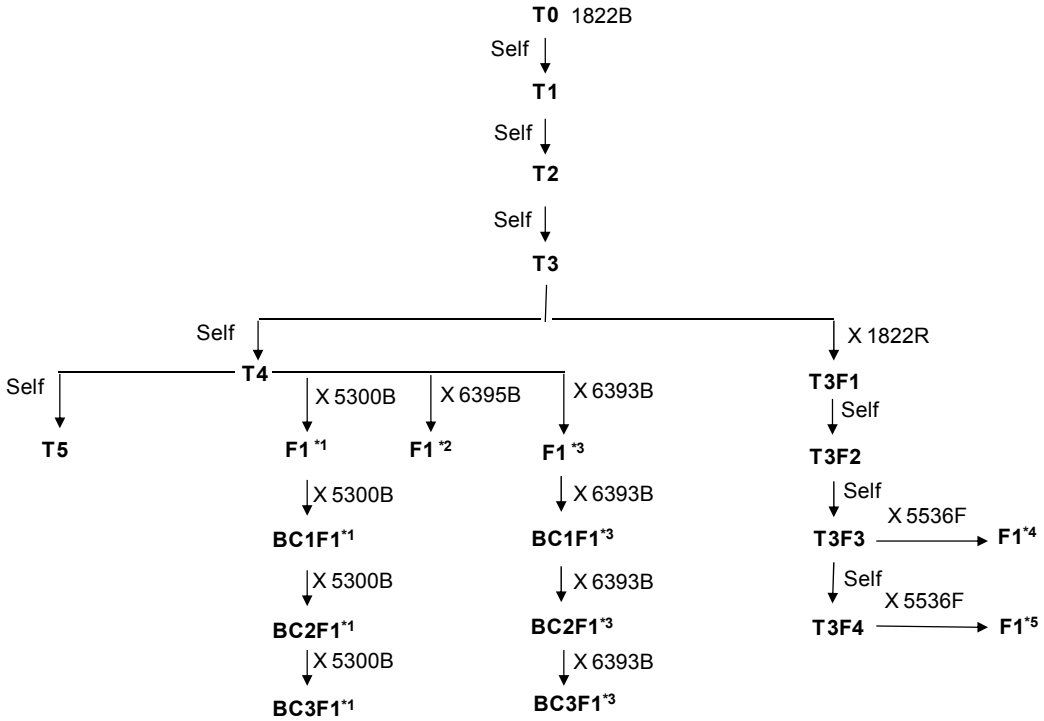

Figure 2. Breeding Diagram for 73496 Canola and Generations Used for Analyses

Table 1. Generations and Comparators Used for Analysis of 73496 Canola

Analysis	Generation	Comparators
Genetic Characterization (Section V)	T2, T3, F1[*2], T3F2, T3F3	1822B, 1822R, and 6395B
Inheritance (Section V-E)	F1[*1, *3], BC1F1[*1, *3], BC2F1[*1, *3], BC3F1[*1, *3], T3F2	Not applicable
Concentrations of GAT4621 (Section V-B)	F1[*4]	5536F x 1822R
Compositional Assessment (Section VIII)	F1[*4]	5536F x 1822R and commercial lines
Germination and Dormancy (Agronomic Performance) (Section VII-A)	F1[*5]	5536F x 1822R
Field Trial Evaluations (Agronomic Performance) (Section VII-B)	T3 (7 locations) T5 (3 locations)	1822B 1822B

III-B. Selection of Comparators for 73496 Canola

To ensure an accurate evaluation of the effect of the transgene, a proper selection of comparator plants is important for the analysis of a transgenic plant. Control canola lines were used as comparators for 73496 canola (Table 1). For all analyses, the control plants had a genetic background similar to that of the 73496 canola generation used, but did not go through the transformation process (near-isoline).

In addition, non-transgenic commercial canola lines (Table 1) were used to obtain tolerance intervals for the nutrient compositional assessment. The tolerance intervals were used to establish the range of natural variation in key nutrients and anti-nutrients, and to determine the possible biological significance of any observed statistical differences.

IV. Donor Genes and Regulatory Sequences

IV-A. Fragment PHP28181A Used in Transformation

73496 canola was produced by biolistic transformation with the *Hin*d III/*Not* I fragment, PHP28181A, from plasmid PHP28181. The PHP28181A fragment containing the *UBQ10* promoter, the *gat4621* gene, and the *pin*II terminator is shown in Figure 3, and the entire PHP28181 plasmid is shown in Figure 4.

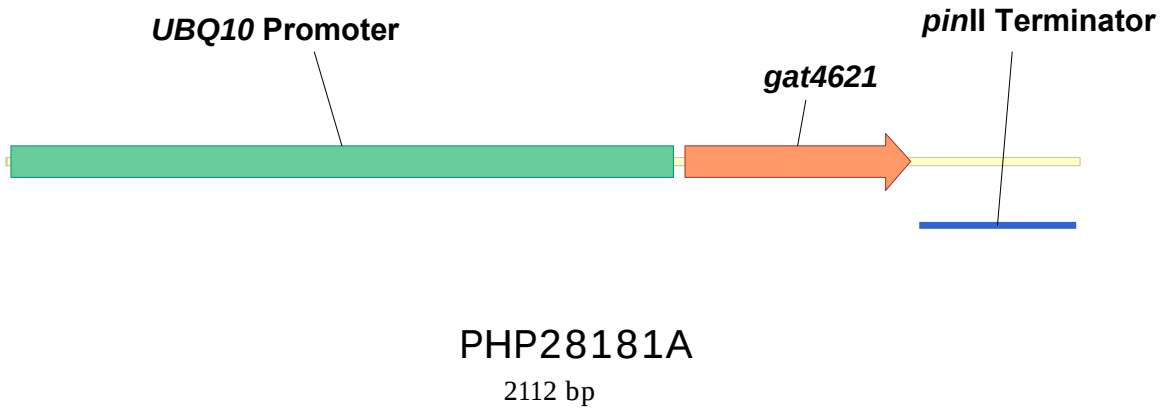

Figure 3. Schematic Diagram of Fragment PHP28181A

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).

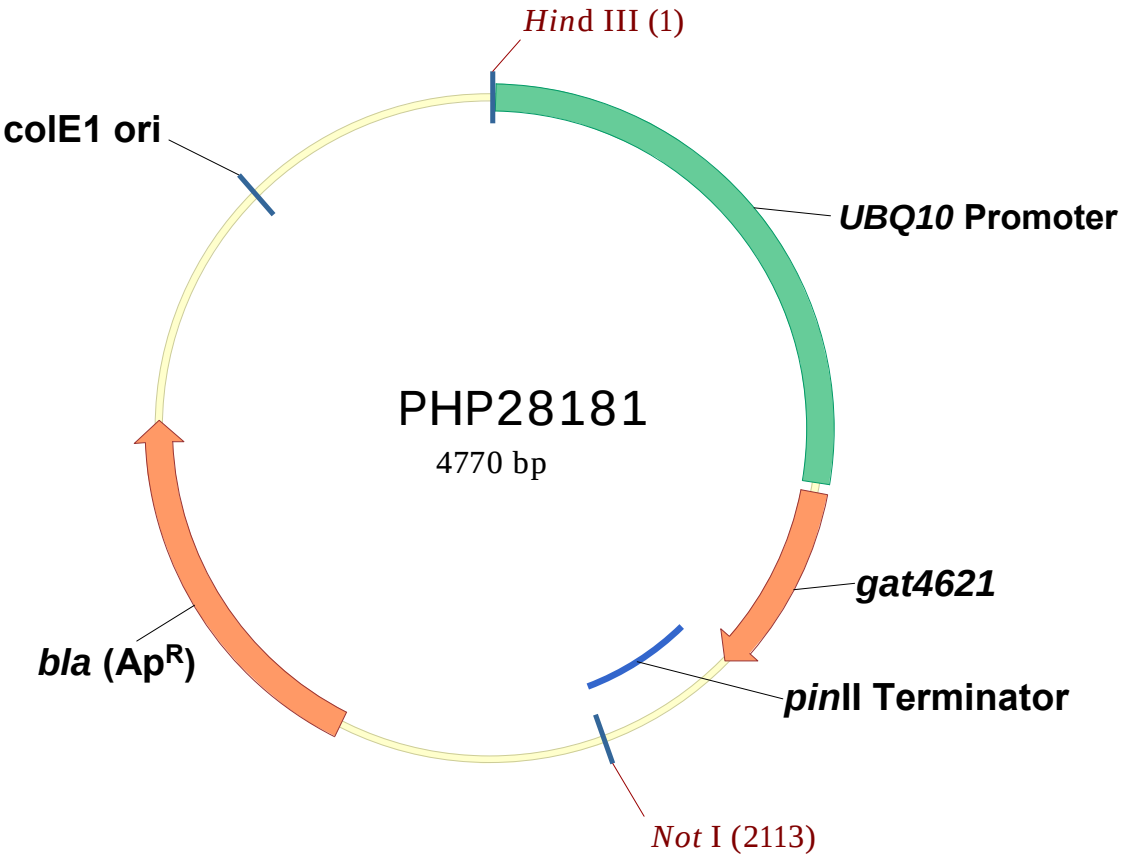

Figure 4. Schematic Diagram of Plasmid PHP28181

Schematic diagram of plasmid PHP28181 indicating the genetic elements. *Hin*d III and *Not* I restriction enzyme sites flank the transformation fragment PHP28181A. The size of the plasmid is 4770 bp.

IV-B. Identity and Source of the *gat4621* Gene Cassette on Fragment PHP28181A

The genetic elements present in the transformation fragment PHP28181A are listed in Table 2. The *gat4621* gene was optimized by a gene shuffling process of glyphosate acetyltransferase genes from *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).

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 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).

Table 2. 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

V. Genetic Characterization of 73496 Canola

V-A. Molecular Analysis Overview

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.

The inserted DNA in 73496 canola was characterized by Southern blot analysis to evaluate the integrity and stability of the inserted *gat4621* cassette. As described earlier in Section III-A, 73496 canola 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 5 and 6, respectively) and outlined in Table 3. 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 analyzed to determine the copy number of each of the genetic elements inserted into 73496 canola and to verify that the integrity of the PHP28181A fragment was maintained upon integration.

The analyses confirmed that a single, intact PHP28181A DNA fragment was inserted into the genome of 73496 canola (Section V-B1 and B2). In addition, these analyses also verified that the inserted DNA remained intact and stably integrated in 73496 canola. All five generations demonstrated identical hybridization patterns. These results confirmed the stability of the inserted DNA in 73496 canola across these five breeding generations. Based on these analyses, schematic maps of the inserted DNA in 73496 canola were determined and are provided in Figure 7.

All five generations were also analyzed 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 both 73496 canola (Section V-B3).

To confirm inheritance of the insertion through traditional breeding, segregation analysis using genotypic and phenotypic assays were conducted (Section V-C). These results indicated that the inserted DNA and the herbicide-tolerance phenotype in 73496 canola 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.

Materials and methods for the molecular and genetic characterization of 73496 canola are described in Appendix 1.

V-B. Results of Molecular Characterization of 73496 Canola by Southern Blot Analysis

The integration pattern of the insertion in 73496 canola 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 73496 canola using probes specific to the *UBQ10* promoter, *gat4621* gene, and *pin*II terminator (Table 3, Figure 5). 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 (Table 3, Figure 6) were used to show that these regions were not transferred to 73496 canola.

Based on the Southern blot analyses described below, it was determined that a single, intact PHP28181A DNA fragment was inserted into the genome of 73496 canola as diagramed in the insertion map (Figure 7) and that no region from the backbone of plasmid PHP28181 was inserted. In addition, these results confirmed the stability of the inserted DNA in 73496 canola across five breeding generations.

B1. Copy Number

The *Nco* I digestion provides information about the number of copies of the PHP28181A DNA fragment that have been integrated into the genome of 73496 canola. The PHP28181A DNA fragment contains a single *Nco* I restriction enzyme site at bp position 1335 (Figure 5) 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 73496 canola 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 5). Predicted and observed fragment sizes for 73496 canola with *Nco* I digestion are provided in Table 4.

The *UBQ10* promoter probe was hybridized to *Nco* I-digested genomic DNA from individual 73496 canola plants of the T2, T3, T3F2, T3F3, and F1*[2] generations (Table 4, Figure 8). A single fragment of greater than 8600 bp was detected in each 73496 canola plant sample (Table 4, Figure 8), 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 7000 bp in each 73496 canola plant sample (Table 4, Figures 9 and 10).

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 73496 canola. The presence of identical hybridization patterns for each probe in all 73496 canola plants of the five generations analyzed demonstrated the stability of the DNA insertion during traditional breeding.

B2. Insertion Integrity

Ssp I digestion was used to verify that the inserted PHP28181A DNA fragment containing the *gat4621* cassette was complete and intact in 73496 canola. Two *Ssp* I sites are present within the PHP28181A DNA fragment (bp positions 157 and 2043; Figure 5). 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 73496 canola with *Ssp* I are provided in Table 4. All three probes are expected to hybridize to an internal fragment of 1886 bp (Table 4, Figure 5). Due to the locations of the *Ssp* I restriction sites within the *UBQ10* promoter and *pin*II terminator elements (Figure 5), these two probes are also expected to hybridize to additional border fragments (Table 4).

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 73496 canola plant sample (Table 4, Figures 11, 12, and 13). The *UBQ10* promoter probe was expected to hybridize to a border band due to the *Ssp* I site within the *UBQ10* promoter element. A faint border band of approximately 2200 bp was detected with the *UBQ10* promoter probe (Table 4, Figure 11). A weakly hybridizing border band of approximately 1100 bp was also detected with the *pin*II terminator probe (Table 4, Figure 13). The weak band resulted from a border fragment including the portion of the *pin*II terminator located 3' to the *Ssp* I site at bp 2043 of PHP28181A (Figure 5) and a second *Ssp* I 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 73496 canola plant sample (Figures 11 and 13).

The presence of the 1886 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 73496 canola. Furthermore, the same hybridization pattern with each probe in the five generations confirmed the stability of the insertion in 73496 canola during traditional breeding.

B3. Plasmid Backbone DNA Analysis

Five generations of 73496 canola were analyzed 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 73496 canola 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 73496 canola.

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 73496 canola or control canola samples, while the expected band of 4770 bp was seen in the plasmid positive control lanes (Figure 14). This confirms that no sequence from the PHP28181 plasmid backbone was inserted into 73496 canola during transformation.

B4. Physical Map of the Inserted DNA in 73496 Canola

Based on the Southern blot analysis, it was determined that a single, intact PHP28181A DNA fragment was inserted into the genome of 73496 canola. Using these data, a physical map of the inserted DNA in 73496 canola showing the applicable restriction enzymes was developed and is provided in Figure 7.

B5. Summary and Conclusions

Southern blot analysis was conducted to characterize the DNA insertion in 73496 canola. The analysis confirmed that a single, intact PHP28181A DNA fragment had been inserted into the canola genome to produce 73496 canola. A single copy of each of the *UBQ10* promoter, *gat4621* gene, and *pin*II terminator genetic elements was present and the integrity of the PHP28181A DNA fragment was maintained upon integration. Identical hybridization patterns were observed across the T2, T3, T3F2, T3F3, and F1*[2] generations, thus demonstrating stability of inheritance of the insertion in 73496 canola during traditional breeding procedures. In addition, Southern blot analysis verified the absence of plasmid backbone sequences in 73496 canola.

Table 3. 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 5 *probe A*	26 to 699 720 to 1286	26 to 699 720 to 1286	674 567
gat4621	*gat4621* gene	Figure 5 *probe B*	1336 to 1770	1336 to 1770	435
*pin*II terminator	*pin*II terminator	Figure 5 *probe C*	1849 to 2082	1849 to 2082	234
Backbone 1	Plasmid between *Not* I and *bla*	Figure 6 *probe 1*	N/A	2120 to 2762	643
bla (Ap^R)	Ampicillin resistance gene	Figure 6 *probe 2*	N/A	2736 to 3566	831
Backbone 2	Plasmid between *bla* and *Hin*d III	Figure 6 *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. The bp positions provided are the positions of each different segment.

Table 4. Summary of Expected and Observed Hybridization Fragments on Southern Blots for 73496 Canola

Probe	Enzyme Digestion	Figure	Expected Fragment Size in 73496 Canola (bp)[a]	Expected Fragment Size in Plasmid PHP28181 (bp)[b]	Observed Fragment Size in 73496 Canola (bp)[c]
UBQ10 Promoter	*Nco* I	8	>1300 (border)	4770	>8600
	Ssp I	11	>200 (border) 1886	2208 1886	~2200 1886[d]
gat4621	*Nco* I	9	>800 (border)	4770	~7000
	Ssp I	12	1886	1886	1886[d]
*pin*II Terminator	*Nco* I	10	>800 (border)	4770	~7000
	Ssp I	13	1886 >100 (border)	1886 676	1886[d] ~1100
Plasmid Backbone[e]	*Nco* I	14	none	4770	none

[a] Size based on map of fragment PHP28181A in Figure 5. Border fragment size rounded to nearest 100 bp.
[b] Size based on plasmid map of PHP28181 in Figure 6.
[c] Observed fragment sizes are approximated from the digoxigenin (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.
[d] Size is same as expected because of equivalent migration with plasmid positive control.
[e] The plasmid backbone probe includes three fragments covering the entire plasmid backbone sequence in a single hybridization solution.

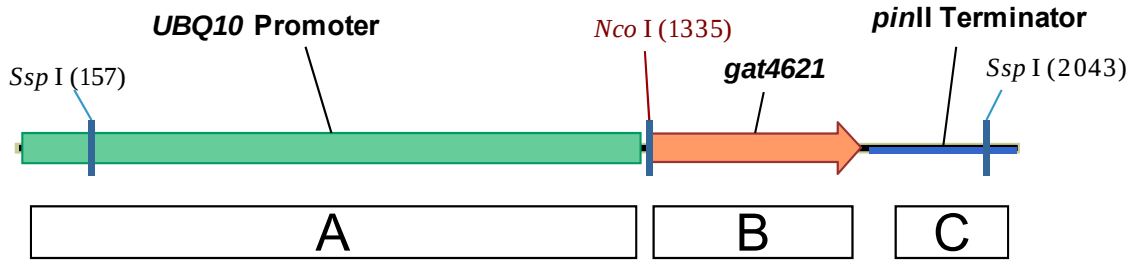

Figure 5. Restriction Enzyme Map of PHP28181A DNA Fragment

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.

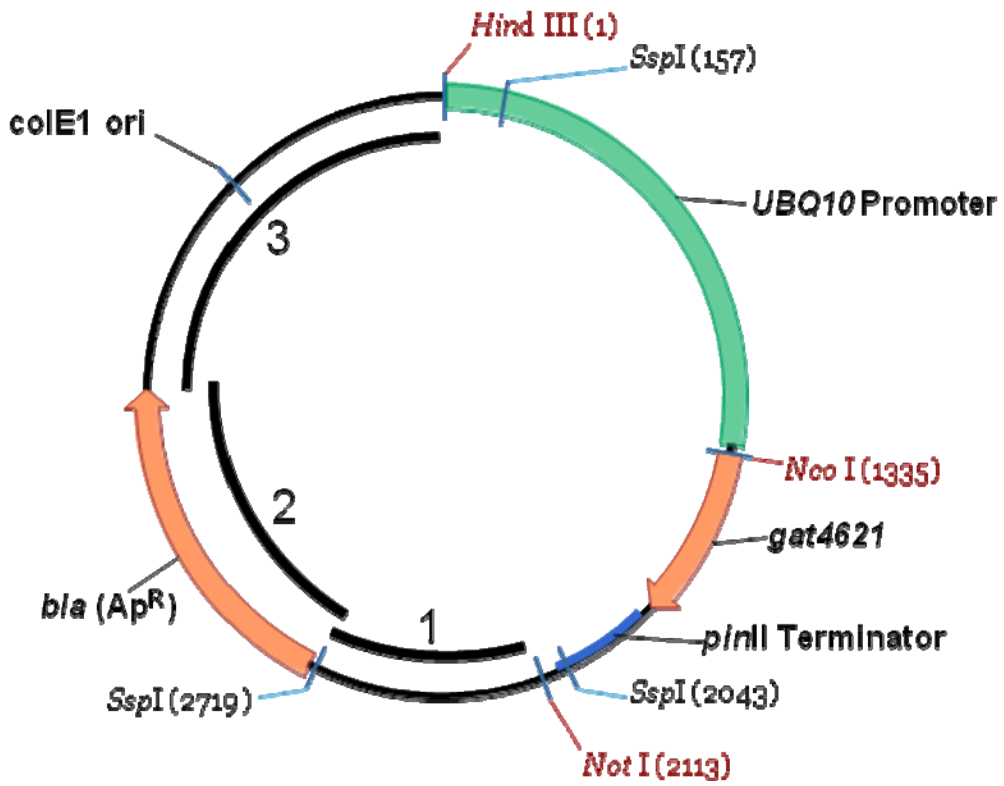

Figure 6. Restriction Enzyme Map of Plasmid PHP28181

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 (Figure 5) used for transformation of canola. 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.

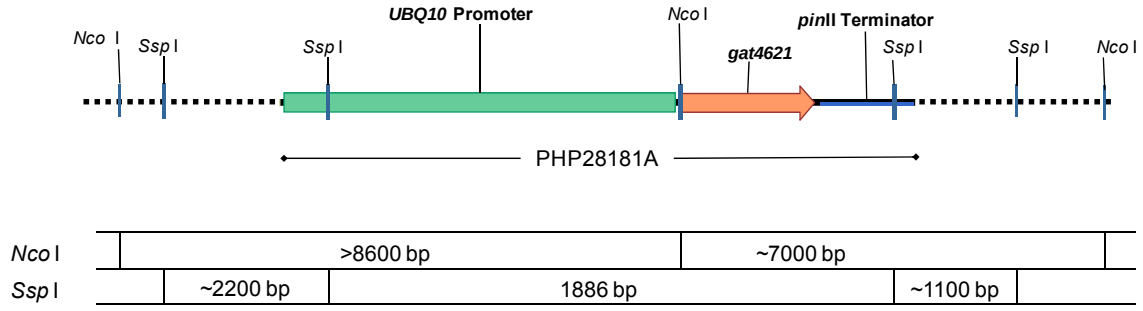

Figure 7. Physical Map of the Insertion in 73496 Canola

Schematic map of the insertion in 73496 canola based on Southern blot analysis. The flanking canola genome is represented by the horizontal dotted line. A single, intact copy of the PHP28181A DNA fragment integrated into the canola genome. *Nco* I and *Ssp* I restriction enzyme sites are indicated with the sizes of observed fragments on Southern blots shown below the map in bp. The locations of restriction enzyme sites outside the PHP28181A DNA fragment are not shown to scale.

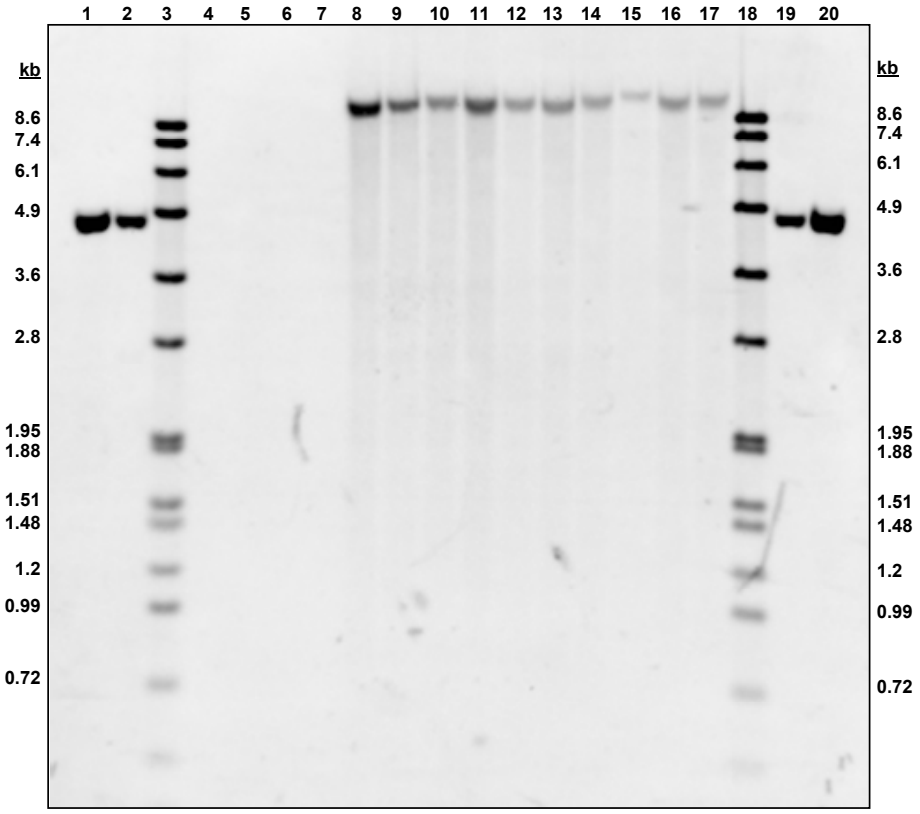

Figure 8. Southern Blot Analysis of 73496 Canola: *Nco* I Digestion and *UBQ10* Promoter Probe

Genomic DNA isolated from leaf tissue from individual plants from five generations of 73496 canola 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	Blank
5	1822B control
6	1822R control
7	6395B control
8	73496 canola / plant 1 (T2 generation)
9	73496 canola / plant 3 (T2 generation)
10	73496 canola / plant 7 (T3 generation)

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

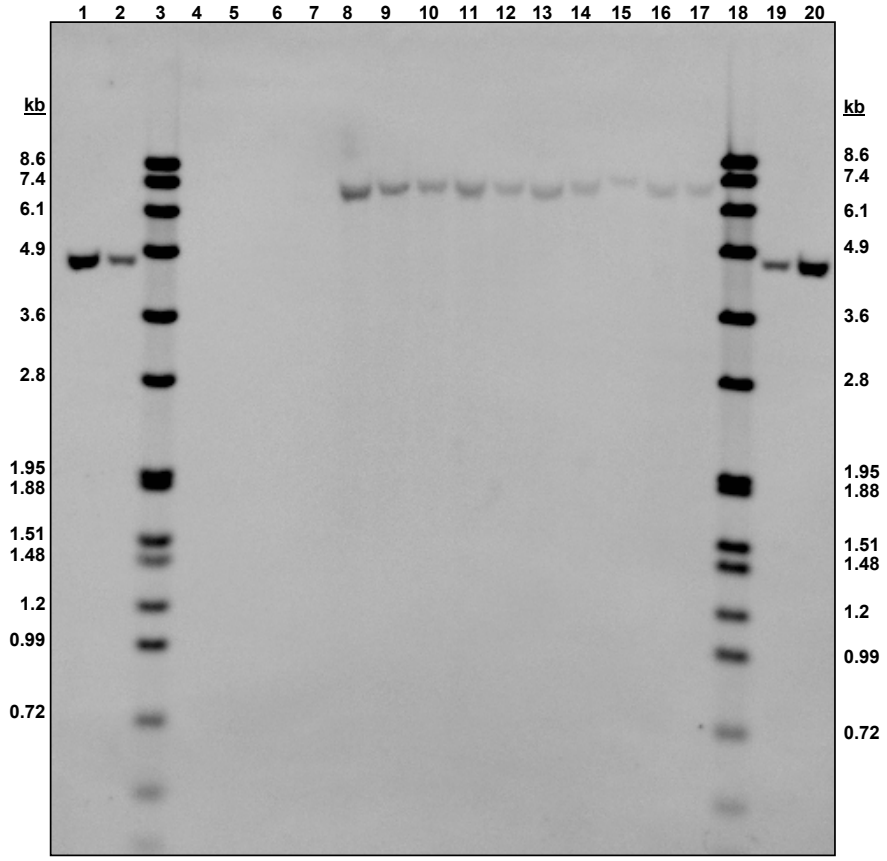

Figure 9. Southern Blot Analysis of 73496 Canola: *Nco* I Digestion and *gat4621* Probe

Genomic DNA isolated from leaf tissue from individual plants from five generations of 73496 canola 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	Blank
5	1822B control
6	1822R control
7	6395B control
8	73496 canola / plant 1 (T2 generation)
9	73496 canola / plant 3 (T2 generation)
10	73496 canola / plant 7 (T3 generation)

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

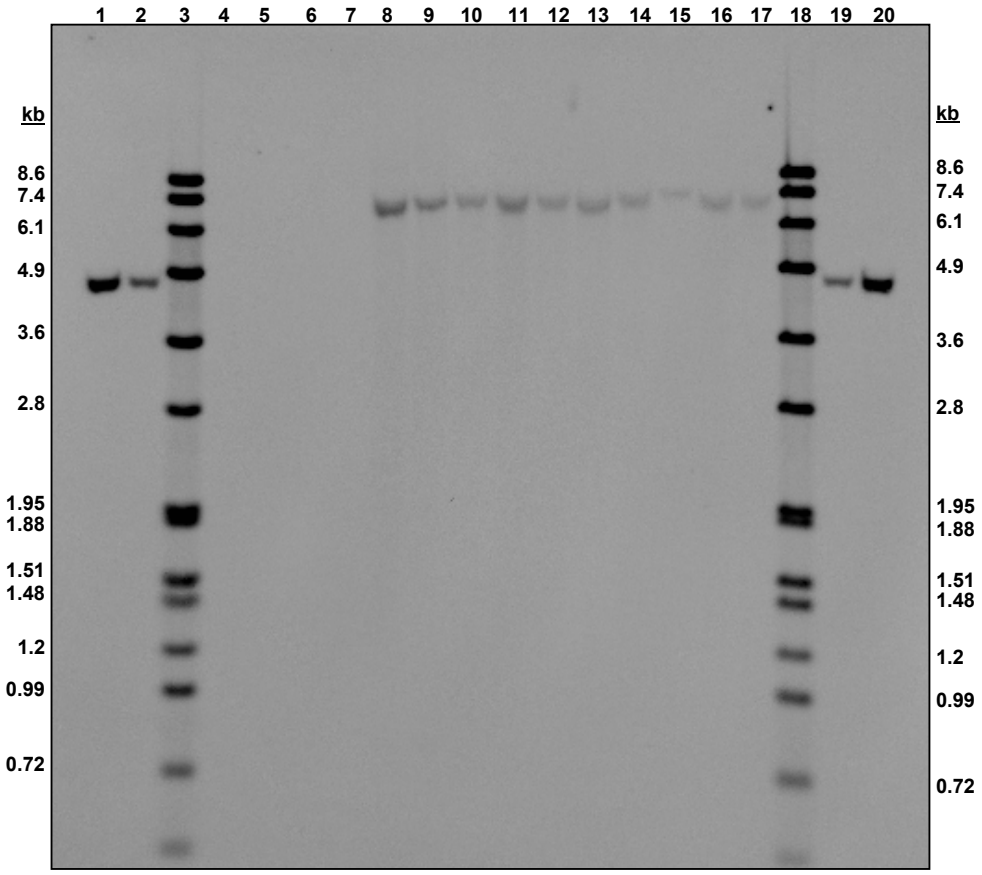

Figure 10. Southern Blot Analysis of 73496 Canola: *Nco* I Digestion and *pin*II Terminator Probe

Genomic DNA isolated from leaf tissue from individual plants from five generations of 73496 canola 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
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	73496 canola / plant 1 (T2 generation)
9	73496 canola / plant 3 (T2 generation)
10	73496 canola / plant 7 (T3 generation)

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

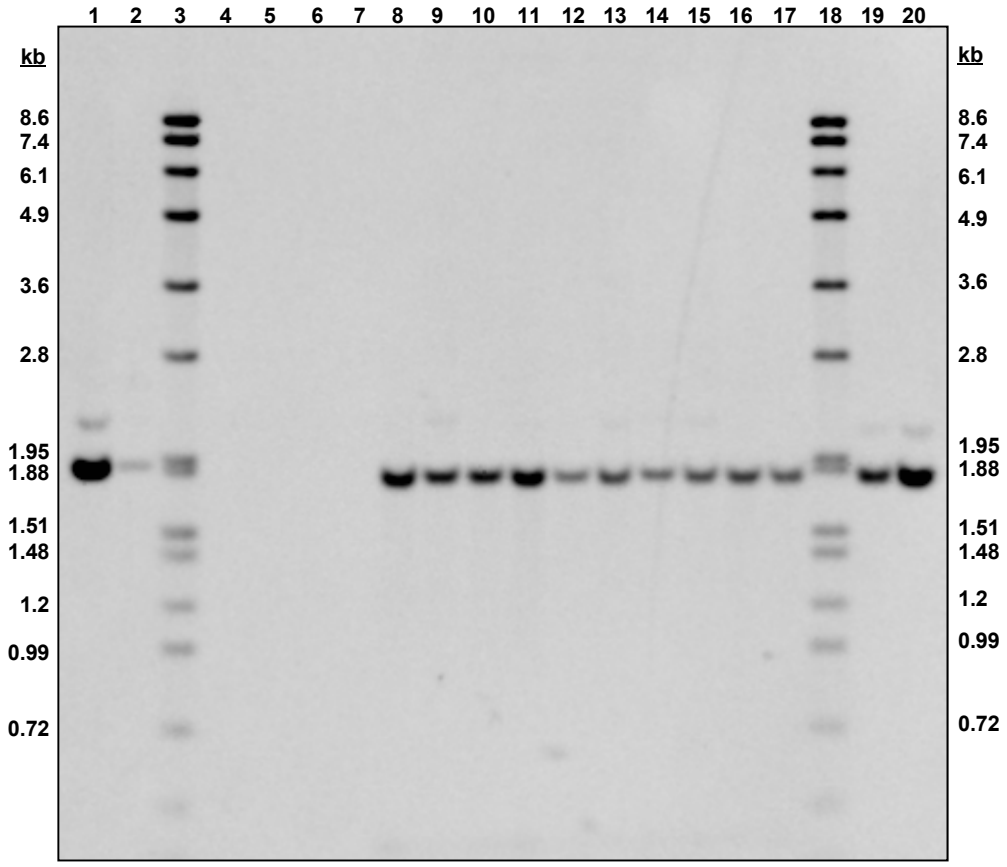

Figure 11. Southern Blot Analysis of 73496 Canola: *Ssp* I Digestion and *UBQ10* Promoter Probe

Genomic DNA isolated from leaf tissue from individual plants from five generations of 73496 canola and control canola lines was digested with *Ssp* 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).
Note: A faint band is visible on the X-ray film at about 2.2 kb in lanes 8 through 17 (may not appear on printed copy).

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	73496 canola / plant 1 (T2 generation)
9	73496 canola / plant 3 (T2 generation)
10	73496 canola / plant 7 (T3 generation)

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

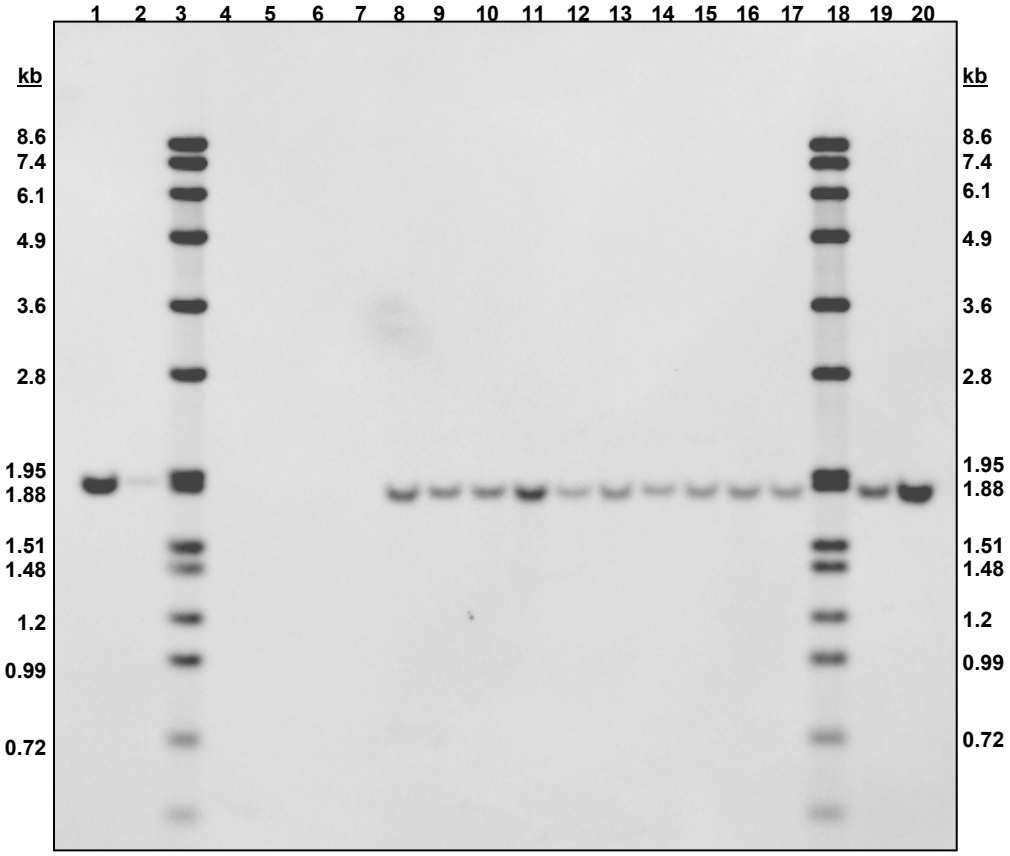

Figure 12. Southern Blot Analysis of 73496 Canola: *Ssp* I Digestion and *gat4621* Probe

Genomic DNA isolated from leaf tissue from individual plants from five generations of 73496 canola and control canola lines was digested with *Ssp* 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	Blank
5	1822B control
6	1822R control
7	6395B control
8	73496 canola / plant 1 (T2 generation)
9	73496 canola / plant 3 (T2 generation)
10	73496 canola / plant 7 (T3 generation)

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

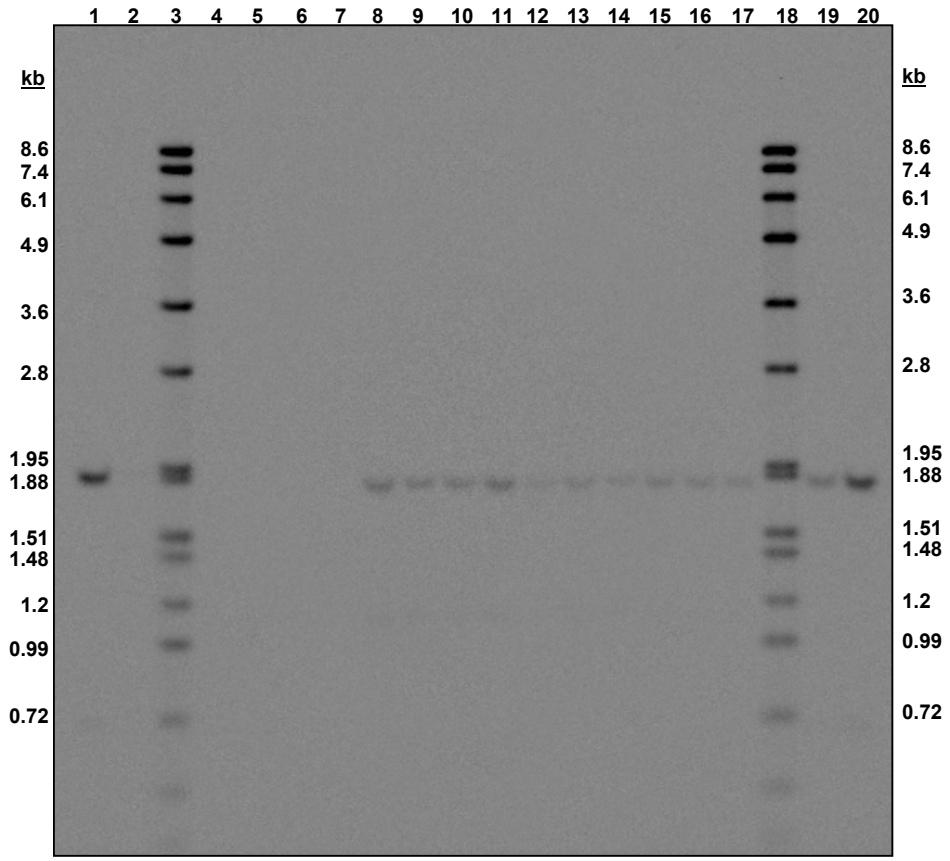

Figure 13. Southern Blot Analysis of 73496 Canola: *Ssp* I Digestion and *pin*II Terminator Probe

Genomic DNA isolated from leaf tissue from individual plants from five generations of 73496 canola and control canola lines was digested with *Ssp* 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).
Note: A faint band is visible on the x-ray film at about 1.1 kb in lanes 8 through 17 (may not appear on printed copy).

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	73496 canola / plant 1 (T2 generation)
9	73496 canola / plant 3 (T2 generation)
10	73496 canola / plant 7 (T3 generation)

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

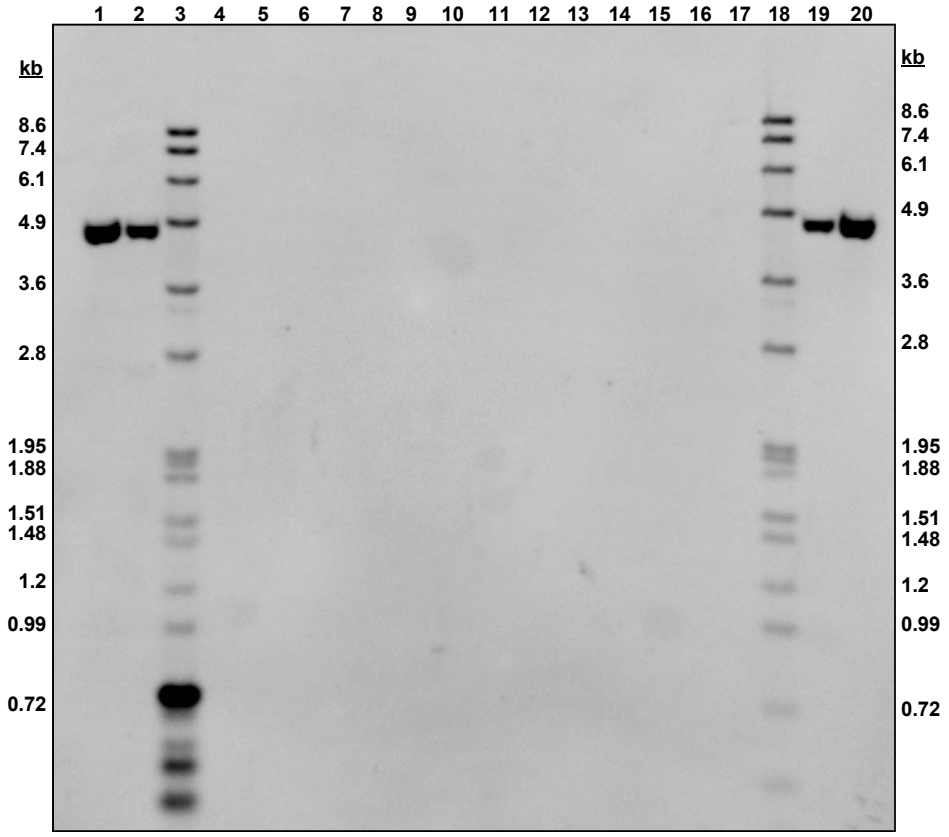

Figure 14. Southern Blot Analysis of 73496 Canola: *Nco* I Digestion and Plasmid Backbone Probe

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

V-C. Inheritance and Genetic Stability of the Introduced Trait in 73496 Canola

Inheritance of the inserted DNA and the herbicide-tolerance phenotype in 73496 canola 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 73496 canola (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 (Section III-A, Figure 2). Those populations with the superscript "*1,*3" designation were pooled populations from two different genetic backgrounds (Figure 2). Chi-square analysis was conducted on the four segregating generations to determine if the observed segregation ratios were consistent with the expected ratios. The assay methods and statistical analysis for the trait inheritance data are described in Appendix 1.

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 5. 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 analyzed. 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 73496 canola, indicating that the observed segregation ratio was consistent with the expected ratio (Table 5).

In the case of the BC1F1^{*1,*3} generation, the observed segregation ratio was not consistent with the expected 1:1 (positive: negative) ratio (Table 5) and was attributed to the hand pollinations that were performed to generate the population. The BC1F1^{*1,*3} population was 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 73496 canola 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 73496 canola generations that were analyzed were either derived from the same progenitor as the BC1F1 genration (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,*3} population was 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 73496 canola 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 5. Summary of Genotypic and Phenotypic Results for 73496 Canola

73496 Canola Generation	Expected Segregation Ratio	Analysis	Observed Values (n = 100[a])		Statistical Analysis	
			Positive	Negative	Chi-Square[b]	P-value[c]
T3F2	3:1	PCR	69	30	1.4848	0.2230
		Herbicide tolerance assay	69	31	1.9200	0.1659
F1[*1,*3]	Non-Segregating	PCR	99	1	NA[d]	
		Herbicide tolerance assay	99	1		
BC1F1[*1,*3]	1:1	PCR	64	36	7.8400	0.0051[e]
		Herbicide tolerance assay	64	36	7.8400	0.0051[e]
BC2F1[*1,*3]	1:1	PCR	55	45	1.0000	0.3173
		Herbicide tolerance assay	55	45	1.0000	0.3173
BC3F1[*1,*3]	1:1	PCR	51	49	0.0400	0.8415
		Herbicide tolerance assay	51	49	0.0400	0.8415

[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] Probability (with 95% confidence) that, for a given 73496 canola generation, the goodness-of-fit between the observed and expected values is greater than the goodness-of-fit between a random sample and the expected values.
[d] Chi-square test is not applicable (NA) for testing a non-segregating population (*i.e.* a nominal proportion of 1.0).
[e] Statistically significant difference, P-value <0.05

V-D. Conclusions on Molecular Characterization and Genetic Stability of 73496 Canola

Southern blot analysis was conducted to characterize the DNA insertion in 73496 canola. The analysis confirmed that a single, intact PHP28181A DNA fragment was inserted into the canola genome. A single copy of each of the *UBQ10* promoter, *gat4621* gene, and *pin*II terminator genetic elements was present and the integrity of the PHP28181A DNA fragment was maintained. Southern blot analysis on five generations confirmed the stability of inheritance of the DNA insertion during traditional breeding procedures. In addition, Southern blot analysis verified the absence of plasmid backbone sequences in 73496 canola.

The inheritance and genetic stability of the inserted DNA in 73496 canola was examined in additional generations by event-specific and gene-specific PCR assays and by evaluation of herbicide-tolerance in the population. Four segregating generations and one non-segregating generation were evaluated for consistency with the expected segregation ratio of the population. The results of this analysis were consistent with the finding of a single locus of insertion of the *gat4621* cassette in 73496 canola that segregated according to Mendelian rules of inheritance. The stability of the insertion and of the herbicide-tolerance phenotype was demonstrated in these populations.

Together, these analyses confirmed the stability of the inserted DNA and its corresponding herbicide-tolerance trait during traditional breeding of 73496 canola.

VI. Characterization of the Introduced GAT4621 Protein

73496 canola was modified to express the GAT4621 protein to confer glyphosate-tolerance. The identity, deduced amino acid sequence, enzymatic activity, derivation, protein characterization, and concentrations of the GAT4621 protein in 73496 canola tissues are described below.

VI-A. The GAT4621 Protein

A1. Identity of the GAT4621 Protein

The GAT4621 protein is a variant of *N*-acetyltransferase protein sequences derived from *Bacillus licheniformis*, a gram positive saprophytic bacterium that is widespread in nature and thought to contribute substantially to nutrient cycling due to the diversity of enzymes produced by members of its species. GAT4621 is 147 amino acids in length and has a molecular weight of 16.5 kDa (Figure 15).

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

Figure 15. Deduced Amino Acid Sequence of the GAT4621 Protein

The introduced *gat4621* coding sequence in 73496 canola is identical to the *gat4621* coding sequence present in the previously approved maize event DP-Ø9814Ø-6 (FDA, 2008; Rood, 2007b; USDA, 2009).

As discussed in Section VI-B, the GAT4621 protein produced in 73496 canola demonstrated physicochemical equivalence to the microbial-produced GAT4621 protein that was used in the protein safety studies that were conducted for maize event DP-Ø9814Ø-6. These bridging data also support the conclusion that the GAT4621 protein expressed in 73496 canola and in maize event DP-Ø9814Ø-6 are equivalent.

A2. Characteristics and Enzymatic Activity of GAT Proteins

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, 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 nonselective, 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 resistance to glyphosate by detoxifying glyphosate to the non-phytotoxic form, *N*-acetylglyphosate (Figure 16). 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). As shown in Figure 16, GAT enzymes acetylate the secondary amine of glyphosate using acetyl coenzyme A as an acetyl donor (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 approved maize (DP-Ø9814Ø-6) (FDA, 2008; Rood, 2007b; USDA, 2009) and soybean (DP-356Ø43-5) events (FDA, 2007; Rood, 2006; USDA, 2008).

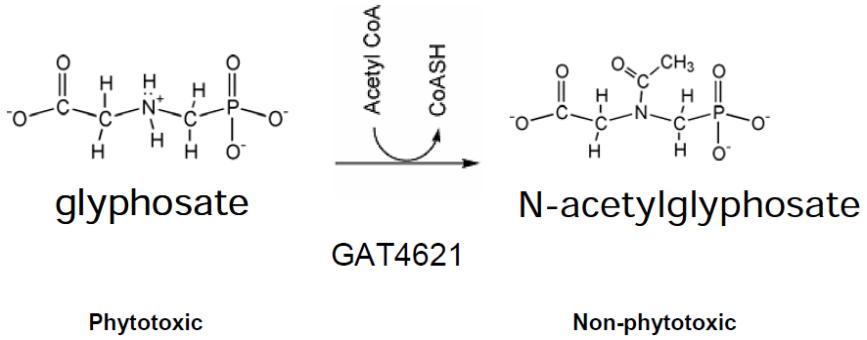

Figure 16. Enzymatic Activity of the GAT4621 Protein

A3. Derivation of the GAT4621 Protein

The derivation of the GAT4621 enzyme was discussed in submissions to the USDA and FDA for maize event DP-Ø9814Ø-6 (FDA, 2008; Rood, 2007b; USDA, 2009) and was discussed in the New Protein Consultation that was provided to FDA (FDA, 2009; Rood, 2007a; Appendix 11). In summary, the gat4621 gene was produced using a DNA shuffling process employing three native gat genes from Bacillus licheniformis as parental templates (Castle *et al.*, 2004). The gat4621 gene variant was the result of 11 rounds of gene shuffling and the encoded GAT4621 enzyme exhibited a 7000-fold increase in catalytic activity relative to the native enzymes, using glyphosate as a substrate (Siehl *et al.*, 2005). The gat4621 gene was further optimized for plant expression by conservative codon substitutions and a codon addition to improve protein production in plants (a GCT codon for alanine was inserted at amino acid position 2).

The GAT4621 protein, encoded by the *gat4621* gene, is 75–78% identical and 90–91% similar at the amino acid level to each of the three native GAT enzymes from which it was derived, compared to 94% identity of each of the native enzymes to each other (Table 6). There are 32 to 36 amino acid changes (22 to 23 of which are conservative) between the shuffled GAT4621 protein and any one of the original three native GAT proteins.

Table 6. Comparison of Amino Acid Sequence Similarity Between Parental GAT Enzymes and GAT4621

	Percent Identity or Similarity of Amino Acid Sequences			
	GAT (strain 401)	**GAT (strain B6)**	**GAT (strain DS3)**	**GAT4621**
GAT (strain 401)	100%	94% Identical	94% Identical	78% Identical 91% Similar
GAT (strain B6)		100%	94% Identical	76% Identical 91% Similar
GAT (strain DS3)			100%	75% Identical 90% Similar
GAT4621				100%

VI-B. Physicochemical Characterization of the GAT4621 Protein

The safety of GAT4621 protein was evaluated and was assessed previously by FDA as part of New Protein Consultation 005 (FDA, 2009; Rood, 2007a; Appendix 11). 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 73496 canola 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 73496 canola 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 are included in Appendix 4.

Utilizing the above analyses, the equivalency of GAT4621 protein expressed in *E. coli* to the protein expressed in 73496 canola 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 73496 canola. Microbial GAT4621 protein was used for *in vitro* digestion and acute toxicology safety assessment studies summarized in Section VI-D.

VI-C. Concentrations of GAT4621 in 73496 Canola

In order to understand the level expression of the GAT4621 protein in 73496 canola 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 73496 canola.

The range of expression of the GAT4621 protein in 73496 canola 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 (Appendix 3 for ELISA materials and methods). 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 73496 canola (Table 7). 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 73496 root was 6.6 ng/mg dry weight. The mean concentration in seed was 6.2 ng/mg dry weight.

Table 7. Concentration of GAT4621 Protein in 73496 Canola Plant Tissues

Tissue	GAT4621 Concentration [ng/mg dry weight ± SD (range)][a]			
	Plant Growth Stage[b]			
	BBCH15	BBCH33	BBCH65	BBCH90
Whole Plant	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	NC[c]	NC	6.6 ± 2.4 (3.9–13)	NC
Seed	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.

VI-D. Summary of the Food and Feed Safety Assessment for the GAT4621 Protein

A food and feed safety assessment was 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 005 and was completed on October 7, 2009 (FDA, 2009; Rood, 2007a). A summary of the information in NPC 005, along with recently updated bioinformatic analyses on GAT4621 are described below. Additional information on 73496 canola, including human and livestock exposure assessments, will be submitted to FDA as part of the consultation process for bioengineered foods.

Canola seed is not commonly consumed without processing and its fractions have different uses for humans and livestock. Canola seed is processed into refined oil which is then used as a human food and livestock feed. The by-product meal fraction from processing is used as livestock feed. Refined oil is the only canola product that is consumed by humans. The oil is free from any protein and considered non-allergenic (Gylling, 2006; Hefle and Taylor, 1999; Moneret-Vautrin and Kanny, 2004), and therefore would not be expected to contain the GAT4621 protein.

Human or livestock consumption of the GAT4621 protein in oil derived from 73496 canola will be negligible. Livestock will consume the GAT4621 protein in meal derived from 73496 canola seed after oil extraction. Any human exposure to GAT4621 protein would be limited to potential occupational exposure to canola seed meal and flour in feed processing facilities, grain mills or on farm. Due to the very low exposure and low risk, the GAT4621 protein in 73496 canola is safe.

Although there will be consumption of the GAT4621 protein by livestock and also human occupational exposure from meal derived from 73496 canola, the GAT4621 protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals, and therefore is safe for human exposure and animal consumption.

1) 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.
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 (EU Commission, 2000; FDA, 2001) and as a probiotic (Alexopoulos *et al.*, 2004a; Alexopoulos *et al.*, 2004b; Kritas *et al.*, 2006). B. licheniformis was determined by 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 (Federal Register, 1997).
3) 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 (Version 10.0, January 2010), which contains the amino acid sequences of 1471 known and putative allergenic proteins. Potential identities between the GAT4621 protein and proteins in the allergen database were evaluated using the FASTA34 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 cutoff = 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.
4) Additional experimental assays were conducted to assess the allerfenic potential of the GAT4621 protein. The GAT4621 protein was assayed for digestibility in simulated gastric fluid and in simulated intestinal fluids and also assayed for the presence of glycosylation. The GAT4621 protein is rapidly hydrolyzed in both simulated gastric and intestinal fluids (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). In addition, the GAT4621 protein in 73496 canola is not glycosylated (Appendix 4). Taken together with the similarity search results, these data indicate that the GAT4621 protein is unlikely to be allergenic to animals or humans.

5) Bioinformatic analyses demonstrated that the GAT4621 protein retains the characteristics found in other N-acetyltransferases that are ubiquitous in plants and microorganisms (Neuwald and Landsman, 1997). GAT4621 contains the definitive motif for the GNAT family of N-acetyltransferases (Marchler-Bauer *et al.,* 2005). This superfamily of enzymes is present in all organisms, including plants, mammals, fungi, algae, and bacteria (Dyda *et al*., 2000).
6) 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 2.2.13 algorithm against Release 176.0 (02/15/10) of the NCBI Entrez Protein dataset. A cutoff *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. The GAT4621 similarity search identified 577 proteins that were within these criteria. 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.
7) There was no evidence of acute toxicity in mice at a target dose of 2000 mg purified protein preparation per kg of body weight (equivalent to approximately 1640 mg of the full-length GAT4621 protein per kg of body weight). Based on expression levels of GAT4621 protein in 73496 canola toasted meal, the highest calculated exposure to GAT4621 protein was for poultry livestock based on a hypothetical total replacement scenario. The margin of exposure for poultry to GAT4621 is greater than 50,000-fold the no observed adverse effect level of 1640 mg/ kg of body weight in mice. Based on these calculations, there is a wide margin of safety for the GAT4621 protein.

VII. Agronomic Performance and Ecological Observations

Agronomic and ecological evaluations were conducted to assess the comparability of 73496 canola to conventional canola. These evaluations form the basis to determine whether 73496 canola is comparable to conventional canola and is therefore no more likely to pose a plant pest risk than conventional canola.

The agronomic evaluations were based on both laboratory experiments and replicated, multi-site field trials conducted by agronomists and scientists who are considered experts in the production and evaluation of canola. To evaluate the agronomic characteristics of 73496 canola, data were collected on representative characteristics that influence reproduction, crop survival, and potential weediness. In each of these assessments, 73496 canola was compared to a near-isoline control that did not carry any recombinant DNA, and, in some experiments, was compared to non-transgenic commercial canola lines. In each experiment, 73496 canola was comparable to the control or commercial comparators.

The ecological evaluations included observed responses to insect and disease stressors during multi-year and multi-site field trials. These observations were made on 73496 canola and control canola and tracked the presence of insect and disease stressors in the field and the plant responses. In each case, 73496 canola responded similarly to the control plants in these trials.

Based on the analyses described below, 73496 canola is comparable to conventional canola and would not pose a greater plant pest risk or increased weed potential than conventional canola.

VII-A. Germination Evaluations

In order to test germination, seeds from the F1[*5] generation (Section III-A, Figure 2) of 73496 canola were tested for germination under warm, cold, and diurnal conditions (Table 8). A near-isoline control was used for comparison. In addition, two commercial canola lines were evaluated to establish a reference range for germination. This range provided a context to determine the biological significance of any statistical differences observed.

Each germination test contained eight replicates of 73496 canola, near-isoline control, and two commercial lines: one 60-seed replicate was counted and weighed for each canola line, with seven additional replicates weighed out based on the weight of the corresponding 60-seed replicate. The "Rules for Testing Seeds", published by the Association of Official Seed Analysis, were used as guidelines for the germination methods and interpretation of results (AOSA, 2007). Each replicate was placed between sheets of moist germination toweling, rolled up, and placed in a growth chamber set to the appropriate test conditions as specified below.

Table 8. Description of Seed Germination Conditions

Warm germination test	• Continuous setting of 25°C and 90% relative humidity for 10 days • Germinated seed counted after 10 days
Cold germination test	• Continuous setting of 10°C and 90% relative humidity for 10 days, followed by three days at a continuous setting of 25°C and 90% relative humidity • Germinated seed counted after 13 days
Diurnal germination test	• Simulates daily weather conditions • Cyclical setting of 10°C and 90% relative humidity for 16 hours and then 25°C and 90% relative humidity for 8 hours, repeated daily for 10 days • Germinated seed counted after 10 days

A statistically significant (P-value = 0.0460) difference in mean germination rate was observed between 73496 canola and the control canola in the warm germination test, with a lower mean germination rate in 73496 canola compared to the control canola (Table 9). However, for 73496 canola and the control canola, the range of germination rate values was within the reference range of commercial canola values. In the cold germination test, no statistically significant differences in mean germination rate were observed between 73496 canola and the control canola (Table 9). In the diurnal germination test, no statistically significant differences in mean germination rate were observed between 73496 canola and the control canola (Table 9).

Table 9. Summary of Germination Results for 73496 Canola

Statistic	73496 Canola	Control Canola	Reference Range[a]
Warm			
Mean[b]	96.4%	98.7%	84% - 100%
Range[c]	93% - 100%	96% - 100%	
P-value[d]	0.0460[a]		
Cold			
Mean	98.8%	98.4%	88% - 100%
Range	93% - 100%	97% - 100%	
P-value	0.7127		
Diurnal			
Mean	98.9%	99.8%	92% - 100%
Range	95% - 100%	98% - 100%	
P-value	0.1635		

[a] Reference range denotes the lowest and highest germination rates across reference canola lines
[b] Mean percentage
[c] Range denotes the lowest and highest germination rates
[d] Statistically significant difference; P-value <0.05

Germination rates in 73496 canola under warm, cold and diurnal growing conditions were comparable to those of the control canola. A slightly lower germination rate was observed for 73496 canola seed under warm growing conditions that was statistically significant. However, this difference was small and the entire range of germination rate values for 73496 canola and the control canola under warm growing conditions were within the corresponding range of reference for commercial canola germination rates.

The data provided here support the conclusion that 73496 canola is comparable to conventional canola with respect to germination.

VII-B. Field Trial Evaluations

73496 canola has been field tested in the United States since 2007 as authorized by USDA permits listed in Appendix 6. The list compiles a number of test sites in diverse regions of the U.S. including the major canola-growing areas. Agronomic data were collected to assess agronomic comparability as it relates to plant pest potential. In addition, extensive testing has also been conducted in Canada in major canola-growing areas, primarily because Pioneer's canola breeding facilities are located in Canada and Canada is the primary market. A table of testing under authorized Canadian permits is also provided in Appendix 6.

Throughout the development process, 73496 canola was observed for unexpected differences in responses to abiotic stress (*e.g*, drought, excess moisture, temperature extremes, *etc.*). Monthly observations for response to naturally occurring abiotic stressors indicated that 73496 canola and near-isoline control were similar with respect to their response to abiotic stress.

Agronomic data were collected from 73496 canola and control canola in two experiments (denoted A and B) that were conducted at 10 total field locations in 2008 and 2009 (Table 10; Figure 17). The trial locations provided a range of environmental and agronomic conditions representative of the major canola growing regions in the U.S. and Canada, where commercial sales of 73496 canola are expected. Agronomic practices used to prepare and maintain each field site were characteristic of each respective region. While the majority of the sites were from Canadian field trial sites, these Canadian locations are considered representative of suitable canola growing areas within the U.S. Zone maps have been created by the U.S. EPA (EPA, 1996) and Canada Pest Management Regulatory Agency (PMRA, 1998) that divide North America into regions where climate conditions are similar (Figure 18, Panels A and B). These zone maps are used to determine where field trials should be placed when conducting studies for establishing tolerances (*e.g.* maximum pesticide residue levels) associated with domestic pesticide registrations. Thus, field trials conducted within the same zone are considered interchangeable. The zones for each of the field trials conducted are provided in Table 10 and show that the zone designations for trials conducted in Canada are representative of U.S. zones.

A statistical analysis of agronomic data was conducted to test for differences in the mean values between the 73496 canola and the near-isoline control (Appendix 2 for statistical model). When numerous comparisons are being made, it is important to control the rate of false positive results. Since the introduction of the false discovery rate (FDR) approach in the mid-1990's, it has been widely employed across a number of scientific disciplines, including genomics, ecology, medicine, plant breeding, epidemiology, dairy science and signal/image processing (*e.g.*, Pawitan *et al.*, 2005; Spelman and Bovenhuis, 1998). A false positive result occurs when two means are deemed significantly different when, in fact, they are not. If one uses a 5% type I error rate for each agronomic characteristic measured, then the number of false positives increases as the number of characteristics increase. In order to help manage the false positive rate, the 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. This resulted in the false positive rate being held to 5%. Both adjusted and unadjusted P-values are provided for the agronomic data.

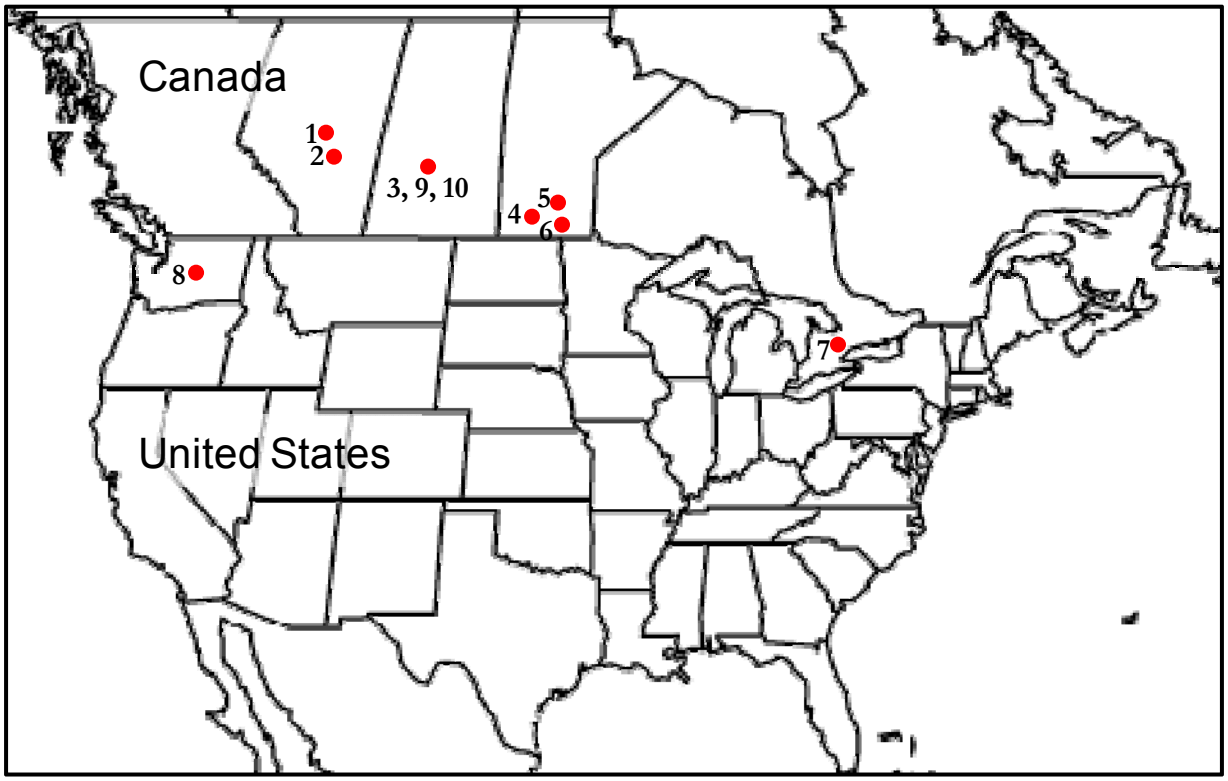

Figure 17. Map of Locations for Agronomic Data Collection for 73496 Canola

Table 10. Site Locations for 2008 and 2009 Field Trial Experiments

Map Site Number	Country	Location	Zone
Experiment A: 2008 Field Trials			
1	Canada	Riviere Qui Barre, AB	14
2		Nisku, AB	14
3		Saskatoon, SK	7/14
4		Crystal City, MB	5/14
5		Rosebank, MB	5/14
6		Morden, MB	5/14
7		Georgetown, ON	5
Experiment B: 2009 Field Trials			
8	U.S.	Ephrata, WA	11
9	Canada	Saskatoon, SK	7/14
10		Saskatoon, SK	7/14

A. Canada PMRA Zones

B. U.S. EPA Zones

Figure 18. Maps Indicating Zones with Similar Growing Conditions for Field Trials

Panel A: Zones established by PMRA in Canada with similar climate conditions for residue field trials (PMRA, 1998). *Panel B*: Zones established by the U.S. EPA with similar climate conditions for residue field trials (EPA, 1996).

Experiment A contains data from seven locations planted in Canada during the 2008 growing season (Table 10, Figure 17). The purpose of Experiment A was to evaluate the agronomic characteristics and yield of 73496 canola. Seed of the T3 generation of 73496 canola was used (Section III-A, Figure 2). The control plants were near-isoline control canola.

The following characteristics were measured: early growth, plant height, days to flower, days to maturity, and yield. Descriptions of the characteristics and their measurement are found in Table 11.

Each site included a randomized complete block design containing three blocks, with each block containing one plot of 73496 canola and one plot of the control canola. Procedures employed to control the introduction of experimental bias in this study were as follows: non-systematic selection of trial areas and plot areas within each site, randomization of the canola entries within each block, and uniform maintenance treatments across each plot area.

Results of Experiment A are summarized in Table 12.

For characteristics early growth, plant height, days to maturity, and yield, no statistically significant differences were observed between the mean values of 73496 canola and the control. One statistical difference was observed between 73496 canola and the control in days to flower (adjusted P-value < 0.05). The mean flowering time was one day longer for 73496 canola; however, the range of individual values for days to flower in 73496 canola was within the range of the control data. Furthermore, this statistical difference was not replicated in the trial conducted in 2009. Because the time to flowering is not a significant parameter for weediness, this statistical difference is not biologically meaningful to the determination of plant pest potential.

The results from Experiment A indicate that 73496 canola is agronomically comparable to the near-isoline control.

Table 11. Experiment A: 2008 Field Trial Agronomic Characteristics Measured

General Characteristic	Characteristic Measured	Number of Sites	Data Description	Scale
Vegetative Parameter	Early Growth	3 of 7	Overall plant health and amount of soil surface area covered by leaves at the 4-leaf to 6-leaf stages	1-9 Scale where 1=Unhealthy/weak looking plants with small leaf coverage; 9=healthy/strong-looking plants with large leaf coverage
	Plant Height	7 of 7	10 plants per plot at physiological maturity. Height was measured from the soil surface to the tip of the highest pod.	Centimeters
Reproductive Parameter	Days to Flower	7 of 7	Number of days from the planting date to the date when approximately 50% of plants had produced flowers.	Days
	Days to Maturity	6 of 7	Number of days from the planting date to the date when approximately 50% of plants had reached physiological maturity.	Days
	Yield	7 of 7	Weight (corrected for moisture content of seed) harvested from all plants in a given plot	kg/ha

Table 12. Experiment A: Summary of Agronomic Characteristics of 73496 Canola Across Seven Locations

Agronomic Characteristic[a] (unit of measurement)		Control Canola	73496 Canola
Early Growth (1-9 scale)	Mean[b]	6.50	7.37
	Range[c]	6 - 7	6 - 8
	P-Value[d]		0.0395
	Adjusted P-Value[e]		0.0790
Days to Flower (days)	Mean	44.6	45.7
	Range	40 - 50	42 - 49
	P-Value		0.000218
	Adjusted P-Value		0.00131[f]
Days to Maturity (days)	Mean	93.0	92.8
	Range	87 - 106	89 - 105
	P-Value		0.468
	Adjusted P-Value		0.562
Plant Height (centimeters)	Mean	89.5	91.6
	Range	65 - 115	60 - 118
	P-Value		0.355
	Adjusted P-Value		0.532
Yield (kg/ha)	Mean	2310	2140
	Range	1020-3500	1140-2910
	P-Value		0.0311
	Adjusted P-Value		0.0790

[a] Refer to Table 11 for descriptions of each agronomic characteristic measured.
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across sites.
[d] Non-adjusted P-value
[e] False Discovery Rate (FDR) adjusted P-value
[f] Statistically significant difference, adjusted P-value <0.05

Experiment B contains data from three locations planted in the U.S. and Canada during the 2009 growing season (Table 10, Figure 17). The purpose of Experiment B was to evaluate the agronomic characteristics and yield of 73496 canola. Seed from the T5 generation was used (Section III-A, Figure 2). The control plants were near-isoline control canola

The following agronomic characteristics were measured in each block: early population, seedling vigor, days for flowering, plant height, disease incidence, insect damage, lodging, days to maturity, shattering, final population, and yield data. Descriptions of the characteristics and their measurement are found in Table 13.

Each site included a randomized complete block design containing four blocks, with each block containing 73496 canola and control canola.

Results of Experiment B are summarized in Table 14.

No statistically significant differences were observed between 73496 canola and control canola mean values for early population, final population, seedling vigor, days to flowering, flowering duration, disease incidence, insect damage, lodging, days to maturity, shattering, yield, or plant height.

The results from Experiment B indicate that 73496 canola is agronomically comparable to the near-isoline control.

Table 13. Experiment B: 2009 Field Trial Agronomic Characteristics Measured

General Characteristic	Characteristic Measured	Evaluation timing[a]	Data Description	Scale
Germination / Emergence	Early Population	BBCH 11-13	Number of plants emerged per plot (two 1 square meter quadrants) during true leaf (rosette) development	Actual count per plot
	Seedling Vigor	BBCH 11-13	Visual estimate of average vigor of emerged plants per plot during true leaf (rosette) development	From 1 to 9, where 1=short plants with small thin leaves, and 9=tall plants with large robust leaves
Vegetative Parameters	Plant Height	BBCH 79	Height from the soil surface to the tip of the highest pod when nearly all pods on the plants are at full size	Height in cm
	Lodging	BBCH 87-89	Visual estimate of lodging severity when >70% of pods are fully ripe	From 1 to 9, where 1=plants lying flat, and 9=plants standing straight
	Shattering	BBCH 87-89	Visual estimate of seed shattering when >70% of pods are fully ripe	From 1 to 9, where 1=high seed shattering, and 9=no seed shattering
	Final Population	Harvest	Total number of plants per plot	Actual count per plot

[a] Based on Lancashire *et al.* (1991) for canola growth stages.

Table 13. Experiment B: 2009 Field Trial Agronomic Characteristics Measured (continued)

General Characteristic	Characteristic Measured	Evaluation timing[a]	Data Description	Scale
Reproductive Parameters	Days to flowering	BBCH 57-62	From the time of planting to the first open flowers.	Days
	Flowering duration	BBCH 69	Number of days from the date when the first flowers were open to the date when flowering ended on approximately 50% of the plants.	Days
	Days to Maturity	BBCH 86-87	Number of days from planting to the date when between 60-70% of pods are ripe.	Days
	Yield	At harvest	Weight (corrected for moisture content of seed) harvested from all plants in a given plot	kg/ha
Ecological Interactions	Disease Incidence	BBCH 71-79	Visual estimate of foliar disease incidence during plant pod elongation.	Ranging from 1-9 where 1 = poor disease resistance or high infection; 9 = best disease resistance or low infection.
	Insect Damage	BBCH 71-79	Visual estimate of insect damage during plant pod elongation.	Ranging from 1-9 where 1 = poor insect resistance or high damage; 9 = best insect resistance or low damage.

[a] Based on Lancashire *et al.* (1991) for canola growth stages.

Table 14. Experiment B: Summary of Agronomic Characteristics of 73496 Canola Across Three Locations

Agronomic Characteristic[a] (unit of measurement)		Control Canola	73496 Canola
Early Population (number of plants)	Mean[b]	108	116
	Range[c]	50 - 184	41 - 207
	P-Value[d]		0.496
	Adjusted P-Value[e]		0.661
Final Population (number of plants)	Mean	87	82
	Range	29 - 158	23 - 164
	P-Value		0.691
	Adjusted P-Value		0.691
Seedling Vigor (1-9 scale)	Mean	5	5
	Range	3 - 8	3 - 8
	P-Value		0.422
	Adjusted P-Value		0.632
Days to Flowering (number of days)	Mean	47	47
	Range	35 - 60	35 - 59
	P-Value		0.327
	Adjusted P-Value		0.561
Flowering Duration (number of days)	Mean	27	28
	Range	22 - 37	22 - 38
	P-Value		0.116
	Adjusted P-Value		0.279
Disease Incidence (1-9 scale)	Mean	7	7
	Range	6 - 9	6 - 8
	P-Value		0.0851
	Adjusted P-Value		0.279
Insect Damage (1-9 scale)	Mean	6	7
	Range	3 - 9	3 - 9
	P-Value		0.165
	Adjusted P-Value		0.329
Lodging (1-9 scale)	Mean	8	8
	Range	7 - 9	6 - 9
	P-Value		0.624
	Adjusted P-Value		0.691

[a] Refer to Table 13 for descriptions of each agronomic characteristic measured.
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across sites for 73496 canola and near-isoline control.
[d] Non-adjusted P-value
[e] False Discovery Rate (FDR) adjusted P-value

Table 14. Experiment B: Summary of Agronomic Characteristics of 73496 Canola Across Three Locations (continued)

Agronomic Characteristic[a] (unit of measurement)		Control Canola	73496 Canola
Days to Maturity (numbers of days)	Mean[b]	113	114
	Range[c]	103 - 132	104 - 133
	P-Value[d]		0.102
	Adjusted P-Value[e]		0.279
Shattering (1-9 scale)	Mean	8	7
	Range	5 - 9	4 – 9
	P-Value		0.646
	Adjusted P-Value		0.691
Yield[g] (kg/ha at 8% moisture)	Mean	3500	2900
	Range	2600 - 4680	1550 - 4280
	P-Value		0.0395
	Adjusted P-Value		0.279
Plant Height (centimeters)	Mean	118	113
	Range	100 - 143	94 - 134
	P-Value		0.0563
	Adjusted P-Value		0.279

[a] Refer to Table 13 for descriptions of each agronomic characteristic measured.
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across sites for 73496 canola and near-isoline control.
[e] Non-adjusted P-value
[e] False Discovery Rate (FDR) adjusted P-value
[f] Yield data from one of the three sites was not included due to an early killing frost that affected seed harvest at maturity.

VII-C. Ecological Observations

Ecological observations (*i.e.*, plant interactions with pest insects and diseases) were recorded for all USDA-APHIS permitted field trials of 73496 canola during the 2007, 2008, 2009, and 2010 growing seasons. In addition, trials were conducted in Canada in 2007, 2008, and 2009 and the ecological observations were recorded. Plant breeders and field staff familiar with plant pathology and entomology observed 73496 canola and control lines at least every four weeks for pest insect and disease pressure and recorded the severity of any stressor seen. Any unexpected differences in response between 73496 canola and various control lines (near-isolines and/or conventional canola lines) were recorded.

The following scale was used when recording observations:

- mild – very little disease or insect injury (<10%) visible
- moderate – noticeable plant tissue damage (10% to 30%)
- severe – significant plant tissue damage (>30%)

A summary of the insect and disease ecological observations for both U.S. and Canada field trials is provided in Appendix 5. In every case, the severity of insect or disease stress on 73496 canola was not qualitatively different from various control lines growing at the same location. These results support the conclusion that the ecological interactions for 73496 canola were comparable to control canola lines with similar genetics or to conventional canola lines.

VII-D. Conclusions on Agronomic Performance and Ecological Observations

73496 canola was observed in laboratory experiments and at 10 field locations in the U.S. and Canada to measure agronomic parameters. Data generated from these studies represent observations that are typically recorded by plant breeders and agronomists to evaluate the characteristics of canola over a broad range of environmental conditions that represent where 73496 canola may be grown. The agronomic characteristics measured are representative of reproduction, survival, and potential weediness. The measured characteristics provide data useful in establishing a basis to assess agronomic comparability and familiarity of 73496 canola compared to conventional canola in the context of ecological risk assessment.

The agronomic data showed no biologically meaningful differences between 73496 canola and control canola (untransformed near-isoline controls and/or commercial canola lines) with respect to early population, vegetative growth, reproductive parameters, yield and ecological interactions. These data support the conclusion that 73496 canola is comparable in agronomic characteristics to conventional canola.

Ecological observations of U.S. and Canadian field trials for responses of 73496 canola to naturally occurring insect and disease stressors showed no unexpected differences from control canola. The assessment of the ecological data detected no biologically significant differences between 73496 canola and control canola lines indicative of a selective advantage that would result in increased weed potential or plant pest risk.

Based on these analyses, 73496 canola is comparable to conventional canola and would not pose a greater plant pest risk or increased weed potential than conventional canola.

VIII. 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. Compositional assessments are performed in accordance with the principles outlined in the OECD consensus document on compositional considerations for canola (OECD, 2001). This document emphasizes quantitative measurements of essential nutrients, and known anti-nutrients and toxicants. These analyses will effectively highlight any compositional changes that may indicate potential safety and anti-nutritional concerns.

For 73496 canola, the following analytes were measured for the comparative assessment: proximates, fiber, 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 (Section III-A; Table 1, Figure 2) of 73496 canola 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 73496 canola to conventional canola. Canola seed was chosen as the test material for the compositional analysis of 73496 canola because and 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 utilized a randomized complete block design with four blocks and each block containing 73496 canola and the control canola. Each plot of 73496 canola 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 73496 canola and the control canola were analyzed for key nutritional components in accordance with the OECD consensus document on compositional considerations for new varieties of canola (OECD, 2001), which included analysis of proximates, fiber, fatty acids, amino acids, vitamins, minerals, glucosinolates, secondary metabolites, phytosterols, and anti-nutrients.

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. The reference lines were planted, harvested, processed, and analyzed using similar methods to those employed for the near-isoline control and 73496 canola. 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 73496 canola and the near-isoline control. For details of the statistical methodology, refer to Appendix 2. 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 73496 canola 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 73496 canola.

VIII-A. Proximate and Fiber Analysis

Analysis of the major constituents of canola seed, or proximates, was used to determine the nutritional properties of seed from 73496 and control canola. No statistically significant differences were observed between 73496 canola and the control canola mean values for any of the proximate and fiber analytes (Table 15).

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

Table 15. Summary Analysis of Proximate and Fiber Results in Canola Seed

Analyte		Control Canola	73496 Canola	Tolerance Interval[a]
% 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[h]	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

VIII-B. Fatty Acid Analysis

Five fatty acids account for more than 96 percent 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 analyze any unintended changes in the concentrations of linoleic and α-linolenic acids, and their key precursors palmitic, stearic, and oleic acids, in seed from 73496 canola.

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 73496 canola 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 16 were identified by OECD as those that should be measured for compositional comparisons in canola (OECD, 2001). 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 16. As expected per the canola definition, erucic acid (C22:1) was not detected in the analysis.

Small but statistically significant differences were observed using the adjusted P-value in concentrations of oleic and linoleic acid between 73496 and control canola (Table 16). However, these fatty acid concentrations were within the respective tolerance intervals derived for commercial canola varieties. Therefore, the small, statistically significant differences observed for certain fatty acids are unlikely to be biologically meaningful.

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

Table 16. Summary Analysis of Fatty Acid Composition in Canola Seed

Analyte		Control Canola	73496 Canola	Tolerance Interval[a]
% Total Fatty Acids				
Lauric Acid (C12:0)	Mean[b]	0.0816	0.0811	0 - 0.216
	Range[c]	0.0568 - 0.105	0.0563 - 0.113	
	CI[d]	0.0639 - 0.104	0.0634 - 0.104	
	P-Value[e]		0.863	
	Adjusted P-Value[f]		0.919	
Myristic Acid (C14:0)	Mean	0.053	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		0.079	
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		0.058	
	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[g]	
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[g]	
(9,15) Isomer of Linoleic Acid (C18:2)	Mean	0.0986	0.0924	0 - 0.524[h]
	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	

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

Analyte		Control Canola	73496 Canola	Tolerance Interval
% Total Fatty Acids				
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	0.069	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		0.079	
Lignoceric Acid (C24:0)	Mean	0.178	0.17	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		0.079	
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] 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] Statistically significant difference; FDR Adjusted P-Value <0.05
[h] Tolerance interval could not be calculated (NC) due to all sample values below the assay LLOQ.

VIII-C. Amino Acid Analysis

Canola seed is generally a good source of essential and non-essential amino acids for most domestic animal species. Total levels of 18 amino acids and 26 free amino acids were measured in 73496 and control seed.

Results are shown in Table 17 for the 18 total amino acids. No statistically significant differences between the 73496 canola and control were noted for any amino acids measured (adjusted P-value).

The levels of 26 free amino acids were also measured (Pioneer data not shown). For each analyte, the measured values for 73496 canola were within statistical tolerance intervals defined by commercial conventional varieties even when a statistically significant difference was observed between 73496 canola and control.

In conclusion, total and free amino acid analysis of canola seed demonstrates that 73496 canola is comparable to conventional canola.

Table 17. Summary Analysis of Amino Acid Composition in Canola Seed

Analyte		Control Canola	73496 Canola	Tolerance Interval[a]
% Dry Weight				
Alanine	Mean[b]	1.14	1.12	0.588 - 1.90
	Range[c]	1.01 - 1.37	0.970 - 1.32	
	CI[d]	1.02 - 1.28	0.996 - 1.25	
	P-Value[e]		0.0446	
	Adjusted P-Value[f]		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 17. Summary Analysis of Amino Acid Composition in Canola Seed (continued)

Analyte		Control Canola	73496 Canola	Tolerance Interval
% Dry Weight				
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	

[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

VIII-D. 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 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 18). These results indicate 73496 canola 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, 2001). Concentrations of δ-tocopherol and total tocopherols were statistically significantly different between 73496 canola and control canola seed samples using the adjusted P-value (Table 18). 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 small, statistically significant differences observed for certain tocopherols are unlikely to be biologically meaningful.

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

Table 18. Summary Analysis of Vitamin Composition in Canola Seed

Analyte		Control Canola	73496 Canola	Tolerance Interval[a]
mg/kg Dry Weight				
Vitamin B1 (Thiamine)	Mean[b]	14.2	14.8	4.92 - 33.1
	Range[c]	9.65 - 21.1	10.6 - 25.8	
	CI[d]	11.8 - 17.2	12.2 - 17.8	
	P-Value[e]		0.611	
	Adjusted P-Value[f]		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	
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[g]	<1.25[g]	0 - 1.67[i]
	Range	<1.25[g]	<1.25[g]	
	CI	NA[h]	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[j]	

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

Analyte		Control Canola	73496 Canola	Tolerance Interval
mg/kg Dry Weight				
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[j]	

[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] Analyte values were below the assay LLOQ. Sample results below the LLOQ were assigned a value equal to the LLOQ.
[h] Statistical analysis was not available (NA).
[i] 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.
[j] Statistically significant difference, FDR adjusted P-Value <0.05.

VIII-E. 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 analyzed in seed samples from 73496 canola and compared with corresponding values from samples of near-isoline control canola seed.

A statistically significant difference was observed in magnesium concentration between 73496 and control canola using the adjusted P-value (Table 19). 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 73496 canola is comparable to conventional canola.

Table 19. Summary Analysis of Mineral Composition in Canola Seed

Analyte		Control Canola	73496 Canola	Tolerance Interval[a]
% Dry Weight				
Calcium	Mean[b]	0.465	0.474	0.172 - 0.939
	Range[c]	0.365 - 0.576	0.397 - 0.572	
	CI[d]	0.406 - 0.532	0.414 - 0.542	
	P-Value[e]		0.457	
	Adjusted P-Value[f]		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[g]	
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	
Copper	Mean	0.000175	0.000185	0 - 0.000772
	Range	<0.000125[h] - 0.000251	<0.000125[h] - 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] 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] Statistically significant difference, FDR adjusted P-Value <0.05.
[h] Analyte values were below the assay LLOQ. Sample results below the LLOQ were assigned a value equal to the LLOQ.

VIII-F. Glucosinolate Analysis

Glucosinolates are considered key toxicants of canola (OECD, 2001). 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 73496 canola was 5.66 µmoles/g dry weight, which is within the acceptable definition for canola (maximum 30 µmoles/g dry weight; OECD, 2001).

A statistically significant difference was observed in progoitrin concentration between 73496 canola and control canola samples based on the adjusted P-value. The range of concentrations of progoitrin in 73496 canola was within the tolerance intervals established using commercial canola varieties (Table 20).

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

Table 20. Summary Analysis of Glucosinolate Composition in Canola Seed

Analyte		Control Canola	73496 Canola	Tolerance Interval[a]
µmol/g Dry Weight				
Glucoiberin	Mean[b]	<0.176[g]	<0.176[g]	NC[i]
	Range[c]	<0.176[g]	<0.176[g]	
	CI[d]	NA[h]	NA	
	P-Value[e]		NA	
	Adjusted P-Value[f]		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[j]	
Epi-Progoitrin	Mean	0.0717	<0.0708[g]	0 - 0.159[k]
	Range	<0.0708[g] - 0.0883	<0.0708[g]	
	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	
Gluconapoleiferin	Mean	0.0339	0.0330	0 - 0.372
	Range	<0.0299[g] - 0.0618	<0.0299[g] - 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[g] - 0.125	<0.0676[g] - 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	

Table 20. Summary Analysis of Glucosinolate Composition in Canola Seed (continued)

Analyte		Control Canola	73496 Canola	Tolerance Interval
µmol/g Dry Weight				
Glucobrassicinapin	Mean	0.401	0.395	0 - 1.80
	Range	<0.390[g] - 0.475	<0.390[g] - 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[g]	<0.0774[g]	NC
	Range	<0.0774[g]	<0.0774[g]	
	CI	NA	NA	
	P-Value		NA	
	Adjusted P-Value		NA	
Neoglucobrassicin	Mean	0.0466	0.0641	0 - 0.192
	Range	<0.0198[g] - 0.0903	0.0230 - 0.162	
	CI	0.0266 - 0.0815	0.0366 - 0.112	
	P-Value		0.00897	
	Adjusted P-Value		0.0525	
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] 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] 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].
[h] Statistical analysis was not available (NA).
[i] Tolerance interval could not be calculated (NC) due to all sample values below the assay LLOQ.
[j] Statistically significant difference, FDR adjusted P-Value <0.05.
[k] 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.

VIII-G. Secondary Metabolite and Anti-Nutrient Analysis

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

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 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 21).

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

Table 21. Summary Analysis of Secondary Metabolite and Anti-Nutrient Composition in Canola Seed

Analyte		Control Canola	73496 Canola	Tolerance Interval[a]
% Dry Weight				
Tannins-Soluble	Mean[b]	0.113	0.109	0.0521 - 0.297
	Range[c]	0.0768 - 0.180	0.0777 - 0.176	
	CI[d]	0.0840 - 0.152	0.0812 - 0.147	
	P-Value[e]		0.415	
	Adjusted P-Value[f]		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[g]	
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	
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	

Table 21. Summary Analysis of Secondary Metabolite and Anti-Nutrient Composition in Canola Seed (continued)

Analyte		Control Canola	73496 Canola	Tolerance Interval
% Dry Weight				
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] 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] Statistically significant difference, FDR adjusted P-Value <0.05.

VIII-H. 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 (Appendix 7). 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 73496 canola and control canola.

H1. Acetylated Amino Acids in Seed and Whole Plant Samples

Concentrations of the five acetylated amino acids were measured in seed samples of 73496 canola and control canola. Results are provided in Table 22 below. As expected based on catalytic efficiency, NAA and NAG were the two most abundant in 73496 canola. Mean concentrations of NAA and NAG were 1480 µg/g dry weight and 32.8 µg/g dry weight, respectively, for 73496 canola seed samples (Table 22). Although the mean concentrations of NAT and NAS were elevated (statistically significant) in 73496 canola 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 22). There was no statistically significant difference in NAGly concentration measured in 73496 canola seed samples relative to the control line.

Table 22. Concentrations of Acetylated Amino Acids in Canola Seed

Analyte		Control Canola	73496 Canola Treated	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]	0.693 - 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[g]	
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[g]	
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[g]	

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

Concentrations of the five acetylated amino acids were measured in whole plant samples derived from greenhouse-grown 73496 canola and control canola. Concentrations of all measured acetylated amino acids except NAS were elevated in whole plant samples of 73496 canola plants compared to control (Table 23). Consistent with results obtained for seed samples obtained from 73496 canola, NAA and NAG were the two most abundant with mean concentrations of 4560 µg/g and 26 µg/g, in whole plant samples, respectively.

Table 23. Concentrations of Acetylated Amino Acids in Canola Whole Plant Samples

Analyte		Control Canola	73496 Canola
		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.

H2. 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 73496 canola and control canola.

As expected, based on data from whole seed samples, NAA and NAG were the two most abundant acetylated amino acids in 73496 canola meal. Mean concentrations of NAA and NAG were 2872 µg/g and 62.3 µg/g, respectively, for 73496 canola toasted meal from seed with hulls (*i.e.*, seed coats) (Table 24). 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 73496 canola seed either with or without hulls.

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 (Table 24).

Table 24. Concentrations of Acetylated Amino Acids in Processed Fractions Produced from 73496 Canola and Control Canola

Fraction	Canola Line	Concentration (µg/g)[a]				
		NAA[b]	NAG	NAT	NAS	NAGly
Toasted Meal (with hulls)	73496	2872	62.3	0.585	1.20	0.151
	Control	2.31	1.36	0.161	1.46	0.129
Toasted Meal (without hulls)	73496	3013	66.0	0.788	2.27	0.224
	Control	2.58	1.71	0.226	1.76	0.180
RBD Oil (with hulls)	73496	ND[c]	ND - <LLOQ	ND - <LLOQ	ND	ND - <LLOQ
	Control	ND	ND	<LOQ	ND	ND - <LLOQ
RBD Oil (without hulls)	73496	ND - <LLOQ[d]	<LLOQ	ND	ND	ND -<LLOQ
	Control	ND	ND	ND	ND	ND

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

VIII-I. Conclusions on the Compositional Analysis of 73496 Canola

Among the numerous compositional analyses that were carried out, concentrations of most analytes were not significantly different between 73946 canola 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.

The concentrations of NAA, NAG, NAS, NAT, and NAGly were measured in samples of seed, whole plant, and processed fractions derived from 73496 canola. 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 8). The safety of these substances has also been evaluated in published studies as described in Appendix 8.

Based on these data we have concluded that 73496 canola was compositionally equivalent to the near-isoline control canola used as the relevant comparator and to commercial canola varieties.

IX. Assessment of Plant Pest Risk for 73496 Canola

In this section, the plant pest risk of 73496 canola is discussed with respect to the potential environmental impact of the presence of the GAT4621 protein in 73496 canola, the fate of the *gat4621* gene in humans and animals, the weediness potential of 73496 canola, and the impact of gene flow from 73496 canola to crop relatives or wild relatives of canola.

IX-A. Environmental Assessment of the Transgenic Protein

The GAT4621 protein present in 73496 canola is identical to the GAT4621 protein present in previously authorized maize event DP-Ø9184Ø-6 (FDA, 2008; USDA, 2009). The GAT4621 protein was reviewed by FDA in a New Protein Consultation submitted to US FDA on January 31, 2007 and completed on October 7, 2009.

The GAT4621 protein is unlikely to pose a hazard to the environment. The GAT4621 protein sequence is derived from *N*-acetyltransferase protein sequences from *Bacillus licheniformis*, a gram positive saprophytic bacterium that is ubiquitous in soil. The GAT4621 protein is a member of the GNAT acetyltransferase superfamily that contains more than 10,000 representatives from plants, animals, bacteria, and fungi, all of which share a highly conserved GNAT motif (Section VI) (Vetting *et al.,* 2005). GAT4621 is 75-78% identical and 90-91% similar at the amino acid level to the translated protein sequences of each of the three original *gat* genes from *Bacillus licheniformis* from which the *gat4621* gene was derived. GAT4621 retains the acetyltransferase enzyme function of the native proteins. In addition, the GAT4621 protein is highly unlikely to be a toxin (Section VI).

Based on the facts above, there are not likely to be any adverse environmental effects due to the presence of the GAT4621 protein in 73496 canola.

IX-B. Fate of Transgenic DNA in Humans and Animals

Transgenic DNA is no different from other DNA consumed as part of the normal diet. Genetically engineered organisms have been used in drug production (Thayer, 2005) and microbial fermentation (cheese) since the late 1970's (Maryanski, 1995; National Centre for Biotechnology Education, 2006). More than 2.47 billion cumulative acres (1 billion hectares) of engineered food and feed crops have been grown and consumed worldwide from 1996 to 2010 (ISAAA, 2010). The FDA has not reported any significant concerns with bioengineered food and feed currently on the market. The EPA has exempted from a tolerance DNA that encodes currently registered plant incorporated protectants because of a lack of toxicity (Federal Register, 2001).

Studies in humans and animals following the fate of DNA once consumed have shown that the majority of DNA is degraded in the gastrointestinal tract. There is evidence that DNA can move from the gastrointestinal tract lumen to other areas of the body, but this is considered to be a normal occurrence and no risks have been identified as a result of absorption (Duggan *et al.*, 2003; Einspanier *et al.*, 2001).

In conclusion, there are not likely to be any adverse effects due to the presence of the *gat4621* gene and associated transgenic DNA in 73496 canola.

IX-C. Weediness Potential of 73496 Canola

Canola is not considered a noxious or invasive weed in the United States (USDA-NRCS, 2010). Weed species typically spread easily in disturbed areas or within crops and generally have a range of life history characteristics in common that enables them to rapidly colonize and persist in an ecosystem. Several characteristics of weeds have been described by Baker (1974) and are indicated in Table 25. In general, these characteristics do not apply to cultivated canola varieties.

Table 25. Common Characteristics of Weeds

Characteristic	Example	Applies to canola varieties?
Dissemination of seed	Long continuous seed production	No. As a cultivated crop, canola has been selectively bred to ensure seed production is timely to allow for efficient harvest. Additionally, canola is an early successional ruderal (*i.e.*, capable of growing in disturbed habitats) which is incapable of regenerating in undisturbed habitats (Crawley *et al.*, 1993). Populations were observed to be seed limited in a study conducted on motorway verges by Crawley and Brown (1995).
	Special adaptations for short and long-distance dispersal	No. However, the small size of canola seeds and their high numbers on post-harvest fields may facilitate some wind dispersal (Lutman, 1993). The dispersal distance will depend on wind strength, the amount of debris on the ground and the moisture content of the material. It is reasonable to expect that seeds and pods of low moisture content may be transported within the field to adjacent fields, or outside agricultural areas.
	High seed output under favorable conditions	Yes. However due to selective breeding, crop plants only functional optimally under managed agricultural conditions, such as high soil fertility or low plant competition. These conditions rarely occur in natural habitats, resulting in poor fitness of canola plants (*i.e.* reduced recruitment, low survivorship, poor competitive ability, low seed production) (OGTR, 2002).
Dormancy of seed	Continuous germination and long-lived seeds	Yes. Studies in the Northern Hemisphere have reported viable seeds of canola persisting in disturbed soils for at least 5 years and possibly up to 10 years or more in undisturbed soil (Chadoeuf *et al.*, 1998; Madsen 1962; Pekrun *et al.*, 1997; Vaughan *et al.*, 1976).

Table 25. Common Characteristics of Weeds (continued)

Characteristic	Example	Applies to canola varieties?
Germination of seed/seed survival	Germination and seed production under a wide range of environmental conditions	No. The persistence of canola seeds in a seed bank depends both on seed dormancy and their vertical distribution in the soil, as seeds are more likely to persist at deep rather than shallow depths (Pekrun *et al.*, 1998; Simard *et al.*, 2002). As with many annual weeds, feral canola plants usually are not capable of surviving outside of cultivation and without human intervention for more than a few generations (Andersson and de Vicente, 2010). Still, some old canola cultivars can persist outside of cultivation for up to eight or nine years after they were last cultivated, albeit at low densities (Gulden *et al*., 2003; Légère *et al*., 2001; Simard *et al*., 2002; Squire *et al*., 1999).
Competitiveness	Choking growth or production of allelochemicals	No. Canola is not known to produce allelochemicals or otherwise strongly compete with other plants. Canola is known to be strongly affected by weed competition during crop establishment (Berglund *et al.*, 2007) and is unable to invade established natural habitats (OGTR, 2002).
	Ability to regenerate from severed rootstock	No. Canola is not known to regenerate from severed rootstock.
	Rapid seedling growth	No. Young canola seedlings are very sensitive to early weed competition (Berglund *et al.*, 2007). Canola is a poor competitor and not regarded as an environmentally hazardous colonizing species. Unless the habitat is regularly disturbed, or seed replenished from outside, canola will be displaced by other plants (OGTR, 2002).
	Rapid growth to reproductive stage	No. As a crop, canola has been selectively bred to develop to maturity for the purpose full yield potential. This is in contrast to rapid growth to reproductive stage for the purpose of dispersing pollen, as with weed species.

Table 25. Common Characteristics of Weeds (continued)

Characteristic	Example	Applies to canola varieties?
	Self-compatible, but not obligatorily self-pollinated or apomictic	Yes. Fertilization of ovules usually results from self pollination, although out-crossing rates of 20-30% have been reported (Rakow and Woods, 1987). The floral biology of canola is such that it is suited to cross pollination by insect vectors, but can be pollinated by wind (Cresswell *et al*., 2004; Hayter and Cresswell, 2006; Hoyle *et al*., 2007).
Stress tolerance	High tolerance or plasticity of climatic and edaphic variation	No. Canola grows well in dry environments and can tolerate moderately saline soil conditions (Bañuelos *et al*., 1997; Stricker *et al*., 1997). However, although tolerant of some drought stress, seed yields of *Brassica napus* are known to decrease due to drought stress (Jensen *et al*., 1996; Kumar and Singh, 1998) and are most susceptible to heat and drought stress during flowering (Berglund *et al*., 2007).

It is generally accepted that most crop plants, including canola, have undergone many years of selective breeding and domestication and therefore, they function optimally only under managed agricultural conditions. Similar conditions rarely occur in natural habitats, resulting in poor fitness of canola plants outside of a managed field (OGTR, 2002). Although canola has a number of life history traits in common with those usually associated with weeds (Table 25), it is a poor competitor and is not regarded as a colonizing species (Salisbury, 2002). Unless the habitat is regularly disturbed or seeds are replenished from outside, canola will be displaced by other plants (Salisbury, 2002).

The agronomic characteristics and germination data (Section VII) provide evidence that the genetic modification resulting in 73496 canola did not alter any major characteristics of the plant that would allow for development of weedy characteristics different from other canola varieties. Furthermore, the herbicide-tolerance trait conferred by *gat4621* gene does not provide a selective advantage in unmanaged ecosystems, but rather only in settings where glyphosate is being applied for weed control. 73496 canola is susceptible to other herbicides, so control of volunteers could be achieved by use of alternative herbicides, or by non-chemical methods that may be part of the weed management best practices.

There is no evidence that herbicide-tolerant varieties of canola are more invasive or more persistent in disturbed habitats than their unmodified counterparts, nor does canola survive well in the wild (Crawley *et al*., 1993; Crawley *et al*., 2001), therefore it is highly unlikely to invade other habitats (Salisbury, 2002). Additionally, because there are many different traits that lead to weediness in plants (*e.g.*, those described in Table 25), one can assume that these are likely to be polygenic traits and changes to these traits would not be easily conferred by adding a single herbicide tolerance gene.

Herbicide-tolerant canola, including varieties tolerant to glyphosate, has been cultivated since the mid-90's (Devine, 2005). To our knowledge, there is no record of herbicide-tolerant canola varieties

demonstrating weedy characteristics as a result of their herbicide tolerance characteristics. Based on Pioneer's detailed characterization, we expect 73496 canola to be similar to other herbicide-tolerant canola with regard to weediness characteristics.

IX-D. Gene Flow Assessment

Gene flow is the movement of genes via pollen flow or seed dispersal; pollen-mediated gene flow could be a potential route for transfer of traits from canola to other related crops or to wild relatives. The introgression of a gene (*e.g.*, the introduced *gat4621* gene) into another species is limited by several variables including pollen viability and dispersal, synchrony of flowering or pollen production, wind speed and direction, topography and surrounding vegetation, temperature, humidity, relative density of donor and receptor plant populations, sexual incompatibility, and genetic instability (Eastham and Sweet, 2002; Gliddon *et al*., 1999; Ingram, 2000; Thompson *et al*., 1999; Warwick *et al*., 2009). In order to evaluate the potential for gene flow from 73496 canola, the geographic areas of canola production, the crop relatives of canola, the wild relatives of canola, and the barriers to gene flow are discussed further below. In order to be conservative in the analysis of possible gene flow, crop or wild relative species were considered for further examination if the species had geographical prevalence in canola production areas and hybridization with *B. napus* was possible.

D1. Geographic Areas of Canola Production

Canola is grown in the U.S. and the primary states of cultivation include North Dakota (approximately 90% of total production), Minnesota, Idaho, Washington, Montana, Oklahoma, and Oregon (USDA-ERS, 2010; USDA-NASS, 2009; USDA-NASS, 2010). Spring canola (typically planted in March and harvested around September to October) in the U.S. is primarily grown in areas of North Dakota (90% of production acreage), Minnesota, and the Pacific Northwest (*e.g*. Idaho, Washington, and Montana), due to canola's poor performance in warm temperatures. Winter canola (typically planted in September and harvested around June to July) in the U.S. is grown on fewer acres than spring canola but can be grown in a broader range of environments if winters are mild. In recent years, winter canola variety trial programs are run in the Southeast (Alabama, Arkansas, Virginia, Georgia), the Midwest (Illinois, Kentucky, Michigan, Ohio, Pennsylvania), and the Great Plains (Wyoming, Colorado, Nebraska, Kansas, Oklahoma, Texas, Missouri); these states also have reported canola production (Brown *et al.*, 2008; USDA-NASS, 2009). Winter canola is often planted in rotation with wheat, so weed control practices are applied to some extent ahead of the canola planting (*e.g.*, Brown *et al.*, 2008), which could limit the likelihood for presence of wild relatives in and around prospective winter canola fields.

D2. Crop Relatives of *Brassica napus*

The genus *Brassica* contains several important crop species that are used for a variety of purposes. *Brassica napus* and *B. rapa* are the most important for their use as oilseed crops (Ellstrand, 2003a). *Brassica* and the related genus *Raphanus* include vegetables for human consumption, *e.g*., swede (*B. napus*), turnip and Chinese cabbage (*B. rapa*), and cauliflower, cabbage, broccoli, and others (*B. oleracea*), Indian mustard (*B. juncea*), and radish (*R. sativus*). Three *Brassica* species and one species in the related genus *Sinapis* are 'mustards': *B. carinata* (Ethiopian mustard), *B. juncea* (Indian mustard), *B. nigra* (black mustard) and *Sinapis alba* (white mustard) (FitzJohn *et al.*, 2007). Cultivation of *B. carinata* as an oilseed and vegetable crop is largely restricted to Ethiopia and India (Hemingway, 1995; Stewart, 2002). Some forms of *B. napus*, *B. oleracea*, *B. rapa* and *R. sativus* are also grown as fodder crops (FitzJohn *et al.*, 2007).

The three *Brassica* species forming the foundation of the Triangle of U (Nagaharu, 1935) are *B. rapa, B. nigra,* and *B. oleracea*. These three species are diploid, and their respective genomes have been distinguished from one another and are described as A, B, and C (∅stergaard and King, 2008). They can be hybridized sexually with varying degrees of difficulty. Figure 19 describes the likelihood for *B. napus* crossing successfully with other cultivated species. As *B. napus* and *B. rapa* may easily cross, other crosses with *B. napus* are difficult to nearly impossible based on genome characteristics (Myers, 2006; Nagaharu, 1935; OGTR, 2002). The resulting amphidiploid hybrids, *B. juncea*, *B. napus*, and *B. carinata*

contain AB, AC, and BC genomes, respectively. Research has shown that *B. napus* and *B. rapa* outcross readily with each other; these species can also outcross, albeit more rarely, with a wide range of wild and cultivated species including *B. oleracea*, *B. nigra*, and *R. sativus* (summarized in OGTR, 2002). *Brassica napus* and *B. juncea* share a common set of chromosomes, enhancing the likelihood of interspecific hybridization and gene flow (Myers, 2006).

Brassica napus and *B. juncea* can self-fertilize (Rakow and Woods, 1987), but *B. rapa* is self-incompatible (Warwick *et al.*, 2003). The A genome is common to the three major oilseed *Brassica* species, explaining the success of interspecific crossing, and the ability to transfer genes among these species (Figure 19).

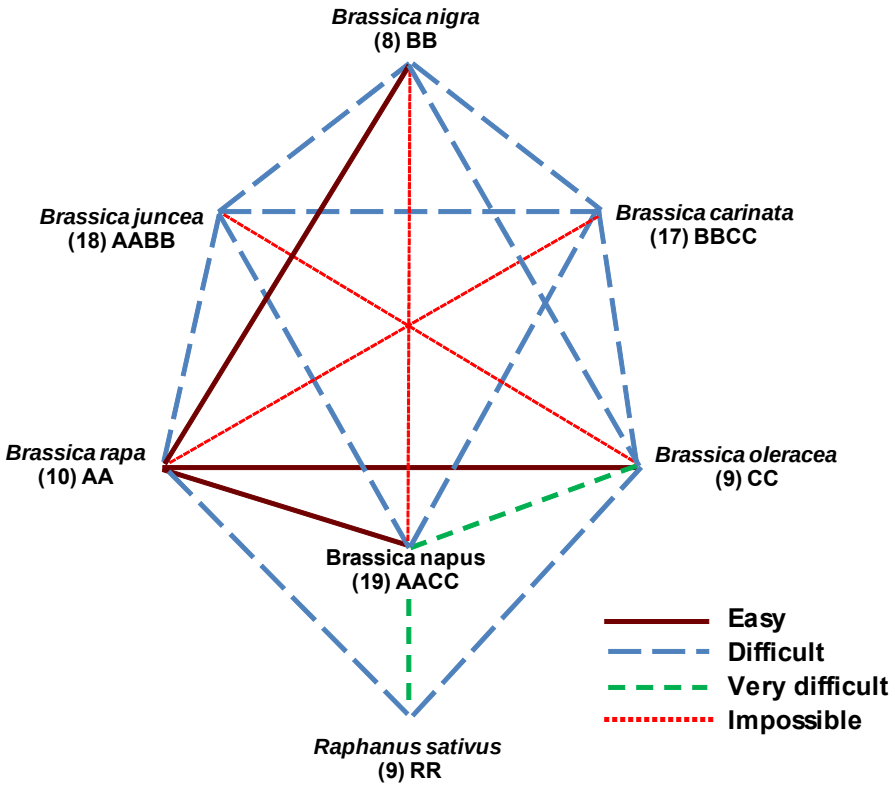

Figure 19. Genome Relationships Among Cultivated Brassicaceae

A modified "Triangle of U" showing genome relationships among cultivated Brassicaceae adapted from Nagaharu (1935) and Myers (2006). Genomes are represented by letters, and haploid genome chromosome numbers are described in parentheses. The three diploid species possess three basic genomes, A, B, and C. Lines represent ease with which species can be crossed. These genomes are combined in the three tetraploid species in pair-wise combinations, *i.e.* AB, AC, and BC.

D3. Wild Relatives of *Brassica napus*

Hybridization between crops and their wild relatives (either the same or different species) has occurred in agricultural history (Ellstrand and Schierenbeck, 2000; Raybould and Wilkinson, 2005). The majority of important crops have wild relatives (CAST, 2007; Ellstrand *et al.*, 1999), and gene flow to wild species from crops occurs at higher frequency than might be expected (Ellstrand *et al.*, 1999). However, the probability of gene flow from crops to related weed species is habitat-dependent and variable between populations (Cureton *et al.*, 2006).

The Brassicaceae family contains a number of major weeds, including those in the genera *Sinapis, Capsella, Thlaspi, Erucastrium, Raphanus*, and others (OECD, 1997). Concerns have been raised about the potential for the transfer of transgenes from the cultivated oilseed *Brassica* species to their weedy relatives in Europe and North America where *Brassica* crop species are widely grown. Some *Brassica* crops and their wild relatives will hybridize only under artificial conditions in laboratories or highly contrived field conditions; others will hybridize at very low rates under natural conditions (Table 26; Barton and Dracup, 2000; Raybould, 1999). However, major barriers to introgression exist, including spatial and temporal isolation, sexual incompatibility, or lack of fertile progeny, thereby reducing the likelihood of gene flow.

Table 26 describes crop and wild relative species that occur in the U.S. to some extent, and the likelihood for hybridizations with canola. In order to be conservative in the analysis of possible gene flow, species were considered for further examination if the species had geographical prevalence in canola production areas and at least one reported successful hybridization (hand pollination or in the field) with *B. napus*. If information was not known about hybridization, the species was also examined further if present in primary canola-growing areas. The species that met these criteria are highlighted in Table 26 and discussed individually in section D5.

Table 26. List of Species with Reports of Hand-Pollinated or Spontaneous Hybridization with *B. napus*

Species Name[a]	Crop	Weed Classification[b]	Hand Pollination (successes:failures)[c]		Spontaneous and Natural Hybridization[c]		Presence in Winter Canola Growing Areas[d]	Presence in Spring Canola Growing Areas[d]
			B.napus as Male	*B.napus* as Female	*B.napus* as Male	*B.napus* as Female		
Brassica carinata	✓	X	4:1	7:0				
Brassica elongata		✓					✓	✓
Brassica fruticulosa		✓	0:1	1:1			✓	X
Brassica juncea	✓	✓	25:1	13:4			✓	✓
Brassica nigra	✓	✓	2:2	4:2	X	X	✓	✓
Brassica oleracea	✓	X	3:11	9:17	✓	X	✓	✓
Brassica rapa	✓	✓	55:8	84:0	✓	✓	✓	✓
Brassica tournefortii		✓	0:1	1:1			✓	X
Camelina sativa	✓	X	0:1	0:1			✓	✓
Capsella bursa-pastoris		✓	0:1	0:1			✓	✓
Coincya monensis		✓					✓	✓
Conringia orientalis		✓	0:1	0:1			✓	✓
Diplotaxis erucoides		X	1:1				✓	✓
Diplotaxis muralis		✓	3:0	1:1			✓	✓
Diplotaxis siifolia		X	0:3	0:1			X	X
Diplotaxis tenuifolia		✓	0:3	1:1			✓	✓
Eruca vesicaria (E.sativa)		✓	2:0				✓	✓
Erucastrum gallicum		✓	0:1	1:0	X		✓	✓
Hirschfeldia incana		✓	1:2	1:2	✓	✓	✓	X
Moricandia arvensis		X	0:2	0:2			X	X
Myagrum perfoliatum		X	0:1	0:1			✓	X
Raphanus raphanistrum		✓	0:4	3:2	✓	✓	✓	✓
Raphanus sativus	✓	✓	1:5	1:2			✓	✓
Rapistrum rugosum		✓		1:0			✓	✓
Rorippa islandica		✓		1:0			X	X
Sinapis alba	✓	✓	0:6	1:2	X		✓	✓
Sinapis arvensis		✓	1:10	5:8	X	✓	✓	✓
Sisymbrium irio		✓	0:1	0:1			✓	X
Sisymbrium orientale		✓					✓	✓

✓ = Yes, to some degree
X = No
[a] Species highlighted in purple have at least one report of successful hybridization with *B. napus*
[b] Weed Science Society of America or USDA NRCS list of noxious weeds
[c] Andersson and de Vicente, 2010; FitzJohn *et al*., 2007
[d] USDA PLANTS Database; Criteria to distinguish growing areas for winter and spring canola were obtained from Brown *et al.* (2008) and are summarized in the following section.
Blank cells = no data available

D4. Gene Flow Assessment Considerations

In general, it is difficult for a transgene to become established in a wild relative or recipient population. In order for successful introgression of a transgene in a recipient population, several conditions must exist and several steps must take place before this would occur (Devos *et al.*, 2009). These are as follows:

1. There must at least be a partial overlap in flowering periods between the transgenic canola and the relative.
2. The relative must grow within the physical range of viable pollen dispersal of the transgenic canola, and viable pollen grains must reach the recipient stigma.
3. Viable and fertile interspecific F_1 hybrids must be produced, and the transgene successfully integrated into the recipient plant genome.
4. The transgene must be transmitted through successive backcross generations.
5. The transgene must be stabilized in the genome of the relative through backcrossing over multiple generations.
6. The introgressed transgene must provide a fitness advantage such that it persists in the relative's populations.

In order to evaluate the likelihood of gene flow of 73496 canola to the species highlighted in Table 26, additional information related to canola flowering synchrony and flowering characteristics, pollen dispersal mechanisms and pollinator biology are discussed below in order to provide context for the gene flow assessment.

<u>Flowering synchrony and flower characteristics</u>

Winter canola can grow in a broader geographic range than spring canola, thereby increasing the number of wild relative species with which geographic overlap is possible (Table 26). However, the flowering period may not overlap. Planting of winter canola occurs in the fall, and the plants overwinter in the rosette stage (Boyles *et al.*, 2009; Boyles, 2010; Wysocki *et al.*, 2005). Rosettes resume growth in the spring as temperatures increase, and (Boyles *et al.*, 2009; Boyles, 2010; Wysocki *et al.*, 2005) Depending on location, flowering for winter canola generally occurs in April to May, with harvest in June to July (Boyles *et al.*, 2009; Boyles, 2010; Wysocki *et al.*, 2005). As an example, *Brassica tournefortii* generally flowers from February to May in Arizona, in December to January in California, and January to March in Texas (summarized by Halvorson, 2003), thereby limiting the likelihood for flowering synchrony with winter canola.

In self-compatible species (*e.g*. *B. napus*, *B. juncea*) (Rakow and Woods, 1987), pollination typically occurs prior to flower opening, thus limiting exposure of the recipient stigma to pollen from another flower or species. Finally, canola crops produce $5x10^{12}$ pollen grains per hectare (Chèvre *et al.*, 1999). Therefore, due to a general lack of mobility of pollen, any pollen coming from outside the immediate field would be competing with this large volume of pollen (Salisbury, 2002), thereby decreasing the likelihood for successful pollination by pollen from outside the immediate field.

<u>Pollen dispersal mechanisms</u>

B. napus is primarily a self-pollinating crop, and the majority of large-scale outcrossing studies with fertile canola indicate outcrossing rates of <2% (Salisbury, 2002). However, worst-case estimates of outcrossing suggest levels between 12 to 47% (Becker *et al.*, 1992; Kapteijns, 1993; Rakow and Woods, 1987). The pollen, which is heavy and sticky, can also be transferred from plant to plant through physical contact with neighboring plants, via pollinator insects and, to a smaller extent, by wind (Cresswell, 1994; Cresswell *et al.*, 2002; Williams, 1984). In general, windborne pollen may make little to no contribution to long distance pollination (OGTR, 2002).

Approximately half of canola pollen travels less than three meters from the source; the vast majority of pollen travels less than 10 meters, with the amount of pollen decreasing as the distance from the pollen source increases (Scheffler *et al.*, 1993; Thompson *et al.*, 1999). Pollinating insects, in particular

honeybees and bumblebees, play a major role in *B. napus* pollination and are believed to be involved in the transfer of pollen over long distances (OGTR, 2002; Salisbury, 2002).

Pollinator biology

Insects are the primary pollen vectors (Hayter and Cresswell, 2003), and insect foraging behavior is complex. The dynamics of bee-mediated pollen movement depend on a number of factors including spatial arrangement of plants, environmental conditions, plant density, availability of pollen, and the size and location of the receiving populations (Ellstrand *et al.*, 1989; Klinger *et al.*, 1992; Levin and Kerster, 1969; Rieger *et al.*, 2002).

In situations with abundant flowers, such as in a cultivated field, individual honey bees generally collect nectar and pollen from flowers in the same or immediately adjacent plants (Cresswell, 1999; OGTR, 2002; Pierre, 2001; Ramsay *et al.*, 1999). Occasionally, bees may travel much further and some pollen transfer may occur over longer distances, for example a maximum flight distance of four kilometers has been reported in the literature (Ramsay *et al.*, 1999; Thompson *et al.*, 1999). However, the majority of bee flights are less than one meter, thereby limiting the likelihood of long distance pollen dispersal via insect pollinators (summarized by OGTR, 2002).

Honeybees forage during daylight and are unlikely to carry viable pollen grains to impact fertilization beyond 12 hours (Kraai, 1962). Honeybees are sensitive to weather events and barometric pressure, and respond to these events by decreasing foraging distances (APHIS, 1998). The distance and success of pollen-mediated gene flow is dependent on its dispersal in space by either wind or insects, and on the length of time the pollen grain remains viable (OGTR, 2002). Canola pollen viability gradually decreases after four to five days in natural circumstances depending on environmental conditions, particularly temperature and humidity (Andersson and de Vicente, 2010; Rantio-Lehtimäki, 1995).

Likelihood of hybridization

Reproductive compatibility among *Brassica* crops is complex. Because experimental hybridization studies are designed to optimize the likelihood of successful hybridization, they create bias toward positive reports of hybridization between species that may be unlikely to cross in natural conditions (FitzJohn *et al.*, 2007). The ease with which a crop and its wild relatives can hybridize through manual cross-pollination reveals little about the potential influence of pre-pollination and other ecological barriers (Arnold, 1997; FitzJohn *et al.*, 2007; Grant, 1994).

Many studies have been conducted to gather experimental data to infer the likelihood of hybridization using a variety of techniques: experimental crosses (manual hand pollination), spontaneous crosses (non-assisted crosses under field conditions), and *in vitro* methods (*e.g.*, embryo rescue). Cross-compatibility varies with the particular genotype used and with the polarity of the cross (*i.e.*, dependent upon which species was the maternal parent; Arnold, 1997). Most combinations are unsuccessful and where crosses are successful, rates of hybrid production are typically very low (Table 26; FitzJohn *et al.*, 2007; Warwick and Black, 1993).

Hybridization of *B. napus* with cultivated and wild *Brassica* species such as *B. carinata*, *B.elongata, B.fruticulosa, B. incana, B.nigra, B. tournefortii*, have been attempted and in most cases the production of hybrids requires considerable human intervention and is only successful if artificial hybridization techniques are used (*e.g.*, embryo rescue) (Andersson and de Vicente, 2010). All resulting F1 hybrids are sterile (Andersson and de Vicente, 2010). *Brassica carinata*, *B.nigra*, and *B. tournefortii* are able to form hybrids with *B. napus* after hand pollination however, they have significant barriers to introgression such as pollen dehiscense prior to the flower opening, sexual incompatibility, reduced hybrid fertility, or sterility, making gene exchange with *B. napus* extremely unlikely (Andersson and de Vicente, 2010). Even among the closely related *Brassica* species (Nagaharu, 1935), hybridization with *B. napus* is highly unlikely. For example, although *B. napus* and *B. rapa* share the A genome, Scott and Wilkinson (1998) reported only 7% of *B. rapa* populations had any hybrids, indicating that 93% of *B. rapa* populations had no hybrids, and hybridization rates were low (0.4-1.5%) in field situations; further, less than 2% of hybrid

seedlings survived (Scott and Wilkinson, 1998). Other published hybridization rates are equally low, *e.g*. <3% for *B. juncea* (Bing *et al.*, 1991; Jørgensen *et al.*, 1996), <0.003% for *R. raphanistrum* (Chèvre *et al.*, 1999; Chèvre *et al.,* 2000), <0.000034% for *S. arvensis* (Lefol *et al.*, 1996). In many cases of attempted hybridizations with *B. napus* as male donor, no hybrids were produced (Salisbury, 2002).

D5. Assessment of Gene Flow Potential from 73496 Canola to Relevant Species

Gene flow assessments were conducted for those species highlighted in Table 26 on the basis of overlapping geographic ranges with canola production and reports of at least one successful hybridization (by hand pollination or in the field) with *B. napus*. If information was not known about hybridization, the species was also examined further if present in primary canola growing-areas.

Crop Species:

Brassica carinata (Ethiopian mustard)

Although some reports of successful hand pollinations are reported for *B. napus* and *B. carinata*, no hybridizations in the field have been reported (Andersson and de Vicente 2010), and no distribution information is available for *B. carinata* in the U.S. (USDA Plants Database, 2010). Several barriers to introgression exist, *e.g*. reduced hybrid fertility or sterility (Andersson and de Vicente, 2010).

Brassica juncea (Indian or brown mustard*)*

B. juncea can occur throughout the U.S. (USDA Plants Database, 2010) and is both a crop and a weed. *B. juncea* (AABB genome) has a common set of chromosomes with *B. napus* (AACC genome), which enhances the likelihood of interspecific hybridization and gene flow (Andersson and de Vicente, 2010). Spontaneous hybridization has been reported at <3% for *B. juncea* (Bing *et al.*, 1991; Jørgensen *et al.*, 1996), although hybridization is less successful when *B. napus* is the female parent (Jørgensen *et al.*, 1998). In general, F1 hybrids are rarely more fit than highly selected and well adapted counterparts, and low initial frequencies, reduced fitness and viability, and competitive disadvantage with respect to parents can lead to hybrid extinction (Wolf *et al.*, 2001). The pollen and seed fertility of the F1 hybrids is typically less than 30% (Bajaj *et al.*, 1986; Choudhary and Joshi, 1999; Frello *et al.*, 1995; GhoshDastidar and Varma, 1999; Prakash and Chopra, 1988; Roy, 1980; Sacristán and Gerdemann, 1986), however spontaneous backcrossing progeny with improved fertility have been documented (Bing *et al.*, 1991; Bing *et al.*, 1996; Jørgensen, 1999). Backcrossing and subsequent gene introgression from *B. napus* to *B. juncea* could be expected, although infrequently (Andersson and de Vicente, 2010).

Brassica nigra (Black mustard)

B. nigra can grow throughout regions of the U.S. (USDA Plants Database, 2010) and is classified as both crop and weed. Hybrids between *Brassica nigra* and *B. napus* have been reported to occur at a low frequency when produced under controlled conditions (Bing *et al.*, 1991; Heyn, 1977). However, under field conditions no hybrids resulted from co-cultivation of *B. nigra* and *B. napus* (Bing *et al.*, 1996).

Brassica oleracea (*e.g.* cauliflower, cabbage, broccoli)

B. oleracea has a more limited distribution than other *Brassica* spp. (*i.e.* CA, CT, DC, GA, IA, IL, KY, LA, MA, MO, ME, MI, MD, MT, NM, NY, OH, OR, PA, RI, TX, VA, WA) (USDA Plants Database, 2010). Hybridization of *B. napus* with *B. oleracea* can be achieved by hand pollination (Eastham and Sweet, 2002; Scheffler and Dale, 1994). Although *B. oleracea* and *B. napus* share a common set of chromosomes (Figure 19), the frequency of successful crosses is very low, and embryos often abort at early stages of development (Chiang *et al.*, 1977; Honma and Summers, 1976; Mattsson, 1988). Thus far, no viable hybrid seeds have been obtained from crosses between *B. napus* and *B. oleracea* without the assistance of embryo rescue or ovule culture (Ayotte *et al.*, 1987; Myers, 2006; Quazi, 1988; Takeshita *et al.*, 1980). Spontaneous hybrids have been reported in wild *B. oleracea* populations in the

UK (Ford *et al.*, 2006). No information is available thus far on the fertility of the natural hybrids reported (Andersson and de Vicente, 2010).

Brassica rapa (Turnip, Chinese cabbage)

B. rapa is a closely related species of canola (amphidiploid), which has a similar life history to canola, but with a shorter growing season. *B. napus* (AACC) and *B. rapa* (AA) have a common set of chromosomes, making interspecific outcrossing more common (*e.g*. Bing *et al.*, 1991). Gene flow measurements by Scott and Wilkinson (1998) reported only 7% of *B. rapa* populations had any hybrids, indicating that 93% of *B. rapa* populations had no hybrids, and hybridization rates were low (0.4-1.5%) in field situations. Importantly, less than 2% of hybrid seedlings survived (Scott and Wilkinson, 1998).

Hybridization frequencies are higher when *B. rapa* occurs as a weed within canola crops, but varies significantly with experimental design. Where natural interspecific hybrids occur, hybrids have reduced fertility and low seed set (average 2-5 per pod) compared with the parents (Jørgensen and Andersen, 1994). Reduced dormancy of *B. rapa* x canola hybrids relative to the persistent wild *B. rapa* (Jørgensen *et al.*, 1999), coupled with the reduced fertility of the inter-specific hybrid (Jørgensen *et al.*, 1999) makes it very unlikely that populations of these hybrids will persist.

Raphanus sativus (radish)

R. sativus can be found throughout the U.S. (USDA Plants Database, 2010). Spontaneous hybridization between a male sterile *B.napus* line and a cultivated variety of *R. sativus* line has been reported (Ammitzboll and Jørgensen, 2006). However, all F1 offspring were found to be hybrids with low pollen fertility (0%-15%) (Ammitzboll and Jørgensen, 2006). Although hybridization can be achieved by hand pollination or through sophisticated methods, such as ovule culture and embryo rescue (Gupta, 1997; Huang *et al.*, 2002; Metz *et al.*, 1995; Rhee *et al.*, 1997; Sundberg and Glimelius, 1991) all artificially produced F1 hybrids have shown to be male sterile (Andersson and de Vicente, 2010).

Sinapis alba (See *Other Highlighted Species* section below).

Weed Species:

Brassica fruticulosa

This species only occurs in California (USDA Plants Database, 2010), therefore risk is limited to canola production in California. Additionally, only one report of successful hand pollination has occurred, and no reports of hybridization in the field are described (Andersson and de Vicente, 2010).

Brassica tournefortii

B. tournefortii grows in the southwestern U.S. (*e.g*. NV, NM, AZ, TX, CA) (USDA Plants Database, 2010), therefore geographic overlap is limited to winter canola production, however as previously discussed, asynchrony of flowering is probable in this case (Boyles *et al.*, 2009; Boyles, 2010; Halvorson, 2003; Wysocki *et al.*, 2005).

Hybridization of *B. napus* has been attempted with *B. tournefortii*. In most cases, the production of F1 hybrids requires considerable human intervention and is only successful if artificial hybridization techniques are used. *B. tournefortii* is able to form hybrids with *B.napus* after hand pollination. However, they have significant barriers to introgression such as pollen dehiscence prior to the flower opening, sexual incompatibility, reduced hybrid fertility, or sterility making gene exchange with *B.napus* extremely unlikely (Andersson and de Vicente, 2010)

Diplotaxis muralis

D. muralis occurs throughout regions of the U.S. (USDA Plants Database, 2010). Ringdahl *et al.* (1987) examined the crosssability of *D. muralis* and *B. napus* using conventional crossing techniques, using *B. napus* as the male parent. They were able to produce viable F1 hybrids after hundreds of hand pollinations conducted (Ringdahl *et al.*, 1987). On the other hand, Fan *et al.* 1985 investigated male sterility in backcross populations from hybrids between *D. muralis* and *B.napus*, using *D. muralis* as the female parent. Male sterility of the F1 populations was caused by an extra chromosome derived from *D.muralis* (Fan *et al.*, 1985).

Erucastrum gallicum

E. gallicum can be found throughout regions of the U.S. (USDA Plants Database, 2010), however studies conducted by Warwick *et al.* (2003) indicated that gene flow from commercial fields of *B. napus* to *E. gallicum* has very low probability of occurrence.

Hirschfeldia incana

H. incana can be found in CA, HI, NV, OR (USDA Plants Database, 2010). Spontaneous hybridization between male-sterile *B. napus* and *H. incana* in field has been reported in both directions (Chèvre *et al.*, 1996; Eber *et al.*, 1994; Lefol *et al.*, 1995). The resulting triploid F1 hybrids are often vigorous and at least as competitive as their wild parent (Eber *et al.*, 1994; Lefol *et al.*, 1996). However, they are usually male sterile and produce very little seed (1 seed per plant) under controlled conditions (Lefol *et al.*, 1996). Hybridization between these two species is easy and recurrent, but introgression rarely occurs (Darmency and Fleury, 2000).

Raphanus raphanistrum

R. raphanistrum occurs throughout regions of the U.S. (USDA Plants Database, 2010), and there is a possibility for successful hybridization; however, the frequency of such hybridization is very low (*e.g.* <0.003%; Ammitzboll and Jørgensen, 2006; Baranger *et al.*, 1995; Chèvre *et al.*, 1996; Chèvre *et al.*, 1999; Chèvre *et al.*, 2000; Darmency *et al.*, 1995; Eber *et al.*, 1994; Rieger *et al.*, 2001;), and hybrids show very low fertility (Andersson and de Vicente, 2010).

Spontaneous hybridization between *B. napus* and *R. raphanistrum* may occur in the wild. Low frequencies of F1 hybrids have been reported and they are allotriploids, which show very low fertility (Baranger *et al.*, 1995; Chèvre *et al.*, 1996; Chèvre *et al.*, 1998; Darmency *et al*, 1998; Kerlan *et al.*, 1992; Pinder *et al.*, 1999; Thalmann *et al.*, 2001; Warwick, 2009). F1 hybrids also commonly show decreased fitness in terms of reduced seedling emergence, a significant emergence delay, and lower survival rate than both parents. (Guéritaine *et al.*, 2003).

Rorippa islandica

Although intergeneric hybridization between *R. islandica* and *B. napus* is possible (Bijral and Sharma, 1995), the distribution of this species in the U.S. is restricted to Washington D.C. (USDA Plants Database, 2010) therefore the potential for gene flow and hybridization is negligible.

Sinapis arvensis

S. arvensis occurs throughout the U.S. (USDA Plants Database, 2010) and is a weed frequently found in canola fields in weed surveys (Zollinger *et al.*, 2003). However, greenhouse studies conducted by Moyes *et al.* (2002) have confirmed the low probability of hybridization between *B. napus* and *S. arvensis.* Low frequencies (<0.00003%) of hybrids were obtained when *S. arvensis* was the receptor (female) (Lefol *et al.*, 1996). Although a low level of interspecific crosses could occur (Leckie *et al.*, 1993), the likelihood of gene flow between *B. napus* and *S. arvensis* is extremely remote, and there is general agreement that no gene introgression will occur (Bing *et al.*, 1991; Downey, 1999; Eber *et al.*, 1994). Hybrids obtained

between *S.arvensis* and *B. napus* (Moyes *et al.*, 2002) had greatly reduced fertility or were completely sterile (Andersson and de Vicente, 2010).

Other Highlighted Species

Of the remaining highlighted species (Table 26), *C. monesis* occurs in CA and the eastern U.S. in states with very limited canola production (USDA Plants Database, 2010). *B. elongata* is present in CO, NV, and OR, and *S. orientale* occurs in the southwestern U.S. (USDA Plants Database, 2010). Crosses between *B. napus* and a number of species belonging to other genera have been attempted using artificial hybridization methods, but the crosses either failed or resulted in partially or completely sterile F1 progeny. Among those species are: *Diplotaxis erucoides, Diplotaxis tenuifolia, Eruca vesicaria (E. sativa), Erucastrum gallicum, Rapistrum rugosum, Sinapis alba, Sisymbrium orientale* (Andersson and de Vicente, 2010).

Related Weeds Frequently Found in Agricultural Fields

In addition to examination of geographic range and likelihood of hybridization, it is important that abundance of weed species in agricultural fields is considered in the risk assessment. Weed abundance in agricultural fields is partially determined by crop production practices, and weed species prominence can be significantly impacted by cropping systems and cultivation practices (CFIA, 1994). In North Dakota weed surveys, some Brassicaceae weed species that are frequently found include *Thlaspi arvense*, *Descurainia sophia,* and *Descurainia pinnata* (Zollinger *et al.*, 2003). The probability of hybridization and gene flow to these species is unknown.

D6. Conclusions on the Potential Plant Pest Risk of 73496 Canola

73496 canola does not pose a plant pest risk. The GAT4621 protein and its source organism, *B. licheniformis*, are familiar and have a history of safe use. The transgenic DNA in 73496 canola is as safe for consumption as any other DNA. *B. napus* is not considered a noxious or invasive weed in the U.S. Although it does demonstrate some weedy characteristics, canola has undergone many years of selective breeding and domestication and is a poor competitor with other species. In addition, the agronomic characteristics and germination data for 73496 canola demonstrated no change that would allow for development of weedy characteristics different from other canola varieties. The *gat4621* gene does not provide a selective advantage in unmanaged ecosystems, rather only in settings where glyphosate is being applied for weed control. Other commercially available herbicides, such as glufosinate or imidazolinones, as well as mechanical means can be used in crop settings for volunteer control.

Gene flow from 73496 canola was evaluated thoroughly with respect to plant pest risk. Although crops and certain wild/weedy relatives have exchanged genes for centuries, the concern with genetically modified crops is that the acquisition of transgenes may increase the fitness of recipient plants (Ellstrand *et al.*, 1999; Ellstrand, 2003b) and the potential weediness or invasiveness in the crop itself or in its wild or weedy relatives as a result of transgene movement (Warwick *et al.*, 2009). Successful hybridization of *B. napus* and a wild/weedy relative is highly unlikely. However, should such unlikely events of successful hybridization and stable introgression of the *gat*4621 gene from 73496 canola into wild/weedy relative populations occur, the herbicide-tolerance trait would only provide selective advantage in situations in which the weedy hybrid was in contact with the herbicide (*i.e.*, in an agricultural field).

The introduced *gat4621* gene in 73496 canola did not change the ability of the plant to interbreed with other plant species. Furthermore, the evaluation of agronomic and phenotypic properties of 73496 canola, including those characteristics associated with reproductive biology, indicated no unintended changes likely to affect the potential for gene flow from 73496 canola to sexually compatible species.

The consequences of gene flow and introgression of the glyphosate-tolerant trait from 73496 canola to the same or sexually compatible species is anticipated to be the same as for existing commercial glyphosate-tolerant canola varieties. If glyphosate-tolerant individuals arise through interspecific or intergeneric hybridization, the tolerance will not confer any competitive advantage to these plants unless

selected by glyphosate herbicide. This would only occur in managed ecosystems where glyphosate-containing herbicides are applied for broad-spectrum weed control, or in plant varieties developed to exhibit glyphosate tolerance and in which glyphosate is used to control weeds. As with glyphosate-tolerant canola volunteers, these individuals, should they arise, would be controlled using other available chemical or mechanical means.

Although gene flow from 73496 canola to relatives is possible, it will not result in increased weediness or invasiveness of these relatives based on the agronomic and ecological assessments (Section VII; Appendix 5). Large-scale cultivation of herbicide-tolerant canola has occurred for nearly 15 years in Canada and the United States. To date, there are no reports of problems with interspecific crosses and introgression of herbicide-tolerant genes into cultivated or wild relatives of canola (Andersson and de Vicente, 2010).

Appendix 1. Materials and Methods for Genetic Characterization of 73496 Canola

1.1. Southern Blot Characterization of 73496 Canola

Southern blot analysis was conducted to characterize the DNA insertions in 73496 canola. Individual plants of the T2, T3, T3F2, T3F3, and F1[*2] generation were analyzed by Southern blot to determine the number of each of the genetic elements of the expression cassette that were inserted and to verify that the integrity of the PHP28181A fragment was maintained upon integration. The integration patterns of the insertion in 73496 canola was investigated with *Nco* I and *Ssp* I restriction enzymes. Southern blot analysis was conducted on individual plants of the five generations to confirm stability of the insertion across generations and to verify the absence of backbone sequences from plasmid PHP28181.

1.1.1. Test Material

Seeds from the T2, T3, T3F2, T3F3, and F1[*2] generations of 73496 canola were planted and leaf tissue harvested from individual plants was used for genomic DNA extraction.

1.1.2. Control Material

Seeds from the unmodified canola varieties 1822B, 1822R, and 6395B were planted and leaf tissue harvested from individual plants was used for genomic DNA extraction.

1.1.3. Reference Material

Plasmid DNA from PHP28181 was prepared from *E. coli* (Invitrogen, Carlsbad, CA) and was used as a positive control for Southern blot analysis to verify probe hybridization and to verify sizes of fragments internal to the plasmid. The plasmid stock was a copy of the plasmid used for transformation to produce 73496 canola and was digested with restriction enzymes to confirm the plasmid map. The probes used in this study were derived from plasmid PHP28181 or from a plasmid containing equivalent genetic elements.

DNA molecular weight markers for gel electrophoresis and Southern blot analysis were used to determine approximate molecular weights. For Southern analysis, DNA Molecular Weight Marker VII, digoxigenin (DIG) labeled (Roche, Indianapolis, IN), was used as a size standard for hybridizing fragments. ΦX174 RF DNA/*Hae* III Fragments (Invitrogen, Carlsbad, CA) was used as a molecular weight standard to determine sufficient migration and separation of the fragments on the gel.

1.1.4. Genomic DNA Extraction

Genomic DNA was extracted from leaf tissue harvested from individual plants as described above. The tissue was pulverized in tubes containing grinding beads using a Geno/Grinder™ (SPEX CertiPrep, Inc., Metuchen, NJ) instrument and the genomic DNA was isolated using a urea-based procedure (modification from Chen and Dellaporta, 1994). Approximately 1 gram of ground tissue per sample was extracted with 5 ml Urea Extraction Buffer (7 M urea, 0.34 M NaCl, 0.05 M Tris-HCl, pH 8.0, 0.02 M EDTA, 1% N-lauroylsarcosine) for 15-18 minutes at 37°C, followed by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and one extraction with water saturated chloroform. The DNA was precipitated from the aqueous phase by the addition of 1/10 volume of 3 M NaOAc (pH 5.2) and 1 volume of isopropyl alcohol, followed by centrifugation to pellet the DNA. After washing the pellet twice with 70% ethanol, the DNA was dissolved in 0.5 ml distilled water and treated with 10 μg ribonuclease A for 15 minutes at 37°C. The sample was then washed with 70% ethanol. After drying, the DNA was re-dissolved with 0.5 ml distilled water and stored at 4°C.

1.1.5. Quantitation of Genomic DNA

Following extraction, the DNA samples were quantified on a spectrofluorometer using PicoGreen® reagent (Molecular Probes, Inc., Eugene, OR) following a standard procedure. The DNA was also visualized on an agarose gel to confirm quantitation values from the PicoGreen® analysis and to determine DNA quality.

1.1.6. Identification of the 73496 Canola Plants Used for Southern Blot Analysis

Phenotypic analysis of 73496 canola plants and control plants was carried out by the use of lateral flow devices able to detect the GAT4621 protein to confirm the absence or presence of the GAT4621 protein in material used for Southern blot analysis.

Leaf extract were prepared by grinding leaf punches to homogeneity in 400 µl of SEB6 extraction buffer (Agdia, Inc., Elkhart, IN). Lateral flow devices (Agdia) were placed in the homogenate and allowed to develop. After incubation, the results were read from the lateral flow devices. A single stripe indicated a negative result and a double stripe indicated the sample was positive for the GAT4621 protein.

Genotypic analysis of the 73496 canola and control canola plants was carried out by real-time polymerase chain reaction (PCR) using assays specific for the DNA insertion. A leaf sample was taken from each test and control plants for event-specific PCR analysis. DNA was extracted from each leaf sample using the Extract-N-Amp™ Plant PCR kit using the described procedure (Sigma-Aldrich, St. Louis, MO).

Real-time PCR was performed on each DNA sample utilizing an ABI PRISM® 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA). TaqMan® probe (Applied Biosystems, Inc.) and primer sets (Integrated DNA Technologies, Coralville, IA) were designed to detect target sequences from the insertion in 73496 canola. In addition, a second TaqMan® probe and primer set for a reference canola endogenous gene was used to confirm the presence of amplifiable DNA in each reaction. The assay analysis consisted of real-time PCR determination of qualitative positive/negative calls. The extracted DNA was assayed using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Inc.). Initial incubation was at 95°C for 10 minutes followed by 40 cycles as follows: 95°C for 15 seconds, 60°C for one minute.

Positive or negative determination for each plant was based on comparison of the threshold cycle (C_T) of the insertion target PCR to that of the canola endogenous reference target. If the event-specific and endogenous PCR targets amplified above C_T, then the plant was scored as positive for the DP-Ø73496-4 event. If the endogenous target amplified and the event target did not, then the plant was scored as negative. For all assays, if neither target amplified for a particular sample, it was determined to be a poor quality sample or failed run and the assay was repeated.

A subset of 73496 canola plants that were identified as containing the inserted DNA and expressing the GAT4621 protein from the five generations described previously were selected for Southern blot analysis.

1.1.7. Digestion of DNA for Southern Blot Analyses

Genomic DNA samples extracted from selected 73496 canola and control canola plants were digested with restriction enzymes following a standard procedure. Approximately 4 µg of genomic DNA was digested using 20-50 units of enzyme according to manufacturer's recommendations. The digestions were carried out at 37°C for approximately three hours, followed by ethanol precipitation with 1/10 volume of 3 M NaOAc (pH 5.2) and 2 volumes of 100% ethanol. After incubation at ≤-5°C and centrifugation, the DNA was allowed to dry and then re-dissolved in TE buffer (10mM Tris, 1 mM EDTA, pH 7.5). The reference plasmid, PHP28181, was spiked into a control plant DNA sample in an amount equivalent to approximately one or three gene copies per canola genome and digested with the same enzyme to serve

as a positive control for probe hybridization and to verify sizes of fragments internal to the plasmid on the Southern blot.

1.1.8. Electrophoretic Separation and Southern Transfer

Following restriction enzyme digestion, the resultant DNA fragments were electrophoretically separated by size through an agarose gel. A molecular weight standard [ΦX174 RF DNA/*Hae*III Fragments (Invitrogen)] was used to determine sufficient migration and separation of the fragments on the gel. DIG labeled DNA Molecular Weight Marker VII (Roche), which is visible after DIG detection as described below, was used to determine hybridizing fragment size on the Southern blots.

Agarose gels containing the separated DNA fragments were depurinated, denatured, and neutralized *in situ*, and transferred to a nylon membrane in 20x SSC buffer (3M NaCl, 0.3 M sodium citrate) using the method as described for the TURBOBLOTTER™ Rapid Downward Transfer System (Whatman, Inc., Piscataway, NJ). The DNA was then bound to the membrane by UV crosslinking (Stratalinker, Stratagene, La Jolla, CA).

1.1.9. DNA Probe Labeling for Southern Blot Hybridization

Probes for the *UBQ10* promoter, *gat4621*, and *pin*II terminator were used to detect genes and elements within the insertion. Probes covering the backbone region of plasmid PHP28181 were used to verify absence of plasmid backbone DNA in 73496 canola. DNA fragments of the probe elements were generated by PCR from plasmid PHP28181 or a plasmid with equivalent elements using specific primers. PCR fragments were electrophoretically separated on an agarose gel, excised and purified using a gel purification kit (Qiagen, Valencia, CA). DNA probes were generated from these fragments by PCR that incorporated a DIG labeled nucleotide, [DIG-11]-dUTP, into the fragment. PCR labeling of isolated fragments was carried out according to the procedures supplied in the PCR DIG Probe Synthesis Kit (Roche).

1.1.10. Probe Hybridization and Visualization

Labeled probes were hybridized to the target DNA on the nylon membranes for detection of the specific fragments using the procedures essentially as described for DIG Easy Hyb solution (Roche). After stringent washes, the hybridized DIG-labeled probes and DIG-labeled DNA standards were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche). Blots were exposed to X-ray film for one or more time points to detect hybridizing fragments and to visualize molecular weight standards bound to the nylon membrane. Images were digitally captured by detection with the Luminescent Image Analyzer LAS-3000 (Fujifilm Medical Systems, Stamford, CT). Digital images were compared to original X-ray film exposures as verification for use in this submission. The sizes of detected bands were documented for each digest and each probe.

1.1.11. Stripping of Probes and Subsequent Hybridizations

Following hybridization and detection, membranes were stripped of DIG-labeled probe to prepare the blot for subsequent re-hybridization to additional probes. Membranes were rinsed briefly in distilled, de-ionized water and then stripped in a solution of 0.2 M NaOH and 0.1% SDS at 37°C with constant shaking. The membranes were then rinsed in 2x SSC and used directly for subsequent hybridizations. The alkali-based stripping procedure effectively removes probes labeled with the alkali-labile DIG.

1.2. Mendelian Inheritance and Genetic Stability of the Trait in 73496 Canola

Inheritance of the inserted DNA and the herbicide-tolerance phenotype in 73496 canola was evaluated to ensure stability of the trait during the plant breeding process and to confirm the trait was at a single genetic locus. A phenotypic and genotypic analysis of individual plants of segregating and non-segregating generations was conducted. The presence of the DP-Ø73496-4 event insertion was determined by event- and *gat4621* gene-specific endpoint PCR analysis performed on leaf punches from seedlings of each generation. Plant phenotypic analysis was conducted by treating the plants with herbicide and determining the level of herbicide injury to the plants.

1.2.1. Test Material

One-hundred plants from segregating generations of 73496 canola (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 in Section III-A, Figure 2. Those populations with the superscript "*1,*3" designation were pooled populations from two different genetic backgrounds (Section III-A, Figure 2). The plants from the T3F2 generation were expected to segregate 3:1 (positive:negative for the presence of the inserted DNA and the herbicide-tolerance phenotype), and the plants from the BC1F1, BC2F1, and BC3F1 generations were expected to segregate 1:1 (positive:negative). Leaf tissue samples were collected for conducting the genotypic analysis described below.

1.2.2. Genotypic Analysis

Leaf punch samples collected from 73496 canola plants were analyzed using qualitative endpoint PCR analysis to confirm the presence or absence of the DP-Ø73496-4 event insertion and the *gat*4621 gene.

The PCR results for one 73496 canola plant (generation T3F2) were inconclusive and the plant had already been discarded so no plant material was available for retesting; therefore, the PCR results for this plant was not included in subsequent statistical analysis.

1.2.3. Phenotypic Analysis

After sample collection, all plants were treated with a mixture containing the herbicide Touchdown HiTech[a] (Syngenta Crop Protection, Inc., Greensboro, NC), non-ionic surfactant, and ammonium sulfate. Glyphosate constitutes 52.3% (by weight) of Touchdown HiTech in the form of a monopotassium salt, equivalent to 5 pounds glyphosate acid equivalents per gallon (0.6 kilograms glyphosate acid equivalents per liter). The spray mixture was applied at an approximate rate of 16 fluid ounces per acre (1.2 liters per hectare) of Touchdown HiTech, 3.5 pounds per acre (3.9 kilograms per hectare) of ammonium sulfate, and 0.25% (by volume) of non-ionic surfactant per volume of spray solution. Spray volume was approximately 23 gallons per acre (215 liters per hectare).

Each plant was visually evaluated 9 days after herbicide application for the presence of herbicide injury, and was identified as presenting an herbicide-tolerant phenotype (plant exhibited no herbicidal injury) or an herbicide-susceptible phenotype (plant exhibited severe herbicide injury). After herbicide injury evaluation, all plants were discarded.

1.2.4. Statistical Analysis of Trait Inheritance Data

Statistical analyses were conducted using SAS software, Version 9.2 (SAS Institute, Inc., Cary, NC). A Pearson's chi-square (χ^2 test at 95% confidence) was conducted for each segregating generation of 73496 canola to determine the goodness-of-fit of the observed segregation ratio to the expected segregation ratio. This value was not calculated for the non-segregating populations. The Pearson test statistic was conducted separately for both the genotypic (*i.e.*, PCR) results and phenotypic (*i.e.*, herbicide tolerance) results, and was computed using the following equation:

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[a] Registered trademark of a Syngenta Group Company

$$\chi^2 = \frac{(O_{pos} - E_{pos})^2}{E_{pos}} + \frac{(O_{neg} - E_{neg})^2}{E_{neg}}$$

Where O_{pos} denotes the total observed positive frequencies, E_{pos} denotes the total expected positive frequencies, O_{neg} denotes the total observed negative frequencies, E_{neg} denotes the total expected negative frequencies, and χ^2 follows a chi-square distribution with one degree of freedom (df).

A chi-square value >3.84 (χ^2(df=1)) has a P-value <0.05 and indicates a significant difference between the observed and expected frequencies. A chi-square value ≤3.84 (χ^2(df=1)) has a P-value ≥0.05 and indicates that the observed data can be interpreted as consistent with the expected ratio.

Appendix 2. Description of Statistical Analyses Performed for Agronomic and Nutrient Composition Data

2.1. Statistical Analysis of Germination Data

Statistical analyses were conducted to evaluate and compare germination rates of 73496 canola and a near-isoline control canola using version 9.1 of SAS[b] software (SAS Institute, Inc., 100 SAS Campus Drive, Cary, NC 27513, USA). Statistical analyses were conducted separately for each of three germination tests (warm, cold, and diurnal).

2.1.1. Mixed Model for Statistical Analysis

For a given germination test, data were analyzed using a Generalized Linear Mixed Model (GLMM), assuming Binomial distribution with the "logit" link function:

GLMM model on the linear predictor scale: $\eta_{ij} = \mu_i + r_{j(i)}$ **Model 1**
$r_{j(i)}\ iid \sim N(0, \sigma^2_R)$

where μ_i denotes the mean response for the ith entry (fixed effect) and $r_{j(i)}$ denotes the effect of the jth replication within the ith entry (random effect nested within fixed effect), and *iid* denotes independently and identically distributed.

Binomial Distribution: $y_{ij}|r_{j(i)} \sim \text{Binomial}\ (n_{ij}, \pi_{ij})$

where y_{ij} denotes the number of germinated seed in the jth replication of the ith entry, j = 1, 2, …, 8 (each replication contained a total of 46~72 seed), n_{ij} denotes the total number of seed in the jth replication of the ith entry, and π_{ij} denotes the probability of a seed being germinated in the jth replication of the ith entry.

"Logit" link function: $\eta_{ij} = \text{logit}(\pi_{ij}) = \log\left(\frac{\pi_{ij}}{1-\pi_{ij}}\right)$

which transformed the parameter π_{ij} into a linear predictor, denoted as n_{ij}. Logit link is default for Binomial data.

2.1.2. Least Squares Mean (LS-Mean) Calculations

For a given germination test, the mean (i.e. least squares mean, also referred to as LS-Mean) value and standard error was estimated from Model 1 for each of 73496 canola and the control canola.

2.1.3. Statistical Comparisons and Interpretations

To test for differences in LS-Mean values between 73496 canola and the control canola, a p-value was calculated for each germination test. For a given comparison, a p-value of < 0.05 was considered to indicate a significant difference.

2.2. Statistical Analyses of Agronomic Characteristic, Yield, and Nutrient Composition Data

Statistical analyses were conducted to evaluate and compare agronomic characteristics and yield of 73496 canola and near-isoline control canola across seven sites during the 2008 growing season (referred to as Experiment A), and across three sites during the 2009 growing season (referred to as Experiment B). Statistical analyses were also conducted to evaluate and compare nutrient composition of seed derived from 73496 canola and near-isoline control canola across six sites during the 2009 growing season.

[b] Registered trademark of the SAS Institute, Inc.

Analysis was conducted separately for each study using SAS software (version 9.1 of SAS was used for analysis of Experiment A, and version 9.2 was used for Experiement B and the nutrient composition data). Days to maturity data were not collected at one site and early growth data were not collected at four sites for Experiment A. Yield data collected at one site were not included in the respective analysis for Experiment B.

2.2.1. Mixed Models for Across Sites Analysis

Default Model

For a given agronomic characteristic or composition analyte, data were analyzed using the following linear mixed model:

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$ **Model 2**

$\ell_j \sim iid\ N(0, \sigma^2_{Site})$, $r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0, \sigma^2_{Ent\times Site})$, and $\varepsilon_{ijk} \sim iid\ N(0, \sigma^2_{Error})$

where μ_i denotes the mean of the ith entry (fixed effect), ℓ_j denotes the effect of the jth site (random effect), $r_{k(j)}$ denotes the effect of the kth block within the jth site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect) and ε_{ijk} denotes the effect of the plot assigned the ith entry in the kth block of the jth site (random effect or residual). Notation $\sim iid\ N(0, \sigma^2_a)$ indicates random variables that are identically independently distributed (*iid*) as normal with zero mean and variance σ^2_a.

Residual maximum likelihood (REML) estimation procedure was used to generate estimates of variance components, and entry means (i.e. LS-Means) across sites were then estimated with standard errors.

Model Allowing Negative Covariance

When the REML estimate for the entry by site variance component $\sigma^2_{Entry\times Site}$ from Model 2 was zero, the random part of the mixed model was changed to a compound symmetry (CS) model that allows for negative covariance (Littell *et al*., 2006).

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + \varepsilon_{ijk}$ **Model 3**

$\ell_j \sim iid\ N(0,\sigma^2_{Site})$, $r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep})$

In the CS model, the residual variance structure for the ijth entry by site combination is defined as:

$$V(\varepsilon_{ij}) = \sigma^2_{CS}\begin{bmatrix} 1 & \rho & \dots & \rho \\ \rho & 1 & \dots & \rho \\ \dots & \dots & \dots & \dots \\ \rho & \rho & \dots & 1 \end{bmatrix}$$

where $\varepsilon_{ij} = [\varepsilon_{ij1}\ \varepsilon_{ij2} \dots \varepsilon_{ijK}]$ is defined as the vector of residuals for the ith entry at the jth site, σ^2_{CS} denotes the residual variance in the CS structure and ρ denotes the correlation between residuals for the same entry at the same site, $\rho = Cov(\varepsilon_{ijk}, \varepsilon_{ijk'})/\sigma^2_{CS}$.

REML estimation procedure was used to generate estimates of variance and covariance components, and entry means (i.e. LS-Means) across sites were then estimated with standard errors.

Model Considering Lower Limit of Quantification (LLOQ)

For the analytes copper, glucoalyssin, gluconapoleiferin, glucoraphanin, and neoglucobrassicin, some sample values were below the assay LLOQ. Sample results below the LLOQ were treated as left-censored observations at the respective assay LLOQ value. Data were analyzed using the following linear mixed model:

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$ **Model 4**

$\ell_j \sim iid\ N(0, \sigma^2_{Site}),\ r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep}),\ (\mu\ell)_{ij} \sim iid\ N(0, \sigma^2_{Entry\times Site}),\ \varepsilon_{ijk} \sim iid\ N(0, \sigma^2_{Error})$

Where μ_i denotes the mean of the ith entry (fixed effect), ℓ_j denotes the effect of the jth site (random effect), $r_{k(j)}$ denotes the effect of the kth block within the jth site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect) and ε_{ijk} denotes the residual for the observation obtained from the plot assigned to the ith entry in the kth block of the jth site.

The conditional likelihood for each observation, given the random effects, was formulated according to the status of the observation (i.e. observed or left-censored) (Thiébaut and Jacqmin-Gadda, 2004):

$$f(y_{ijk} \mid \theta) = \begin{cases} P(y_{ijk} = y^*_{ijk}) = \phi(\dfrac{y^*_{ijk} - \mu_{ijk}}{\sigma}) & observed \\ P(y_{ijk} \le c) = \Phi(\dfrac{c - \mu_{ijk}}{\sigma}) & left-censored \end{cases}$$

Where θ denotes the vector of all random effects, μ_{ijk} denotes the mean of y_{ijk}, ϕ denotes the standard normal density function, Φ denotes the standard normal cumulative distribution function, y^*_{ijk} denotes the observed sample value of y_{ijk}, and c denotes the assay LLOQ value.

The conditional likelihood function is a product of all individual conditional likelihoods, and the marginal likelihood function is formed when the conditional likelihood function is integrated over all random effects. Maximum likelihood (ML) procedure was then used to generate estimates of variance components and entry means (i.e. LS-Means) across sites.

2.2.2. Least Squares Mean (LS-Mean) and Confidence Interval (CI) Calculations

For each agronomic characteristic or composition analyte, the LS-mean value across sites was estimated from the corresponding statistical model for herbicide-treated 73496 canola and the control canola. The 95% CI for each of the entry means was formed by:

LS-Mean ± $t_{0.975,v}$ × standard error of LS-Mean

where $t_{0.975,v}$ denotes the upper 0.025 percentage point of a t-distribution with v degrees of freedom. The degrees of freedom were determined by Kenward-Roger method (Kenward and Roger, 1997).

2.2.3. Statistical Comparisons and Interpretations

The first step in the evaluation was to test for differences in LS-Mean values between herbicide-treated 73496 canola and the control canola. The FDR method of Benjamini and Hochberg (Benjamini and Hochberg, 1995; Westfall *et al.*, 1999) was applied as a post-hoc procedure to account for multiple comparisons due to multiple endpoints (i.e. agronomic characteristics or composition analytes) and p-values were adjusted accordingly. Significant differences were established if an adjusted p-value <0.05.

For nutrient composition analytes where a statistically significant difference (adjusted p-value <0.05) was identified in the across sites analysis, the respective range of individual values was compared to a

tolerance interval. Tolerance intervals containing 99% of the values for corresponding analytes of the conventional canola population with 95% confidence level (Graybill, 1976) were derived from data collected from seven conventional (i.e. non-modified) commercial canola lines grown at five field locations in canola-growing areas of the U.S. and Canada over two growing seasons. The selected canola lines represent non-modified canola population with a history of safe use, and the selected environments (i.e. site and year combinations) represent canola growth under a wide range of environmental conditions (i.e. soil type, temperature, precipitation, and irrigation) and canola maturity group zones.

2.3. Special Considerations

2.3.1. Agronomic Data Considerations

Plant Height

Plant height was measured on 10 plants per plot. Plot average (i.e. average of 10 plants) was treated as the response for the across sites analysis.

2.3.2. Nutrient Composition Data Considerations

Transformation

A natural logarithmic "ln(y)" transformation was performed for the raw data of all nutrient composition analytes before statistical analyses. For each analyte, residuals from the across sites analysis were examined for validation of the normality and constant variance assumptions. The assumptions were reasonably satisfied for most analytes after "ln(y)" transformation.

For the analyte tryptophan, residual distribution skewed to the left and therefore, a square "$(y)^2$" transformation was performed to the raw data instead. The model assumptions were reasonably satisfied for this analyte after "$(y)^2$" transformation.

The statistical comparisons were conducted based on the transformed data. The estimated mean values and the confidence limits were then back-transformed to the original data scale for reporting purposes.

Fatty Acids

For some fatty acids analytes, absolute sample values were detected below the assay LLOQ. When sample values were converted from absolute values to relative proportions (i.e. percent of total fatty acids), sample results that were below the LLOQ were assigned a zero value to reflect a negligible proportion. However, these zeros were not "true" zeros, but were some unknown small positive values. Even though the origination of these zero values was the LLOQ values, it was not straight-forward to determine an LLOQ value on the relative proportion scale for each analyte. Therefore, Model 4 was not used to analyze fatty acids analytes with zero values; instead it was deemed appropriate to treat these zero sample values as missing values and analyze the non-missing data using Model 2 or Model 3.

Partial LLOQ Sample Values

For a given analyte (including non-fatty acids and fatty acid analytes), the number of samples below the assay LLOQ value determined whether a statistical analysis was conducted. The following rules were implemented.

- If < 80% of samples for each entry were below the LLOQ, then statistical analysis was conducted using the appropriate mixed model.

- If ≥ 80% of samples for a single entry within the study were below the LLOQ, then mixed model across sites analysis was not conducted. However, if the numbers of samples below the LLOQ were not even between the control canola and herbicide-treated 73496 canola, such observation

would be discussed. Descriptive statistics (i.e. arithmetic means and ranges) were calculated for analytes that were not statistically analyzed using mixed model analysis.

2.4. SAS Procedures

SAS PROC GLIMMIX was used to fit Model 1, SAS PROC MIXED was used to fit Model 2 and Model 3, and SAS PROC NLMIXED was used to fit Model 4. All three procedures generated LS-Means and 95% confidence intervals, and provided statistical comparisons (i.e. p-values). SAS PROC MULTTEST was used to provide adjusted p-values.

Appendix 3. Materials and Methods for Determination of GAT4621 Protein Concentrations

Plant tissue concentrations of the GAT4621 protein were determined using enzyme-linked immunosorbent assays (ELISA) developed at Pioneer Hi-Bred International, Inc., Johnston, IA, USA.

3.1. Plant Material

73496 canola plants of the F1*4 generation (refer to Section III-A, Figure 2), plus near-isoline control canola plants, were grown concurrently at six field trial locations near: Portage la Prairie, MB; Saskatoon, SK (grain samples not included from this site); Minto, MB; Elm Creek, MB; Ephrata, WA; and Velva, ND. Trials were planted in a randomized complete block design containing four blocks per treatment and were managed according to standard local agronomic practices, with the exception that the 73496 canola plants were treated with two applications of glyphosate herbicide (Touchdown HiTech®), at the first true leaf stage and the five-leaf stage, respectively. Plant tissue samples were collected from impartially selected, healthy individual plants from each block at the following growth stages:

- 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)

3.2. Storage and Processing of ELISA samples

After collection, tissue samples were shipped overnight on dry ice to Pioneer Hi-Bred International, Inc. Whole plant samples, which comprised the entire above-ground portion of the plant, were coarsely homogenized and all samples (whole plant, root, and seed) were lyophilized, and then finely homogenized and stored frozen (≤ -10ºC).

3.3. Protein Extraction from Ground Canola Tissues

Lyophilized, ground tissue samples were weighed into 1.2-ml tubes at the following target weights: 15 mg for whole plant, and 20 mg for root and seed. Each sample was extracted with 0.6 ml of chilled H5 buffer solution (90 mM HEPES pH 7.6, 140 mM NaCl, 1% PVP-40, 0.3% Tween-20, 1% bovine serum albumin, 1% polyethylene glycol, and 0.007% Thimerosal), centrifuged, and the supernatants removed, diluted, and analyzed.

3.4. Determination of GAT4621 Protein Concentrations

Sample extracts were quantitatively analyzed for GAT4621 by sequential double-antibody sandwich ELISA using antibodies specific for the GAT protein. Standards (analyzed in triplicate wells) and diluted sample extracts (analyzed in duplicate wells) were incubated in a plate pre-coated with GAT-specific antibody. Following incubation, unbound substances were washed from the plate. A different GAT-specific antibody conjugated to the enzyme horseradish peroxidase (HRP) was added to the plate and incubated. Unbound substances were washed from the plate leaving the bound GAT4621 protein "sandwiched" between the antibody coated on the plate and the antibody-HRP conjugate. Detection of the bound GAT4621-antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the optical density (OD) of each well was determined using a plate reader. An average of the results from duplicate wells was used to determine the concentration of the GAT4621 protein in ng/mg sample weight.

SoftMax[c] Pro software (Molecular Devices Corporation, Sunnyvale, CA) was used to perform the calculations required to convert the OD values obtained by the microtiter plate reader to tissue protein concentrations. A standard curve was included on each ELISA plate. The equation for the standard curve was generated by the software, which used a quadratic fit to relate the mean OD values to the respective standard concentrations (ng/ml).

Lower limits of quantification (LLOQ) by ELISA were established with each sample matrix and were 0.29 ng/mg dry weight for whole plant samples, and 0.22 ng/mg dry weight for root and seed samples. For each plant tissue type and growth stage, levels of GAT4621 protein in samples from control plants were below the LLOQ.

[c] Registered trademark of Molecular Devices Corporation

Appendix 4. Characterization of the Plant-Expressed and Microbial-Expressed GAT4621 Proteins

4.1. Characterization of the Plant-Expressed GAT4621 Protein

Physicochemical characterization of the GAT4621 protein isolated from 73496 canola 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 73496 canola 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 73496 canola 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 73496 canola.

Utilizing these analyses, the equivalency of GAT4621 protein expressed in *E. coli* to the protein expressed in 73496 canola 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 73496 canola. In addition, the GAT4621 protein produced in 73496 canola is not glycosylated and supports the weight of evidence assessment that the GAT4621 protein is unlikely to be an allergen.

4.2. SDS-PAGE Analysis of the Plant-Expressed GAT4621 Protein

Analysis of GAT4621 protein isolated from 73496 canola 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 4.1).

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

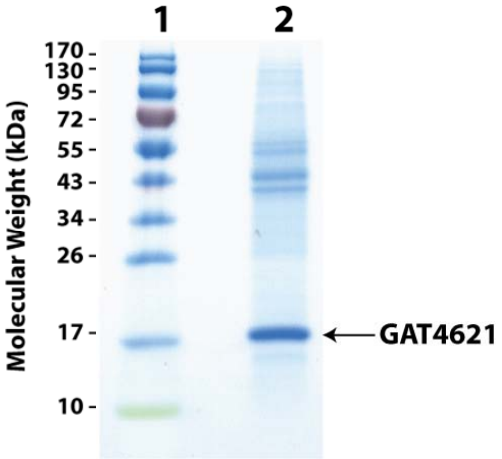

Figure 4.1. SDS-PAGE Analysis of the Plant-Expressed GAT4621 Protein

A sample of the purified GAT4621 protein (~ 1 µg total protein) from 73496 canola (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.

4.3. Western Immunoblot Analysis of the Plant-Expressed and Microbial-Expressed GAT4621 Proteins

Western blot analysis of the GAT4621 protein prepared from 73496 canola 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 4.2).

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

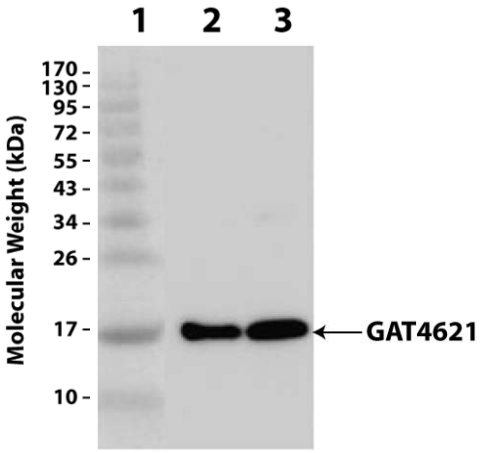

Figure 4.2. Western Immunoblot Analysis of the Plant-Expressed and Microbial-Expressed GAT4621 Proteins

A sample of the purified GAT4621 protein (~ 10 ng total protein) from 73496 canola (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 polyvinylidene difluoride (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.

4.4. 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 73496 canola 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 4.3). 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.

Edman sequencing analysis confirmed that the N-terminal sequences of the 73496 canola-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 4.3. N-Terminal Amino Acid Sequence Analysis of the Plant-Expressed and Microbial-Expressed GAT4621 Proteins

A sample of purified GAT4621 protein from 73496 canola 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 73496 canola-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.

4.5. MALDI-MS Identification of Tryptic Peptides

MALDI-MS analysis of the tryptic peptides for GAT4621 isolated from 73496 canola 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 73496 canola (Figure 4.4). 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.

MAIEVKPINAEDTYDLRHRVLRPNQPIEACMF
ESDLTRSAFHLGGFYGGKLISVASFHQAEHSE
LQGKKQYQLRGVATLEGYREQKAGSSLVKHA
EEILRKRGADMIWCNARTSASGYYRKLGFSEQ
GEVFDTPPVGPHILMYKRIT

Figure 4.4. Deduced GAT4621 Amino Acid Sequence Showing Matching Peptides from MALDI-MS

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

4.6. 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 73496 canola was analyzed using a glycoprotein staining procedure. Plant-expressed GAT4621 protein did not exhibit positive staining with the glycoprotein staining reagent (Figure 4.5, lane 4, Glycoprotein staining panel), while the positive control horseradish peroxidase was stained and clearly visible (Figure 4.5., lane 3, Total protein staining panel).

Based on this analysis, the 73496 canola-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.

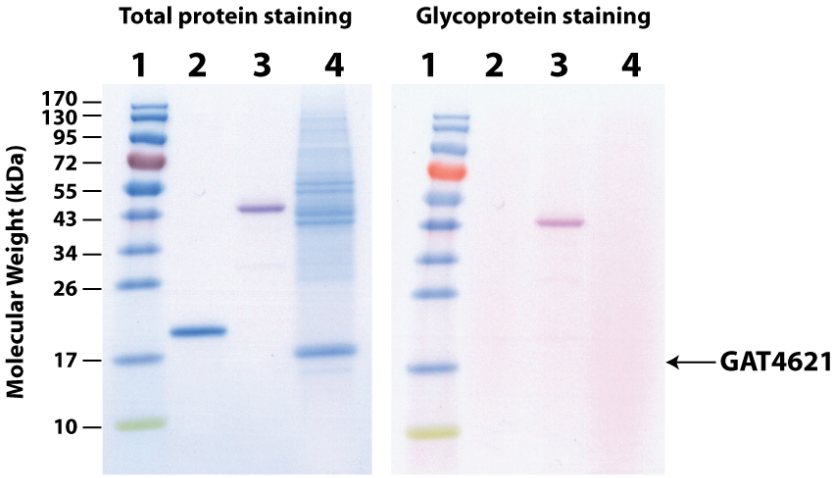

Figure 4.5. Glycosylation Analysis of the Plant-Expressed GAT4621 Protein

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 73496 canola (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.

4.7. Methods for Isolation and Purification of the GAT4621 Protein from Canola Leaf Tissue

The GAT4621 protein was isolated from leaf tissue of greenhouse-grown 73496 canola plants. Each plant was tested to confirm the presence of the introduced trait by event-specific PCR. Leaves were harvested from plants at the five- to six-leaf stage [BBCH 15-16] and stored frozen (-80ºC) until use.

Approximately 80 g of leaf tissue derived from 73496 canola was extracted by homogenizing with a Waring blender in 300 ml Buffer A (50 mM HEPES, pH 7.8, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 0.007% thimerosal) containing Complete Protease Inhibitors (Roche Applied Science, Indianapolis, IN). The homogenate was filtered through four layers of cheese cloth and then centrifuged for 20 minutes at approximately 30,500 *g*. The clarified extract was subjected to 85% saturated ammonium sulfate precipitation. The precipitated proteins were resuspended in approximately 60 ml of Buffer A. This sample was then desalted with 10DG columns (Bio-Rad Laboratories, Hercules, CA) and pre-equilibrated with Buffer B (50 mM MES, pH 7.2, 5% glycerol, 1 mM EDTA). The desalted sample was loaded to an SP Sepharose Fast Flow (GE Healthcare Biosciences, Piscataway, NJ) column (10 ml) equilibrated with Buffer B. The column was washed with the same buffer (approximately 50 ml) and then the bound proteins were eluted with a salt gradient (approximately 100 ml 0-0.5 M NaCl). The GAT4621 fractions were collected, pooled and diluted (1:1) with Buffer A. GAT4621 protein was further purified with an immuno-affinity column (approximately 3 ml) prepared with GAT-specific mouse monoclonal antibody (12C9). The column was sequentially washed with 50 ml each of Buffer A, Buffer C (Buffer A containing 100 mM NaCl and 0.2% Triton X-100) and Buffer D (Buffer A containing 100 mM NaCl and 0.4% Triton X-100). The bound GAT4621 protein was eluted using ImmunoPure IgG Elution Buffer (Thermo Scientific Inc., Rockford, IL). Fractions were collected and the protein concentration was estimated by a modified Bradford assay (Coomassie Plus Protein Assay Reagent, Thermo Scientific, Inc.). The collected fractions were analyzed by SDS-PAGE and western blotting with a GAT-specific antibody (18F9) to confirm the

presence of the GAT4621 protein. The fractions containing GAT4621 were pooled and concentrated using Nanosep 3K concentrators (Pall Corporation, Ann Arbor, MI).

4.8. Methods for Isolation and Purification of the Microbial-Expressed GAT4621 Protein

The GAT4621 protein was expressed in *E. coli* strain BL21(DE3) as a soluble protein and was purified at Aldveron, LLC (Fargo, ND) using cation exchange chromatography, anion exchange chromatography and hydrophobic interaction chromatography, followed by diafiltration into 0.1 M ammonium bicarbonate, pH 7.8. The purified protein was then lyophilized. For characterization, the GAT4621 protein was used in the following form: 1 mg of the lyophilized powder was dissolved in 1 ml of 100 mM KCl, 10% methanol, and 25 mM HEPES, pH 7.2.

4.9. Methods for SDS-PAGE Analysis

SDS-PAGE was performed by first mixing GAT4621 protein samples with Laemmli sample buffer (NuSep Limited, Frenchs Forest, Australia) containing 100 mM dithiothreitol and heating at 100°C for approximately five minutes. The prepared protein samples were loaded into a 10-20% gradient Ready Gel Tris-HCl gel (Bio-Rad Laboratories, Inc., Hercules, CA). PageRuler Prestained Protein Ladder (Fermentas, Inc., Vilnius, Lithuania) molecular weight markers were loaded into the gel to provide a visual estimate of molecular weight. Electrophoresis was conducted using the Ready Gel Cell system (Bio-Rad Laboratories, Inc.) with Tris-glycine running buffer (Fermentas, Inc.) at 150 volts for 60 minutes.

Upon completion of electrophoresis, the gel was removed from the gel cassette and washed three times with deionized water for five to 15 minutes each. The gel was then stained for approximately 60 minutes with GelCode Coomassie Blue stain reagent (Thermo Scientific Inc., Rockford, IL), and washed with deionized water at least four times for approximately ten minutes each or until the background of the gel was clear.

4.10. Methods for Western Immunoblot Analysis

GAT4621 protein samples were run by SDS-PAGE as described in the previous section. The resulting gel was soaked in cathode buffer (60 mM Tris, 40 mM CAPS, 0.1% SDS, pH 9.6) for 10-20 minutes. A polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc.) was briefly placed in 100% methanol followed by immersion in anode buffer (60 mM Tris, 40 mM CAPS, 15% methanol, pH 9.6) for 10-15 minutes. A Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell system (Bio-Rad Laboratories, Inc.) was used to transfer proteins from the gel to the membrane at approximately 120 mA for 30-60 minutes.

Following protein transfer, the membrane was washed four times for five to ten minutes each in Classic Buffer (50 mM Tris-HCl, pH 7.0, 500 mM NaCl, 0.5% Tween-20) and then blocked by incubation in phosphate-buffered saline solution with Tween-20 (PBST solution: 8.1 mM phosphate buffer, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween-20) containing 5% non-fat dry milk for 30-60 minutes. The blocked membrane was washed four times for 5-10 minutes each in Classic Buffer and then incubated for approximately 60 minutes with a GAT-specific mouse monoclonal antibody (18F9) diluted 1:8,000 in PBST containing 5% non-fat dry milk. The unbound antibody was removed from the membrane with four washes of Classic Buffer for 5-10 minutes each. The membrane was then incubated for approximately 60 minutes with a secondary antibody (anti-mouse IgG HRP conjugate, Promega, Madison, WI) diluted 1:10,000 in PBST containing 5% non-fat dry milk, followed by four washes with Classic Buffer for 5-10 minutes each. The washed blot was immersed in phosphate-buffered saline (PBS: 8.1 mM phosphate buffer, pH 7.4, 137 mM NaCl, and 2.7 mM KCl) or PBST solution for at least 5 minutes and then incubated with SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific Inc.) according to the manufacturer's instructions. The signal was detected with Fujifilm Luminescent Image Analyzer (LAS-3000, Fujifilm, Hanover Park, IL).

4.11. Methods for N-Terminal Amino Acid Sequence Analysis

Following SDS-PAGE of the 73496 canola-derived GAT4621 protein sample, the resulting gel was soaked in cathode buffer (60 mM Tris, 40 mM CAPS, 0.1% SDS, pH 9.6) for approximately 20 minutes. A PVDF membrane (Millipore Corp., Bedford, MA) was briefly wetted with 100% methanol, followed by immersion in anode buffer (60 mM Tris, 40 mM CAPS, 15% methanol, pH 9.6) for 10-15 minutes. A Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell system (Bio-Rad Laboratories, Inc.) was used to transfer proteins from the gel to the membrane at 120 mA for approximately 60 minutes. Following protein transfer, the membrane was then stained with GelCode Coomassie Blue stain reagent (Thermo Scientific Inc.) to visualize the GAT4621 protein band. The resulting GAT4621 band was excised and shipped to the Keck Biotechnology Resource Laboratory (Yale University, New Haven, CT) for Edman N-terminal amino acid sequencing using the Procise 494 cLC analyzer (Applied Biosystems, Inc., Foster City, CA) equipped with an online high performance liquid chromatography system.

The microbial-expressed GAT4621 protein sample was sent to Crop Genetics (E. I. du Pont de Nemours and Company, Wilmington, DE) in 25mM HEPES, pH 7.2, 100mM KCl, 10% methanol. The protein was transferred to PVDF membrane using ProSorb Inserts (Applied Biosystems, Foster City, CA) and washed with 200 µl 0.1% trifluoroacetic acid in water. The membrane was removed and used for Edman N-terminal amino acid sequencing with the Procise 494 LC analyzer (Applied Biosystems) equipped with an online high performance liquid chromatography system.

4.12. Methods for MALDI-MS Identification of Tryptic Peptides

Following SDS-PAGE, the 73496 canola-derived GAT4621 protein band was visualized by staining with Coomassie Blue and the band was then excised from the gel. The gel slice containing the protein was placed in a labeled tube and shipped on dry ice via overnight delivery to the Keck Biotechnology Resource Laboratory (Yale University). The protein in the gel slice was digested with trypsin for 18 hours at 37°C. An aliquot of the digest was analyzed by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) on an Applied Biosystems/MDS SCIEX 4800 MALDI TOF/TOF Analyzer in the reflectron mode of operation. Detected peptide peaks were considered a match if the observed experimental mass was within 50 parts per million (ppm) of the theoretical mass of peptides determined via *in silico* trypsin cleavage of the protein sequence. The peptide mass matching was performed using GPMAW 7.1.

Allowances were made for the following potential modifications to the peptides: oxidation of methionine (observed value is 15.995 Da greater than the theoretical value) and modification of cysteine residues by propionamide adduct during SDS-PAGE (observed value is 71.037 Da greater than the theoretical value).

For the microbial-expressed GAT4621, the procedures for excising the protein and trypsin digestion were similar to those described for the 73496 canola-derived protein. The analysis was conducted on an Applied Biosystems/MDS SCIEX 4700 MALDI-Tof-Tof instrument (Applied Biosystems) in the reflectron mode of operation. Detected peptide peaks were considered a match if they were within 100 parts per million (ppm) of the theoretical mass of peptides determined via *in silico* trypsin cleavage of the protein sequence.

Allowances were made for the following potential modifications to the peptides: oxidation of methionine or tryptophan residues (observed value is 15.995 Da greater than the theoretical value) and modification of cysteine residues by acrylamide free radicals during SDS-PAGE (observed value is 71.037 Da greater than the theoretical value).

4.13. Methods for Protein Glycosylation Analysis

A GelCode glycoprotein staining kit (Thermo Scientific Inc.) was used according to the manufacturer's instructions to determine whether the GAT4621 protein was glycosylated. The 73496 canola-derived GAT4621 protein, a positive control protein (horseradish peroxidase) and a negative control protein (soybean trypsin inhibitor) were run by SDS-PAGE as described in the SDS-PAGE analysis section. Following electrophoresis, the gel was fixed with 50% methanol for 30 minutes and washed with 3% acetic acid. The gel was then incubated with the GelCode oxidizing solution for 15 minutes and washed three times with 3% acetic acid. The gel was next incubated with the GelCode glycoprotein staining reagent (Thermo Scientific Inc.) for 15 minutes and then treated with the GelCode reducing reagent, followed by extensive washing with 3% acetic acid and deionized water. Glycoproteins were detected as magenta colored bands on the gel. Following glycoprotein detection, the gel was scanned and the image was captured electronically. The same gel was then stained with Coomassie Blue as described previously to visualize the total protein content of all protein bands, and an image of the gel was captured electronically.

Appendix 5. Ecological Observations for 73496 Canola

Key to "Range of severity" in Tables 5.1 through 5.4:
mild – very little disease or insect injury (<10%) visible;
moderate – noticeable plant tissue damage (10% to 30%);
severe – significant plant tissue damage (>30%).

Table 5.1. United States: Insect Stressor Comparison between 73496 Canola and Controls

Year	Permit Number	State	County	Insect Stressor	Range of Severity[a]	Difference with Control?
2007	07-255-103n	CA	Imperial	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild to Moderate	No
				Green peach aphid (*Myzus persicae*)	Mild to Moderate	No
2008	08-261-103n	CA	Imperial	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
				Green peach aphid (*Myzus persicae*)	Mild	No
				Aphid (*Brevicoryne brassicae*)	Mild	No
2009	09-016-106n	ND	McHenry	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
		WA	Grant	Imported cabbage moth (*Pieris brassicae*)	Mild	No
2009	09-245-104n	CA	Imperial	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
				Green peach aphid (*Myzus persicae*)	Mild to Moderate	No
2010	10-020-103n	ND	Cass	Grasshopper (*Melanoplus sanguinipes)*	Mild to Moderate	No
				Aphid (*Brevicoryne brassicae*)	Moderate	No
				Diamond back moth (*Plutella xylostella)*	Moderate	No
				White cabbage butterflies (*Pieris rapae)*	Mild to Moderate	No
				White cabbage moths (*Mamestra brassicae*)	Mild	No
				White cabbage butterflies (*Pieris rapae)*	Mild	No

Table 5.1. United States: Insect Stressor Comparison between 73496 Canola and Controls (continued)

Year	Permit Number	State	County	Insect Stressor	Range of Severity[a]	Difference with Control?
2010	10-020-103n	ND	Ward	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
			Grand Forks	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
				Diamond back moth (*Plutella xylostella)*	Mild	No
				Aphid (*Brevicoryne brassicae*)	Mild	No
			McHenry	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
		WA	Grant	Green peach aphid (*Myzus persicae*)	Mild	No

[a] Range of severity scores are as follows: Mild – very little insect injury (<10%) visible; Moderate – noticeable plant tissue damage (10% to 30%); Severe – significant plant tissue damage (>30%)

Table 5.2. United States: Disease Stressor Comparison between 73496 Canola and Controls

Year	Permit Number	State	County	Disease Stressor	Range of Severity[a]	Difference with Control?
2007	07-255-103n	CA	Imperial	None reported	N/A	N/A
2008	08-261-103n	CA	Imperial	Downey mildew (*Peronospora parasitica*)	Mild	No
2009	09-016-106n	ND	McHenry	None reported	N/A	N/A
		WA	Grant	Powdery mildew (*Erysiphe polygoni*)	Mild	No
2009	09-245-104n	CA	Imperial	None reported	N/A	N/A
2010	10-020-103n	ND	Cass	White mold/Sclerotinia (*Sclerotinia sclerotiorum)*	Mild to Moderate	No
			Ward	None reported	N/A	N/A
			Grand Forks	White mold/Sclerotinia (*Sclerotinia sclerotiorum)*	Mild	No
				Fusarium wilt (*Fusarium oxysporum*)	Mild	No
				Alternaria (*Alternaria brassicae)*	Mild	No
			McHenry	None reported	N/A	N/A
		WA	Grant	Powdery mildew (*Erysiphe polygoni*)	Mild	No

[a] Range of severity scores are as follows: Mild – very little disease injury (<10%) visible; Moderate – noticeable plant tissue damage (10% to 30%); Severe – significant plant tissue damage (>30%)

Table 5.3. Canada: Insect Stressor Comparison between 73496 Canola and Controls

Year[a]	Province	Nearest City	Insect Stressors	Range of Severity[b]	Difference with Control?
2008	MB	Morden	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
			Lygus bug (*Lygus sp.*)	Mild	No
		Rosebank	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
			Lygus bug (*Lygus sp.*)	Mild	No
		Crystal City	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
		Carman	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
	ON	Georgetown	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
			Imported cabbage moth (*Pieris brassicae*)	Mild	No
			Lygus bug (*Lygus sp.*)	Mild	No
2009	AB	Fort Saskatchewan	Grasshopper (*Melanoplus sanguinipes)*	Mild to Severe	No
			Cutworm (*Euxoa ochrogaster)*	Mild to Severe	No
		Gibbons	Grasshopper (*Melanoplus sanguinipes)*	Mild to Severe	No
			Cutworm (*Euxoa ochrogaster)*	Mild to Severe	No
		Riviere Qui Barre	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
			Thrips (*Thrips tabaci)*	Mild	No
			Cutworm (*Euxoa ochrogaster)*	Mild	No
	MB	Minto	Imported cabbage moth (*Pieris brassicae*)	Mild	No
			Aphid (*Brevicoryne brassicae*)	Mild	No
			Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
			Thrips (*Thrips tabaci)*	Mild	No
		Rosebank	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
		Portage la Prairie	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
		Wellwood	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
		Franklin	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	No
	SK	Dundurn	Alfalfa looper (*Autographa californica)*	Mild	No

[a] Canadian permits: 2008: 08-PHI-294-CAN01, 2009: 09-PHI1-294-CAN01
[b] Range of severity scores are as follows: Mild – very little insect injury (<10%) visible; Moderate – noticeable plant tissue damage (10% to 30%); Severe – significant plant tissue damage (>30%)

Table 5.4. Canada: Disease Stressor Comparison between 73496 Canola and Controls

Year[a]	Province	Nearest City	Disease Stressor	Range of Severity[b]	Difference with Control?
2008	MB	Morden	Sclerotinia (*Sclerotinia sclerotiorum)*	Moderate	No
		Rosebank	Sclerotinia (*Sclerotinia sclerotiorum)*	Mild to Moderate	No
	ON	Georgetown	Sclerotinia (*Sclerotinia sclerotiorum)*	Mild	No
2009	MB	Minto	Sclerotinia (*Sclerotinia sclerotiorum)*	Mild	No
		Franklin	Sclerotinia (*Sclerotinia sclerotiorum)*	Mild to Moderate	No
	SK	Dundurn	Sclerotinia (*Sclerotinia sclerotiorum)*	Mild	No

[a] Canadian permits: 2008: 08-PHI-294-CAN01, 2009: 09-PHI1-294-CAN01
[b] Range of severity scores are as follows: Mild – very little disease injury (<10%) visible; Moderate – noticeable plant tissue damage (10% to 30%); Severe – significant plant tissue damage (>30%)

Appendix 6. USDA and Canadian Field Trials of 73496 Canola

Table 6.1. USDA Field Trials of 73496 Canola[a,b]

Year	Permit Name	Permit Valid Date	State	Number of Counties Where Planted	Acreage
2007	07-255-103n	10/1/2007	California	1	0.00594
2008	08-261-103n	10/28/2008	California	1	0.07128
2009	09-016-106n	2/20/2009	Idaho	1	0.05
			Minnesota	1	0.035
			New Jersey	1	0.01
			North Dakota	4	0.19264
			Washington	5	0.15535
2009	09-245-104n[c]	9/21/2009	California	1	0.55556
2010	10-020-103n[c]	3/2/2010	North Dakota	4	0.50327
			Washington	1	0.304
2010	10-253-111n[c]	10/5/2010	California	1	0.3

[a] Plantings through October 19, 2010 are listed.
[b] In USDA final reports, 73496 canola (event DP-Ø73496-4) is called DP-073496-4 or DP-73496-4.
[c] Final field test report not yet due to USDA.

Table 6.2. Canada Field Trials of 73496 Canola[a]

Year	Permit Name	Permit Valid Date	Province	Number of Locations Planted	Acreage
2008	08-PHI-294-CAN	4/29/2008	Alberta	2	0.28038
			Manitoba	2	0.70093
			Ontario	1	0.22664
			Saskatchewan	1	0.14019
2009	09-PHI1-294-CAN01	4/30/2009	Alberta	4	0.33685
			Manitoba	9	0.85154
			Ontario	2	0.24223
			Saskatchewan	7	0.2479
2010	10-PHI1-294-CAN	4/29/2010	Alberta	5	2.44817
			Manitoba	1	0.15381
			Ontario	2	0.40707
			Saskatchewan	1	0.87204

[a] Plantings through October 19, 2010 are listed.

Appendix 7. Survey of Potential Substrates for GAT4621

In enzyme kinetics, k_{cat} is a measure of the turnover rate or speed of the reaction. The higher the k_{cat}, the faster the enzyme reaction. K_M is the affinity of the enzyme for a substrate or tightness of binding of the substrate to the enzyme. The lower the K_M, the greater the affinity of the enzyme for the substrate. A k_{cat}/K_M ratio is the common way to express the catalytic efficiency of the enzyme. The greater the k_{cat}/K_M ratio, the greater the catalytic efficiency of the enzyme for a given substrate. Specificity of an enzyme can be judged by comparing the values of k_{cat}/K_M for various enzyme substrates.

In order to examine the specific catalytic efficiency of the GAT4621 enzyme expressed in 73496 canola, a substrate specificity study was done with the microbial GAT4621 protein that had previously been determined to be equivalent to plant-derived GAT4621 protein from 73496 canola (Section VI-D and Appendix 4).

A dithiobis-2-nitrobenzoate enzymatic end-point assay was used to detect any enzyme activity in a survey of various substrates (Siehl *et al*., 2005) using assay buffer containing 100 mM KCl to represent physiological conditions in the plant cytosol and chloroplast (Cuin *et al*., 2003; Sanders and Bethke, 2000). Twenty agrochemicals, 21 amino acids and 11 antibiotics were tested as potential substrates for the GAT4621 protein. As expected from a previous survey (Siehl *et al*., 2005) with a Round 11 GAT protein that only differed from GAT4621 in 73496 canola by the absence of an alanine residue in the second position of the protein sequence, no significant activity was seen with the majority of the substrates.

Of the substrates surveyed for activity with GAT4621, only five amino acid substrates indicated low but measurable enzyme activity (sufficiently above the limit of quantitation of the assay): L-aspartate, L-glutamate, L-serine, glycine and L-threonine. The remaining amino acids and substrates surveyed (other than glyphosate) produced levels of end product near or below the limit of quantitation of the end-point assay.

GAT4621 activity on the five amino acids was further characterized using a continuous spectrophotometric assay (Siehl *et al*., 2005) for characterization of kinetic properties (Table 1). The affinity of the GAT4621 enzyme for glycine was so low that K_M values could not be accurately estimated. Therefore, a k_{cat}/K_M ratio could not be calculated.

Table 7.1. k_{cat}/K_M for the GAT4621 Enzyme on Glyphosate and Selected Amino Acids

Substrate	k_{cat}/K_M (+/- standard deviation) min^{-1} mM^{-1}	% k_{cat}/K_M of glyphosate
Glyphosate	1063 (+/- 31.8)	100
Aspartate	12.1 (+/- 1.57)	1.14
Glutamate	8.32 (+/- 0.46)	0.78
Serine	0.57 (+/- 0.05)	0.05
Threonine	0.60 (+/- 0.01)	0.06
Glycine	ND[a]	ND[1]

[a] ND – Not able to determine because the K_M was too high to estimate.

The results of the GAT4621 substrate survey confirmed the results of the earlier published substrate survey for the GAT enzyme. The GAT4621 enzyme was able to use five amino acids as substrates (aspartate, glutamate, serine, threonine and glycine), although inefficiently (~0.05 - 1% of that of GAT4621 on glyphosate). Further kinetic characterization indicated that the K_M of GAT4621 for glycine was too high to estimate (low affinity of the GAT4621 enzyme for glycine as substrate). The level of

catalytic efficiency of GAT4621 on aspartate, glutamate, serine, and threonine was about 1%, 0.8%, 0.05 and 0.06%, respectively, of the activity on glyphosate.

Due to the low levels of GAT4621 enzyme activity on five amino acids as substrates, the concentrations of NAA, NAG, NAS, NAT and NAGly in 73496 and control canola seed was measured in the nutrient composition studies (Section VIII).

Appendix 8. Safety and History of Consumption of Acetylated Amino Acids

8.1 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.

8.2 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 cotranslationally or posttranslationally 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.

8.3 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 odors and flavors 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 to L-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.

8.4 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.

8.4.1 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.

8.4.2 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).

8.4.3 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 signaling pathways by antagonizing phosphorylation of threonine residues (Mukherjee *et al.*, 2007).

8.4.4 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 signaling 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 sulfur 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).

8.4.5 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).

8.5 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 (FDA, 2008; USDA, 2009), these compounds are found in plant species including canola, are not novel, and are normal components of animal diets.

8.6 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 8.1.

Table 8.1. 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

Appendix 9. Herbicide Resistant Weeds

9.1. Evolution of Herbicide Resistant Weeds

Weeds will tend to adapt and circumvent any single control mechanism. Instances of herbicide resistant weeds have occurred for many herbicide classes including acetyl-CoA carboxylase and acetolactate synthase (ALS) inhibitors, glyphosate, hormone, triazines and other photosystem II inhibitors (Table 9.1; Heap, 2010b).

Herbicide resistance usually evolves in only one or two weed species in an area, even though a much larger number of weeds are exposed to the same herbicide selection intensity. Nonetheless, weed resistance to herbicides currently affects hundreds of thousands of fields and the most widely used herbicides (Heap, 2010a). According to a recent survey, more than 348 herbicide resistant weed biotypes (representing 194 species) are confirmed to be present in agricultural fields around the world (Table 9.1; Heap, 2010b). In this survey, several criteria are used to confirm resistance. Among these criteria, a resistant weed must meet certain specific definitions, is confirmed through unbiased experimentation, is able to pass the phenotype to subsequent generations, and has a practical economic impact to growers (Heap, 2005). Resistant weeds often increase the cost of crop production and limit the effectiveness of herbicides that can be used and the crops that can be grown. Growers use a variety of approaches to limit the impact of resistant weeds on crop productivity.

A graph of the number of confirmed herbicide resistant weeds by herbicide class and by year is provided in Figure 9.1. Globally, confirmed glyphosate resistant weeds do not represent the largest class of herbicide resistant weeds (Figure 9.1). One hundred thirty-two herbicide resistant weed biotypes are confirmed in the United States, with 12 of these resistant to the glyphosate (glycine) class of herbicides (Heap, 2010c).

When growers indicate that their "weeds have become resistant," they really mean that the population of resistant weed biotypes has increased to an unacceptable level. The spread of a resistance phenotype depends primarily upon the exposure or selection applied by the herbicide. When an herbicide is applied, most of the susceptible weeds die while the resistant weeds survive, mature, and produce seed. Although the resistant population may be small, repeated application of the same herbicide continues to increase the proportion of resistant weeds in the population.

Not all weed shifts are driven by a genetically based biochemical capacity to survive exposure. For example, weeds with delayed emergence and slower development are also able to avoid exposure to the herbicide (Hilgenfeld *et al*., 2004).

Herbicide resistance can become an ecological problem if the resistant weed biotype replaces the non-resistant biotype in the weed population. Even then, the shift to an herbicide resistant population of weeds has ecological consequence only if the resistant population cannot be controlled with other herbicides or other control practices. This is generally not the case. Many hundreds of cases of resistant weeds have been documented worldwide, but resistance is usually not a limiting factor for crop production.

In spite of the evolution of herbicide resistance in weed populations, US growers continue to have many herbicides and management options for weed control. Even so, growers must always be concerned about herbicide sustainability and the economic consequences of losing any herbicide technology due to the evolution of resistant weeds.

Table 9.1. Herbicide Resistant Weeds Summary Table (Heap, 2010b)

Herbicide Group	Site of Action	Example Herbicide	Total Number of Biotypes
ALS inhibitors	Inhibition of acetolactate synthase ALS (acetohydroxyacid synthase AHAS)	Chlorsulfuron	107
Photosystem II inhibitors	Inhibition of photosynthesis at photosystem II	Atrazine	68
ACCase inhibitors	Inhibition of acetyl CoA carboxylase (ACCase)	Diclofop-methyl	37
Synthetic Auxins	Synthetic auxins (action like indoleacetic acid)	2,4-D	28
Bipyridiliums	Photosystem-I-electron diversion	Paraquat	25
Ureas and amides	Inhibition of photosynthesis at photosystem II	Chlorotoluron	21
Glycines	Inhibition of EPSP synthase	Glyphosate	20
Dinitroanilines and others	Microtubule assembly inhibition	Trifluralin	10
Thiocarbamates and others	Inhibition of lipid synthesis - not ACCase inhibition	Triallate	8
PPO inhibitors	Inhibition of protoporphyrinogen oxidase (PPO)	Oxyfluorfen	4
Triazoles, ureas, isoxazolidiones	Bleaching: Inhibition of carotenoid biosynthesis (unknown target)	Amitrole	4
Chloroacetamides and others	Inhibition of cell division (Inhibition of very long chain fatty acids)	Butachlor	4
Nitriles and others	Inhibition of photosynthesis at photosystem II	Bromoxynil	3
Carotenoid biosynthesis inhibitors	Bleaching: Inhibition of carotenoid biosynthesis at the phytoene desaturase step (PDS)	Flurtamone	2
Arylaminopropionic acids	Unknown	Flamprop-methyl	2
4-HPPD inhibitors	Bleaching: Inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD)	Isoxaflutole	1
Mitosis inhibitors	Inhibition of mitosis / microtubule polymerization inhibitor	Propham	1
Cellulose inhibitors	Inhibition of cell wall (cellulose) synthesis	Dichlobenil	1
Organoarsenicals	Unknown	MSMA	1
Total number of resistant biotypes			348

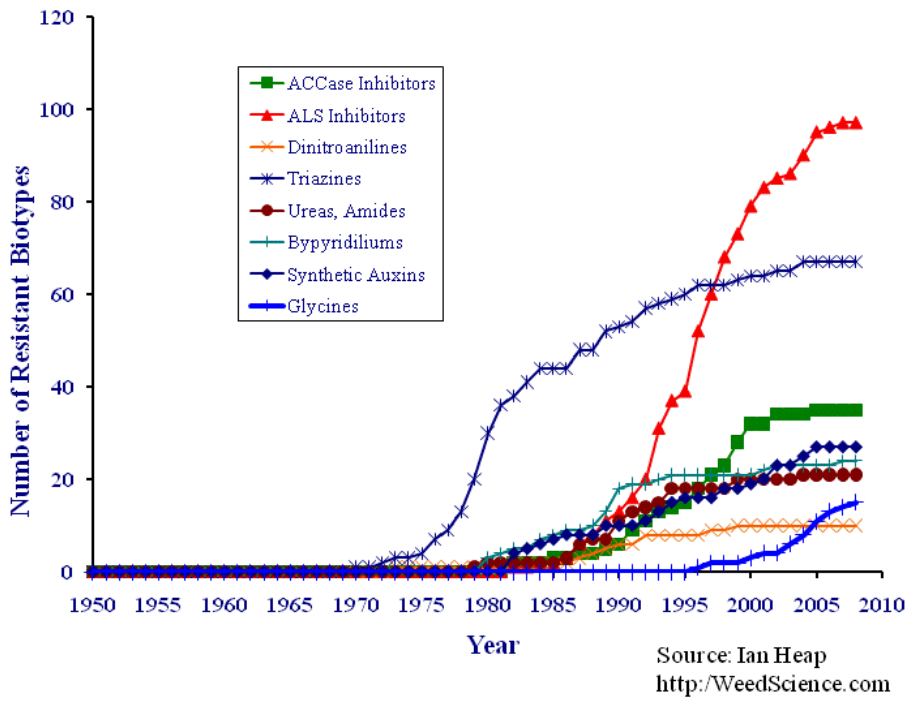

Figure 9.1. Number of Resistant Biotypes of Weeds by Herbicide Class and Year (Heap, 2010d)

9.2. Characteristics of Glyphosate

Glyphosate is a broad spectrum herbicide that was introduced in the 1970s for management of annual, perennial and biennial herbaceous grasses, sedges, and broadleaves, as well as woody brush and trees (Franz *et al*., 1997).

Glyphosate controls plants by inhibiting the enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase). EPSPS is an essential enzyme in the shikimate pathway that ultimately leads to the production of aromatic amino acids (tryptophan, tyrosine, and phenylalanine). The shikimate pathway for synthesizing aromatic amino acids, and therefore the enzyme EPSPS, is found in plants, bacteria and fungi, but not animals.

The structure of glyphosate resembles the structure of the substrate for EPSPS, which is phosphoenolpyruvate (PEP). Therefore, glyphosate competes with PEP for the enzyme's active site and prevents conversion of PEP to the precursor that is required in the synthesis of aromatic amino acids. Aromatic amino acids are essential for many plant processes such as protein synthesis, cell wall formation, pathogen defense and hormone production. At high rates, glyphosate is toxic to virtually all plants.

9.3. Evolution of Resistance to Glyphosate

For more than two decades, the evolution of glyphosate resistance was not perceived as a problem (Bradshaw *et al*., 1997). This opinion was based on the difficulty of discovering a fully functional 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that was insensitive to glyphosate, the inability of plant species to enzymatically deactivate glyphosate, the lack of soil activity of glyphosate (thus reducing the selection pressure due to exposure), and the empirical observation that no resistant weeds had appeared after at least 20 years of use.

Known theoretical ways for weeds to develop herbicide resistance did not seem likely for glyphosate (Jasieniuk, 1995). Over-production of the EPSPS target site did not increase resistance enough for plants to survive glyphosate amounts used in agricultural settings (Kishore and Shah, 1988). The EPSPS modifications that conferred glyphosate resistance in bacteria were inside the enzyme's active site, which reduced its catalytic efficiency and thus would probably reduce plant fitness (Padgette *et al*., 1995). No higher plants could be found metabolically inactivate glyphosate (Dyer, 1994).

However, the views about the ability of weeds to evolve glyphosate resistance changed in 1996 when glyphosate-resistant *Lolium rigidum*, was discovered in Australia (Powles *et al*., 1998; Pratley *et al*., 1999). Since then, glyphosate-resistant biotypes in at least 19 other weed species have been confirmed globally (Heap, 2010a), and it is estimated that more than several million acres are now affected by glyphosate resistant weeds (Owen, 2010).

Another economically significant glyphosate-resistant weed is horseweed (*Conyza canadensis*); it was confirmed in 2000 as the first annual broadleaf species with glyphosate resistance (Heap, 2010a; VanGessel, 2001). Currently, glyphosate-resistant horseweed is estimated to be infesting over several million acres in the U.S. across 17 states (Heap, 2010a).

The molecular basis for weed resistance to glyphosate is not understood for all weeds that have been identified, though some mechanisms have been elucidated. Initial studies of various weed species revealed EPSPS target site insensitivity (Baerson *et al*., 2002). Target site-based resistance has been identified for some weed species, including *Eleusine indica*, *Lolium multiflorum*, and *Lolium rigidum* (Powles and Preston, 2006). Differences in translocation and transport to the chloroplast are also important in some weeds, such as horseweed (*Conzya canadensis*) and *Lolium rigidum* (Feng *et al*., 2004; Lorraine-Colwill *et al*., 2003; Powles and Preston, 2006). Because weed populations can be diverse genetically, any particular weed species may evolve different mechanisms of resistance, as is the case for *Lolium rigidum* (Powles and Preston, 2006). Most recently, EPSPS gene duplication and increased gene expression has been hypothesized to explain glyphosate resistance in *Amaranthus palmeri* (Gaines *et al*., 2010).

The effectiveness, economic benefits, and ease of using glyphosate have led to repeated applications, year after year, in areas where glyphosate tolerant biotech crops are grown. The practice of continuously planted glyphosate-resistant soybean was implicated in the development of glyphosate-resistant horseweed in Delaware (VanGessel, 2001). This intensive use has resulted in a high selection pressure for weeds that inherently are difficult to control with glyphosate (Culpepper, 2004). Eventually, this selection pressure can lead the spectrum of weeds in the fields to a shift to those weeds that inherently can tolerate glyphosate.

As predicted, spectrum shifts to weed populations with endogenous glyphosate resistance have occurred more rapidly than evolved resistance in response to glyphosate exposure (Shaner, 2000). For example, in Iowa, common waterhemp (*Amaranthus rudis*) and velvetleaf (*Abutilon theophrasti*) became a concern in glyphosate-resistant soybean soon after crop commercialization (Owen, 1997).

9.4. Stewardship of Herbicide Tolerant 73496 Canola

In order to best manage their weed populations, growers understandably need to know where to obtain information on whether herbicide resistant weed biotypes have been reported in their area. It is important to understand that when weeds develop resistance to an herbicide, they do so under specific circumstances (heavy reliance on a single herbicide mode of action) at a specific location. Weeds that develop resistance to herbicides are referred to as "biotypes" to indicate that only a sub-type of the species has developed resistance. If the resistant weed biotypes are controlled effectively by mechanical means or use of alternative mode-of-action herbicides, the problem may be limited to a specific locality in a specific year.

Growers can obtain information about resistant weed biotypes reported in their area from many sources, including their local crop protection chemical dealers, crop protection chemical company representatives,

their state Department of Agriculture, University Extension Services, and local crop consultants. The most timely and location specific information can likely be provided by their county extension agent, as well as their local seed and/or chemistry providers. The USDA Cooperative Extension System offices are staffed by one or more experts who provide practical, research-based information to agricultural producers and others in rural areas and communities of all sizes. Some of this information is also available to growers from the university extension services, such as the University of Minnesota Extension (Gunsolus, 2002) and North Dakota State University (http://www.ag.ndsu.edu/weeds/herbicide-resistant-weeds).

The Glyphosate, Weeds, and Crops Website (http://www.glyphosateweedscrops.org/), a website devoted to glyphosate stewardship, also develops and compiles member publications and news reports on managing glyphosate-resistant weeds. DuPont is one member of this website and membership also includes major universities and agricultural institutions.

The weed science community is also a valuable source of information about resistant weed biotypes that have been reported (International Survey of Herbicide Resistant Weeds, www.weedscience.org). They also monitor the evolution of herbicide resistant weeds. However, because the information posted on their website relies on extension agents and weed scientists to self-report, it may not be completely updated with information for the grower's local area.

DuPont’s Pioneer Hi-Bred International, Inc. and Crop Protection Chemicals businesses both have long histories of product stewardship. Examples of these efforts are detailed below.

9.4a. Local Weed Management

In collaboration with university investigators, private consultants, other manufacturers, and growers, DuPont Crop Protection field development personnel conduct hundreds of field trials annually to refine existing recommendations and to investigate potential new active ingredients and herbicide combinations deployed in an Integrated Weed Management context to improve solutions for existing and emerging weed problems. Within Pioneer, field agronomists give presentations to local audiences about resistant weeds and best management practices. In addition, they assist customers in making crop management decisions, including options for managing weeds.

9.4b. Product Labeling

For at least 20 years, all DuPont herbicide labels have carried voluntary statements regarding resistant weed management (Figure 9.2). These labels actively promote an integrated weed management philosophy as seen in the second paragraph:

RESISTANCE

When herbicides that affect the same biological site of action are used repeatedly over several years to control the same weed species in the same field, naturally-occurring resistant biotypes may survive a correctly applied herbicide treatment, propagate, and become dominant in that field. Adequate control of these resistant weed biotypes cannot be expected. If weed control is unsatisfactory, it may be necessary to retreat the problem area using a product affecting a different site of action.

To better manage herbicide resistance through delaying the proliferation and possible dominance of herbicide resistant weed biotypes, it may be necessary to change cultural practices within and between crop seasons such as using a combination of tillage, retreatment, tank-mix partners and/or sequential herbicide applications that have a different site of action. Weed escapes that are allowed to go to seed will promote the spread of resistant biotypes.

It is advisable to keep accurate records of pesticides applied to individual fields to help obtain information on the spread and dispersal of resistant biotypes. Consult your agricultural dealer, consultant, applicator, and/or appropriate state agricultural extension service representative for specific alternative cultural practices or herbicide recommendations available in your area.

Figure 9.2. Resistant Weed Management Label

9.4c. Training and Education of Sales Representatives and Agronomists

DuPont Crop Protection: In addition to the formal academic training of technical, marketing, and sales professionals, DuPont Crop Protection has a mandatory on-line interactive training course with intensive and comprehensive coverage of product stewardship generally and herbicide resistance specifically. This training is administered globally to all employees involved in weed management recommendations and is also provided to other interested parties as a community education contribution. The technical, marketing, and sales professionals are required to be certified in this training by receiving a passing grade of 90% or higher in each of the eight modules. A person unfamiliar with this topic is expected to take about twelve hours to complete the internal version of the training. This is the first training of its kind to be awarded Certified Crop Adviser (CCA) credits by the American Society of Agronomy. The CCA standard allows growers, employers, and other organizations to help manage risk by enabling them to have assurance that a person has the appropriate professional qualifications.

Pioneer: The Pioneer sales force, made up of agronomists, account managers and sales representatives, and customers receive ongoing mandatory and voluntary stewardship training throughout the sales season through a variety of tactics. These include stewardship training sessions administered in person or communicated electronically by Pioneer Stewardship personnel. The Pioneer sales force also utilizes a proprietary computer software system to access information about products, traits, crop management practices, sales transactions and required stewardship documentation. This formal training is in addition to the practical experience of the Pioneer sales force working with growers on the positioning and use of products containing biotech traits, including herbicide resistant traits.

9.4d. Technical Bulletins Provided to Seed Customers and the Public

Direct Mail

Pioneer's Growing Point magazine is mailed to all Pioneer customers, prospects and employees. This magazine contains information about products, marketing programs, production and management practices and advice on agronomic application of traits and technologies.

Information on Websites

- Pioneer's "Growing Point" site (https://www.pioneer.com/growingpoint/login/login.jsp) has an extensive agronomy section, with access to information about many different crops and crop management practices. Technical bulletins such as "Crop Insights" have been published for the past 15 years, and are a good example of how Pioneer makes the latest information available to growers.

- The DuPont Biotechnology website (http://www2.dupont.com/Biotechnology/en_US/) contains an in-depth "Science Knowledge" section addressing "Herbicide Resistant Crops and Weed Management: Scientific Summary and the DuPont Perspective", with a section devoted to Integrated Weed Management, as well as an FAQ section that addresses weed control and Integrated Weed Management. There is also a Scientific Summary on Integrated Weed Management and Herbicide Resistance: (http://www2.dupont.com/Biotechnology/en_US/science_knowledge/herbicide_resistance/moreinfo8.html).

9.4e. Involvement in Industry Groups

DuPont Crop Protection is a long-standing participant in the Herbicide Resistance Action Committee (HRAC), an industry-based group supported by Croplife International. Their stated mission is to "Facilitate the effective management of herbicide resistance by fostering communication and co-operation between industry, government and farmers." They work towards fostering responsible attitudes towards herbicide use, communicating herbicide resistance management strategies and support their implementation through practical guidelines, and they seek active collaboration with public and private

researchers, especially in the areas of problem identification and devising and implementing management strategies.

9.4f. Involvement with Academic Groups

Pioneer and DuPont personnel interact with academic weed scientists in conducting trials at university sites as well as seeking input from them regarding weed management strategies.

9.4g. Customer Satisfaction and Weed Resistance Management Plan

Pioneer and DuPont Crop Protection are committed to active market presence wherever we sell our products. Consistent with our stewardship principles, all of our business teams are required to maintain an active contact with customers and awareness of end-user practices and a capability to respond rapidly to issues arising. DuPont Crop Protection maintains a customer satisfaction database to track and address any complaints, including for resistant weeds. Field employees are trained and provided tools and processes for responding to inquiries regarding product performance (or any potential impacts on human health or environment).

Appendix 10. Environmental Report on the Deregulation of 73496 Canola

After consultation with USDA-APHIS BRS Environmental Risk Analysis Program, Pioneer is electing to submit information related to the NEPA analysis for 73496 canola at a later time. This will ensure that the most recent acreage and marketing trends, as well as the most recent literature and discussion of timely issues related to herbicide-tolerant canola, can be included. The following outline provides a brief summary of the discussion topics for evaluating the environmental impact of the deregulation of 73496 canola. Full information in this section will be submitted when this petition has been evaluated for technical completion or upon USDA request. Pioneer welcomes suggestions from USDA on additional topics that would be helpful for the development of a draft Environmental Assessment.

10.1. Overview of U.S. Canola Production, Market, and Trade

1. U.S. canola acreage, primary states, and trends
2. U.S. imports and exports of canola and canola processed products
3. Presence of HT canola in the marketplace today

10.2. Potential Impact of the Introduction of 73496 Canola on U.S. Canola Production, Market, and Trade

1. U.S. canola acreage and primary states of cultivation
2. U.S. imports and exports of canola and canola processed products
3. Presence of HT canola in the marketplace today

10.3. Current Agronomic Practices for Canola in the U.S.

1. Rotational practices
2. Other agronomic practices (Tillage, Irrigation)
3. Weeds and weed management
4. Herbicide Use
5. Other agricultural chemical use (pesticide, fertilizer)
6. Volunteer management
7. Identity Preservation/ “co-existence”

10.4. Potential Impact of the Introduction of 73496 Canola on Agronomic Practices

1. Rotational practices
2. Other agronomic practices (tillage, irrigation)
3. Weeds and weed management
4. Herbicide Use
5. Other agricultural chemical use (pesticide, fertilizer)
6. Volunteer management
7. Identity Preservation/ “co-existence”

10.5. Additional Potential Impacts of the Introduction of 73496 Canola

10.5.1. Potential Impact on Farming Operations: Organic, Conventional (non-GE), Specialty Products, and Small/Large Farms

1. Organic farming choice/impact
2. Conventional Farming (non-GE) choice/impact
3. Specialty products/IP systems (e.g. condiment mustard, HEAR)

10.5.2. Potential Impacts on Raw or Processed Agricultural Commodities

10.5.3. Potential Impact on Animal and Plant Communities and Biodiversity

10.5.4. Potential Impact on Threatened or Endangered Species

10.5.5. Potential Impact on Water Use, Soil, and Air Quality

10.6. Cumulative Impacts

10.6.1. 73496 Canola Stacking with Other Transgenic Canola

1. Other events on the market and availability-primary consideration would be stacks with herbicide tolerance traits
2. Change in weediness characteristics with stacked herbicide traits
3. Change in gene flow with stacked herbicide traits
4. Change in agronomic practices with stacked herbicide traits
5. Change in herbicide use with stacked herbicide tolerance traits
6. Change in weed resistance development with stacked herbicide traits

10.6.2. Other Glyphosate Tolerant Crops on the Market

Appendix 11. GAT4621 New Protein Consultation

PIONEER.
A DuPont Company

Early Food Safety Evaluation for a Glyphosate N-Acetytransferase Protein: GAT4621

Submitting Company:

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PO Box 1014
Johnston, IA 50131-1014

Submitted by:

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January 31, 2007

No CBI

Table of Contents

Key to Abbreviations 3

1. Name, Description and Function of GAT4621 4
2. Description of the Intended Effect of the GAT4621 Protein 5
3. Identity and Source of Introduced Genetic Material 5
4. Assessment of Allergenicity Potential of GAT4621 8
    - 4.1. Amino Acid Sequence Homology of GAT4621 to Known Protein Allergens 8
    - 4.2. Lability of GAT4621 to Pepsin in Simulated Gastric Fluid (SGF) 8
    - 4.3. Lability of GAT4621 to Pancreatin in Simulated Intestinal Fluid (SIF) 10
    - 4.4 Glycosylation Analysis of the GAT4621 Sequence 12
    - 4.5 GAT4621 Gene Source and History of Exposure 12
    - 4.6. Conclusions on the Allergenicity Potential of GAT4621 12
5. Assessment of Toxicity Potential of GAT4621 12
    - 5.1. Assessment of Amino Acid Homology of GAT4621 to Known Protein Toxins 12
    - 5.2. Results of Mouse Acute Oral Study Using GAT4621 13
    - 5.3. Conclusions on the Toxicity Potential of GAT4621 14
6. Information on History of Safe Consumption of GAT Proteins in Food 14
7. Overall Conclusions 14
8. References 16

Key to Abbreviations

~	approximately
ATCC	American Type Culture Collection
BAR	phosphinothricin acetyltransferase from *Streptomyces hygroscopicus*
B. licheniformis	*Bacillus licheniformis*
DNA	deoxyribonucleic acid
E score	expectation score
E. coli	*Escherichia coli*
GAT	glyphosate N-acetyltransferase
GAT4601	specific GAT protein from 7th round of gene shuffling
gat4601	specific *gat* gene from 7th round of gene shuffling
GAT4621	specific GAT protein from 11th round of gene shuffling
gat4621	specific *gat* gene from 11th round of gene shuffling
GNAT family	GCN5-related family of N-acetyltransferases
ILSI	International Life Sciences Institute
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
NCBI	National Center for Biotechnology Information
OECD	Organisation for Economic Cooperation and Development
PAT	phosphinothricin acetyltransferase from *Streptomyces viridochomogenes*
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SIF	simulated intestinal fluid

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

1. Name, Description and Function of GAT4621

GAT4621 is a glyphosate acetyltransferase protein based on N-acetyltransferase protein sequences from *Bacillus licheniformis*, a gram positive saprophytic bacterium that is widespread in nature. The GAT4621 protein is 147 amino acids in length and has an approximate molecular weight of 17 kDa (Figure 1).

Figure 1. Amino Acid Sequence of the GAT4621 Protein

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

The GAT4621 protein is very similar to the GAT4601 protein, which was the subject of New Protein Consultation 003 submitted to FDA on June 16, 2006. The GAT4601 protein is present in Pioneer's 356043 soybean, which was the subject of BNF0108 submitted to FDA on November 16, 2006.

GAT4601 and GAT4621 are 91% identical and 96% similar (Figure 2). Shaded amino acids represent conservative amino acid differences between GAT4601 and GAT4621, and boxed amino acids represent non-conservative differences. The GAT4621 protein has higher catalytic efficiency for glyphosate than GAT4601, as discussed below on pp. 6-7.

Figure 2. Sequence Comparison of GAT4621 and GAT4601

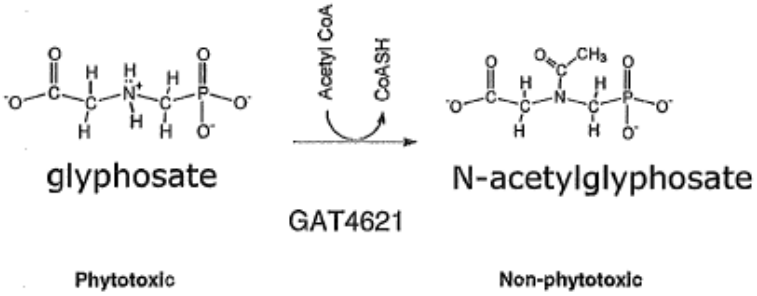
```
                 1                                                 50
GAT4621     (1)  MAIEVKPINAEDTYDLRHRVLRPNQPIEACMFESDLTRSAFHLGGFYGGK
GAT4601     (1)  -MIEVKPINAEDTYELRHRILRPNQPIEACMFESDLLRGAFHLGGFYRGK

                 51                                               100
GAT4621    (51)  LISVASFHQAEHSELQGKKQYQLRGVATLEGYREQKAGSSLVKHAEEILR
GAT4601    (50)  LISIASFHQAEHSELQGQKQYQLRGMATLEGYREQKAGSTLVKHAEEILR

                 101                                          147
GAT4621   (101)  KRGADMIWCNARTSASGYYRKLGFSEQGEVFDTPPVGPHILMYKRIT
GAT4601   (100)  KRGADMLWCNARTSASGYYKKLGFSEQGEIFDTPPVGPHILMYKRIT
```

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 a highly conserved GNAT motif but have high sequence diversity (Vetting *et al.*, 2005). GNAT proteins are known to have a number of metabolic functions including detoxification (Dyda *et al.*, 2000).

2. Description of the Intended Effect of the GAT4621 Protein

Expression of the GAT4621 protein in transgenic crops provides tolerance to the broad spectrum herbicide glyphosate. The GAT4621 protein detoxifies glyphosate to the non-herbicidal form N-acetylglyphosate (Figure 3). This detoxification mechanism is similar to that of the phosphinothricin acetyltransferase (PAT or BAR) enzymes from *Streptomyces*, which detoxify phosphinothricin- or bialaphos-based herbicides by adding an acetyl group (De Block *et al.*, 1987).

Figure 3. Enzymatic Activity of GAT4621



3. Identity and Source of Introduced Genetic Material

In order to develop a GAT protein that would confer commercial levels of tolerance to the herbicide glyphosate when expressed in plants, an in-house collection of several hundred *Bacillus* isolates was screened. A mass spectrometry method was developed to detect low levels of N-acetylglyphosate, the non-phytotoxic end product of glyphosate acetylation. The *Bacillus* isolates were grown to stationary phase, permeabilized, and incubated with glyphosate and acetyl coenzyme A, and the supernatants were screened for the presence of N-acetylglyphosate by mass spectrometry. Several strains of *Bacillus licheniformis* exhibited GAT activity and had the greatest reproducible accumulation of N-acetylglyphosate (http://www.isb.vt.edu/articles/sep0403.htm and Castle *et al.*, 2004).

To isolate the gene encoding GAT, recombinant *E. coli* expressing genomic DNA fragments from *B. licheniformis* were assayed by the mass spectrometry method. DNA sequences of multiple genomic fragments specifying GAT activity from *B. licheniformis* strain B6 and *B. licheniformis* strain 401 (purchased from ATCC—catalog number 14580) were determined, and the corresponding genes were cloned. A polymerase chain reaction (PCR) survey of *B. licheniformis* strains revealed a third gene variant in isolate DS3. The B6, 401 and DS3 *gat* genes were used as parents for fragmentation-based multigene shuffling to create enzymes with improved activity on the substrate glyphosate.

DNA 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.

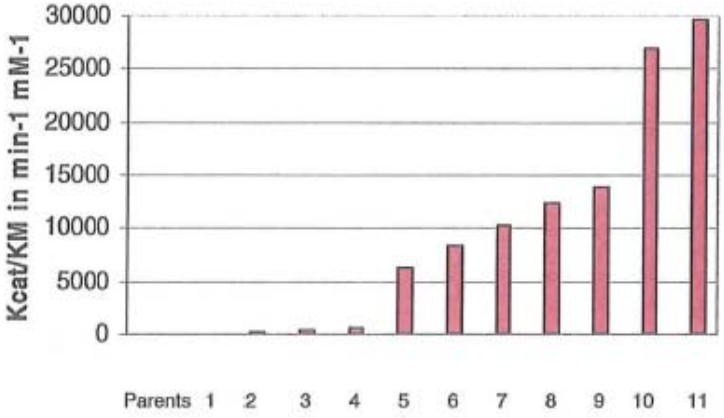
To initiate the first round of gene shuffling, the three native *B. lichenifomis gat* genes were used as parental templates. The initial diversity represented among the three native GAT protein sequences occurred at 12 of the 146 total amino acid positions. Libraries of shuffled gene variants were created, expressed in *E. coli*, and screened. Shuffled variants that resulted in the accumulation of more N-acetylglyphosate than the parental controls were selected for further rounds of shuffling. In each round of DNA shuffling, approximately 5,000 gene variants were screened and 24-48 purified enzymes were analyzed to determine their kinetic properties. Typically, three to twelve improved variants exhibiting a high k_{cat}, a low K_M, or a high k_{cat}/K_M ratio were chosen to be the parents for the next round.

In enzyme kinetics, k_{cat} is a measure of the turnover rate or speed of the reaction. The higher the k_{cat}, the faster the enzyme reaction. K_M is the affinity of the enzyme for a substrate or tightness of binding of the substrate to the enzyme. The lower the K_M, the greater the affinity of the enzyme for the substrate. A k_{cat}/K_M ratio is the common way to express the catalytic efficiency of the enzyme.

Several GAT enzymes from the third round of gene shuffling had rate constants (k_{cat}/K_M ratios) about 100-fold improved over the original enzymes, but the gene variants were unable to confer glyphosate tolerance to transgenic plants. At the fifth round of shuffling, two advances were made: 1) the additional introduction of diversity by PCR incorporation of oligonucleotides based on related DNA sequences from *Bacillus cereus* and *Bacillus subtilis* during the fragment reassembly step that allowed for substitutions at 27 amino acid residues; and 2) a functional pre-screen based on resistance of GAT-expressing *E. coli* to glyphosate (Castle *et al.*, 2004). At the sixth round of shuffling, a spectrophotometric assay was used that allowed the variants to be screened for relative k_{cat} and K_M values without protein purification. At the end of the seventh round of gene shuffling, the *gat4601* gene encoding the GAT4601 protein was identified. An additional oligonucleotide incorporation of sequence diversity based on acetyltransferase proteins with 30-60% identity to GAT was introduced in the eighth round of shuffling.

At the end of the eleventh round of gene shuffling, the rate constant of the best round 11 variant analyzed was approximately 7000-fold improved over the native enzymes (Castle *et al.*, 2004; Siehl *et al.*, 2005). The average k_{cat}/K_M of the parental enzymes was 4.2 min^{-1} mM^{-1}, and the average of the best round 11 variant was approximately 29,600 min^{-1} mM^{-1} (Figure 4).

Figure 4. Kinetic Improvement of GAT Enzymes Through Shuffling



Taken from Siehl *et al.*, 2005.

In order to optimize the gene from the best round 11 variant for plant expression, codon changes in the gene sequence were made to eliminate rare plant codons. These changes did not alter the encoded protein sequence. Additionally, a GCT codon for alanine was inserted at amino acid position 2. This, along with a plant promoter, resulted in a consensus translation initiation site thought to be best for protein production in plants (Joshi *et al.*, 1997). The plant-optimized gene was given the designation *gat4621*.

The GAT4621 protein, encoded by the *gat4621* gene, is 75-78% identical and 90-91% similar at the amino acid level to each of the three native GAT enzymes from which it was derived, compared to 94% identity of each of the native enzymes to each other (Table 1). There are 32-36 amino acid changes (22-23 of which are conservative) between the shuffled GAT4621 protein and any one of the original three native GAT proteins.

Table 1. Comparison of Sequence Identity Between Parental GAT and GAT4621 Proteins

	GAT from strain 401	GAT from strain B6	GAT from strain DS3	GAT4621
GAT from strain 401	100%	94% identical	94% identical	78% identical 91% similar
GAT from strain B6		100%	94% identical	76% identical 91% similar
GAT from strain DS3			100%	75% identical 90% similar
GAT4621				100%

4. Assessment of Allergenicity Potential of GAT4621

No single factor has been recognized as the primary indicator for allergenic potential, and no validated animal model that is predictive of allergenic potential is available. Therefore, a weight-of-evidence approach, which takes into account a variety of relevant factors and experimental observations used to derive an overall assessment of the allergenic potential of a novel protein, was applied to evaluating the allergenic potential of the GAT4621 protein (Codex, 2003). The allergenicity potential assessments are typically based on what is known about food allergens, including the history of exposure and safety of the gene(s) source; molecular structure of the proteins (*e.g.*, amino acid sequence identity to known human allergens); physicochemical properties such as stability to pepsin digestion *in vitro* (Thomas *et al.*, 2004) or other enzymes such as pancreatin; glycosylation status, and an estimate of the exposure of the novel protein(s) to the gastrointestinal tract where absorption occurs (*e.g.*, digestibility, protein abundance in the crop, and food/feed processing effects).

The allergenic potential of GAT4621 was assessed by: 1) bioinformatic comparison of the amino acid sequence of the GAT4621 protein with known protein allergen sequences; 2) evaluation of the stability of the microbially produced and purified GAT4621 protein from *E. coli* using *in vitro* gastric and intestinal digestion models; 3) glycosylation analysis of the GAT4621 sequence; and 4) assessment of the *gat4621* gene source and history of use or exposure.

4.1. Amino Acid Sequence Homology of GAT4621 to Known Protein Allergens

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 (Version 7.0, January 2007), which contains the amino acid sequences of known and putative allergenic proteins. Potential identities between the GAT4621 protein and proteins in the allergen database were evaluated using the FASTA34 sequence alignment program (Pearson and Lipman, 1988) set to the default parameters (word size = 2, scoring matrix = BLOSUM50, gap creation penalty = -10, gap extension penalty = -2, *E* score cutoff = 10). The resulting alignments were returned and reviewed for identities greater than or equal to 35% over 80 or greater residues. None of the alignments met or exceeded the 35% 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. The use of a match of eight contiguous, identical amino acids appears to have some relevance based upon the minimum peptide length for a B cell-binding epitope (Metcalfe *et al.*, 1996). Results of the evaluation showed there were no eight or greater contiguous identical amino acid matches observed with the GAT4621 amino acid sequence.

4.2. Lability of GAT4621 to Pepsin in Simulated Gastric Fluid (SGF)

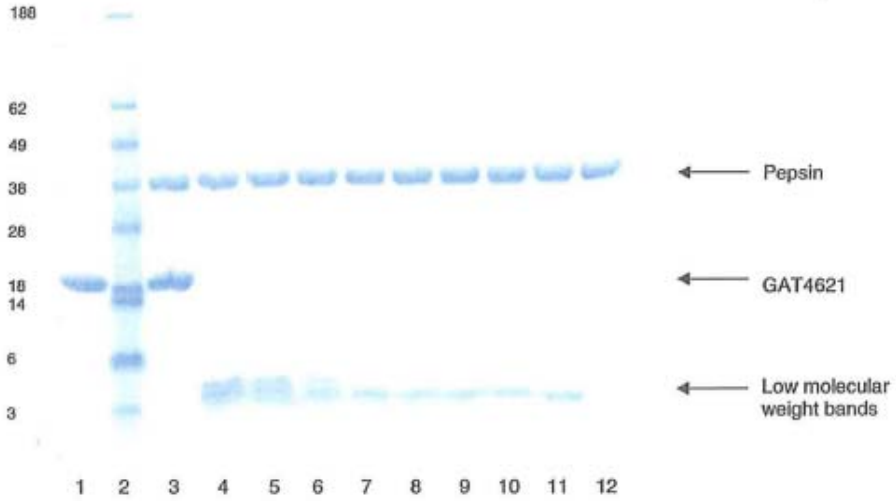
A factor that may increase the likelihood of allergic oral sensitization to proteins is the stability of the protein to gastro-intestinal digestion. Proteins that are highly digestible could be expected to have less opportunity to exert adverse health effects when consumed. The ability of food allergens to remain stable long enough to cross the mucosal membrane of the intestinal tract where absorption occurs is important in the context of a weight-of-evidence approach to understanding a protein's potential allergenic risk (Metcalfe *et al.*, 1996; FAO/WHO, 2001; Codex, 2003; Thomas *et al.*, 2004).

SGF was used to assess the susceptibility of microbially expressed and purified GAT4621 protein 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). The GAT4621, 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 ratio on a weight basis (Thomas *et al.*, 2004).

Results of the SGF study are shown in Figure 5. The GAT4621 parent protein was not detectable at 30 seconds in SGF (Figure 5, 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 5. Lability of GAT4621 to Pepsin in SGF: Scanned Image of SDS-PAGE Gel



Lane	Load Volume (µl)	Sample Identification
1	20	GAT4621 (~2.3 µg) water ~60 minutes
2	13	SeeBlue molecular weight marker
3	20	GAT4621 (~2.3 µg) "Time 0"
4	20	GAT4621 30 seconds
5	20	GAT4621 1 minute
6	20	GAT4621 2 minutes
7	20	GAT4621 5 minutes
8	20	GAT4621 10 minutes
9	20	GAT4621 20 minutes
10	20	GAT4621 30 minutes
11	20	GAT4621 60 minutes
12	20	SGF control (pepsin) ~60 minutes

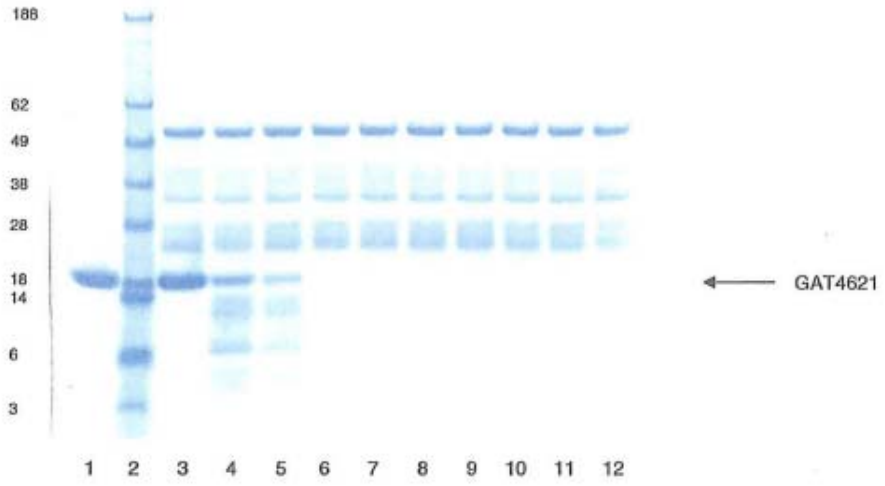
4.3. Lability of GAT4621 to Pancreatin in Simulated Intestinal Fluid (SIF)

The ability of food allergens to remain stable long enough to cross the mucosal membrane of the intestinal tract where absorption can occur is important in the context of a weight-of-evidence approach to understanding a protein's potential allergenic risk. In order to assess lability of the GAT4621 protein in the intestinal tract, microbially expressed and purified GAT4621 protein was incubated in SIF containing pancreatin prepared as described in the United States Pharmacopoeia (Anonymous, 1995) 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 an ~ 40:1 pancreatin to GAT4621 protein ratio on a weight basis. ß-lactoglobulin and BSA were used as controls (data not shown).

Porcine pancreatin contains many enzymes, including amylase, lipase and protease. In Figure 6, the mixture of proteins in pancreatin can be seen as multiple stained protein bands in lane 12.

Results of the SIF study are shown in Figure 6. The GAT4621 protein was not detectable at 5 minutes in SIF (Figure 6, 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 6. Lability of GAT4621 to Pancreatin in SIF: Scanned Image of SDS-PAGE Gel



Lane	Load Volume (µl)	Sample Identification
1	20	GAT4621 (~2.3 µg) water ~60 minutes
2	13	SeeBlue molecular weight marker
3	20	GAT4621 (~2.3 µg) "Time 0"
4	20	GAT4621 30 seconds
5	20	GAT4621 1 minute
6	20	GAT4621 2 minutes
7	20	GAT4621 5 minutes
8	20	GAT4621 10 minutes
9	20	GAT4621 20 minutes
10	20	GAT4621 30 minutes
11	20	GAT4621 60 minutes
12	20	SIF control (pancreatin) ~60 minutes

4.4 Glycosylation Analysis of the GAT4621 Sequence

Allergenic proteins are often glycosylated, but this relationship is not absolute, as numerous proteins that are not considered allergens are glycosylated, while some allergenic proteins are not. However, the absence of glycosylation, in the context of other weight-of-evidence data, provides additional support for the conclusion that a protein is non allergenic.

Microbially produced GAT4621 protein is not glycosylated (data not shown). Data are not yet available for plant-produced protein. However, the GAT4621 protein encoded by the plant transcription unit does not have the amino acid consensus sequence for N-linked glycosylation (asparagine, followed by any amino acid except proline, followed by serine or threonine). Therefore, the plant-expressed GAT4621 is not expected to be post-translationally modified with N-linked glycosylation.

4.5. GAT4621 Gene Source and History of Exposure

The *gat4621* gene, which codes for the GAT4621 protein, is derived from *Bacillus licheniformis*, a ubiquitous gram-positive soil bacteria that 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, protease, pullulanase), biocontrol agents (EU Commission; SCF/CS/ADD/AMI52; July 2000; USFDA; CFSAN; Office of Food Additive Safety; July 2001) and as a probiotic (Kritas *et al.*, 2006; Alexopoulos *et al.*, 2004a and b). *B. licheniformis* does not have a history of causing adverse effects (also see Section 6 below).

4.6. Conclusions on the Allergenicity Potential of GAT4621

Bioinformatic analyses revealed no similarities to known protein allergens for the GAT4621 protein sequence. None of the proteins identified met or exceeded the threshold of greater than or equal to 35% identity over 80 or greater residues. Furthermore, no contiguous stretches of eight or greater amino acids were shared between the GAT4621 protein and proteins in the allergen database. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the GAT4621 protein and known protein allergens. The GAT4621 protein was rapidly hydrolyzed in both simulated gastric and intestinal fluids. Further, the GAT4621 protein is not expected to be N-link glycosylated in plants and its source organism, *B. licheniformis*, has a history of safe use in the food industry. Taken together, these data support the conclusion that the GAT4621 protein is not a potential allergen.

5. Assessment of Toxicity Potential of GAT4621

The potential toxicity of GAT4621 was assessed by bioinformatic comparison of the amino acid sequence of the GAT4621 protein with publicly available protein sequences. Proteins most similar to GAT4621 were manually inspected to identify any that could be potentially toxic to humans or animals. A close match could be an indicator of toxicological potential of GAT4621. In addition, the acute oral toxicity of GAT4621 in mice (dosing via gavage) was evaluated.

5.1. Assessment of Amino Acid Homology of GAT4621 to Known Protein Toxins

A global sequence similarity search of the GAT4621 protein sequence against the NCBI Protein dataset was conducted using the BLASTp algorithm. A sequence file comprising the translation of the *gat4621* gene was queried using the BLASTP 2.2.13 algorithm against Release 157.0 (12/18/06) of the Genpept "nr" dataset, which incorporates non-redundant entries from all GenBank nucleotide translations along with protein sequences from SWISS-PROT (http://www.expasy.org/sprot/), PIR (http://pir.georgetown.edu/), PRF (http://www4.prf.or.jp/en/), and PDB (http://www.wwpdb.org/).

One of the most important parameters to monitor when performing similarity searches is the expectation, or *E* score. This *E* score represents the probability that a particular alignment is due to random chance and can be used to evaluate the significance of an alignment. The calculated *E* score depends on the overall length of the aligned sequences (including inserted gaps), the number of identical and conserved residues within the alignment, and the size of the database (Pearson and Lipman, 1988; Baxevanis, 2005). When examining an alignment between two protein sequences, a very low *E* score is more likely to reflect a true similarity while a high *E* score is more likely to be produced by chance and therefore less biologically relevant.

A cutoff expectation (*E*) score of 1.0 was used to generate biologically meaningful similarity between the GAT4621 protein and proteins in the NCBI GenPept database. Although a statistically significant sequence similarity generally requires a match with an *E* score of less than 0.01 (Pearson, 2000), a cutoff of $E < 1.0$ insures that proteins with even limited similarity will not be overlooked in the search. Low complexity filtering was turned off and the maximum number of alignments returned was set to 2000.

The GAT4621 similarity search identified 225 proteins that were within these criteria. The top three accessions represent GAT gene variants (Castle *et al.*, 2004), while the fourth accession is a closely related acetyltransferase protein from *B. licheniformis*. Thirty-seven other accessions represent putative or predicted acetyltransferases from other *Bacillus* species, such as *B. subtilis*, *B. cereus*, and *B. thuringiensis*. The remaining 184 matching accessions represent both known and putative acetyltransferase proteins from various bacterial, archaebacterial, and eukaryotic species.

None of the similar proteins returned by the search were identified as toxins, demonstrating that GAT4621 protein is unlikely to share relevant sequence similarities with known protein toxins and is therefore unlikely to be a toxin itself.

5.2. Results of Mouse Acute Oral Study Using GAT4621

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, 2001). 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.

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.

5.3. Conclusions on Toxicity Potential of GAT4621

Bioinformatic analyses revealed GAT4621 to be similar to other N-acetyltransferase proteins. No biologically relevant sequence similarities were observed between known protein toxins and the GAT4621 protein sequence. Along with the lack of acute toxicity in mice, these data support the conclusion that the GAT4621 protein is not acutely toxic.

6. Information on History of Safe Consumption of GAT Proteins in Food

The GAT4621 protein retains the characteristics found in other N-acetyltransferases that are ubiquitous in plants and microorganisms (Neuwald and Landsman, 1997). GAT4621 contains the definitive motif for the GNAT family of N-acetyltransferases (Marchler-Bauer *et al.*, 2005). This superfamily of enzymes is present in all organisms, including plants, mammals, fungi, algae, and bacteria (Dyda *et al.*, 2000).

Although GAT4621 is a optimized protein, it is 75-78% identical and 90-91% similar at the amino acid level to the translated protein sequences of each of the three original *gat* alleles from *B. licheniformis* from which *gat4621* was derived. 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. *B. licheniformis* was determined by EPA to present low risk of adverse effects to human health and the environment and was subsequently granted a TSCA section 5(h)(4) exemption (EPA, 1996).

7. Overall Conclusions

Using FDA's guidance for the early food safety evaluation of new proteins in new plant varieties that are under development, the GAT4621 protein was evaluated for its allergenicity and toxicity potential.

The allergenic potential of GAT4621 was assessed by: 1) bioinformatic comparison of the amino acid sequence of the GAT4621 protein with known protein allergen sequences; 2) evaluation of the stability of the microbially produced and purified GAT4621 protein from *E. coli* using *in vitro* gastric and intestinal digestion models; 3) N-linked glycosylation analysis of the GAT4621 sequence; and 4) assessment of the *gat4621* gene source and history of use or exposure.

Bioinformatic analyses revealed no similarities between known protein allergens and the GAT4621 protein sequence. Furthermore, no short (≥ eight amino acids) polypeptide matches were shared between the GAT4621 protein and proteins in the allergen database. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the GAT4621 and known allergens. The GAT4621 protein was rapidly hydrolyzed in both simulated gastric and intestinal fluids. Further, the GAT4621 protein is not expected to be N-link glycosylated in plants and its source organism, *B. licheniformis*, has a history of safe use in the

food industry. Taken together, these data support the conclusion that the GAT4621 protein is not a potential allergen.

Bioinformatic analyses revealed GAT4621 to be similar to other N-acetyltransferase proteins. No biologically relevant sequence similarities were seen between known protein toxins and the GAT4621 protein sequence. There was no evidence of acute toxicity in mice. These data support the conclusion that the GAT4621 protein is not acutely toxic.

The GAT4621 protein retains the characteristics found in other N-acetyltransferases that are ubiquitous in plants and microorganisms. GAT4621 contains the definitive motif for the GNAT family of N-acetyltransferases. This superfamily of enzymes is present in all organisms, including plants, mammals, fungi, algae, and bacteria (Dyda *et al.*, 2000). Although GAT4621 is a optimized protein, it is 75-78% identical and 90-91% similar at the amino acid level to the translated protein sequences of each of the three original *gat* alleles from *B. licheniformis* from which *gat4621* was derived. 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. *B. licheniformis* was determined by EPA to present low risk of adverse effects to human health and the environment and was subsequently granted a TSCA section 5(h)(4) exemption.

Based on the data and information provided in this submission, we have determined that the GAT4621 protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals.

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