Pioneer Petition (11-063-01p) for Determination of Non-regulated Status of 76496 Canola

OECD Unique Identifier:
DP-Ø73496-4

Plant Pest Risk Assessment

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A. Introduction

Pioneer Hi-Bred International, Inc. (referred hereafter as Pioneer) has petitioned the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) for a determination that the genetically engineered (GE) glyphosate herbicide-resistant[1] canola event DP-073496-4 **(**hereafter referred to as 73496 canola**)** is unlikely to pose a plant pest risk and, therefore, should no longer be a regulated article under the APHIS' 7 Code of Federal Regulations (CFR) part 340. This petition was assigned the number 11-063-01p, and is hereafter referenced as Pioneer 2011. APHIS administers 7 CFR part 340 under the authority of the plant pest provisions of the Plant Protection Act (PPA) of 2000 (7 U.S.C. 7701 *et seq.*)[2]. This plant pest risk assessment was conducted to determine if 73496 canola is unlikely to pose a plant pest risk.

APHIS regulations in 7 CFR part 340 regulate the introduction (importation, interstate movement, or release into the environment) of certain GE organisms and products. A GE organism is no longer subject to the plant pest provisions of the PPA or to the regulatory requirements of Part 340 when APHIS determines that it is unlikely to pose a plant pest risk. A GE organism is considered a regulated article under Part 340 if the donor organism, recipient organism, or vector, or vector agent used in engineering the organism belongs to any genera or taxa designated in 7 CFR 340.2 and is also considered a plant pest. A GE organism is also regulated under 7 CFR part 340 when APHIS has reason to believe that the GE organism may be a plant pest or APHIS does not have sufficient information to determine if the GE organism is unlikely to pose a plant pest risk. 73496 canola was produced by biolistic transformation of microspores (Pioneer 2011), and none of the introduced genetic sequences come from plant pest organisms listed in 7 CFR 340.2. Pioneer has conducted introductions of 73496 canola as a regulated article under APHIS-authorized notifications since 2007 (Table 6.1 of Appendix 6, p. 115 in Pioneer 2011), in part, to gather information to support that it is unlikely to pose a plant pest risk.

Potential impacts in this Plant Pest Risk Assessment are those that pertain to plant pest risk characteristics associated with 73496 canola and its progeny and their use in the absence of confinement. APHIS utilizes data and information submitted by the applicant, in addition to current literature, to determine if 73496 canola is unlikely to pose a plant pest risk. APHIS regulations in 7 CFR 340.6(c) specify the information needed for consideration in a petition for nonregulated status. APHIS will evaluate information

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[1] Pioneer has described the phenotype of DP-073496-4 canola as "herbicide tolerant" and historically APHIS has also referred to GE plants with reduced herbicide sensitivity as herbicide tolerant. However, the phenotype would fall under the Weed Science Society of America's definition of "herbicide resistance" since DP-073496-4 canola has an "inherited ability to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type" (WSSA 1998). By the WSSA definition, "resistance [to an herbicide] may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis." Herbicide tolerance, by the WSSA definition, only applies to plant species with an "inherent ability to survive and reproduce after herbicide treatment. This implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant."

[2] Plant Protection Act in 7 U.S.C. 7702 § 403(14) defines plant pest as: "Plant Pest - The term "plant pest" means any living stage of any of the following that can directly or indirectly injure, cause damage to, or cause disease in any plant or plant product: (A) A protozoan. (B) A nonhuman animal. (C) A parasitic plant. (D) A bacterium. (E) A fungus. (F) A virus or viroid. (G) An infectious agent or other pathogen. (H) Any article similar to or allied with any of the articles specified in the preceding subparagraphs."

submitted by the applicant related to plant pest risk characteristics, expression of the gene product, new enzymes, or changes to plant metabolism, weediness of the regulated article, impact on the weediness of any other plant with which it can interbreed, changes to agricultural or cultivation practices that may impact diseases and pests of plants, effects of the regulated article on nontarget organisms, indirect plant pest effects on other agricultural products, and transfer of genetic information to organisms with which it cannot interbreed.

APHIS may also consider information relevant to reviews conducted by other agencies that are part of the 'Coordinated Framework for the Regulation of Biotechnology' (51 FR 23302, June 26, 1986). Under the Coordinated Framework, the oversight of biotechnology-derived plants rests with the APHIS, the Food and Drug Administration (FDA), and the Office of Pesticide Programs of the U.S. Environmental Protection Agency (EPA). Depending on its characteristics, certain biotechnology-derived products are subjected to review by one or more of these agencies. The EPA under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), regulates the distribution, sale, use and testing of pesticidal substances produced in plants and microbes, including those pesticides that are produced by an organism through techniques of modern biotechnology. The EPA also sets tolerance limits for residues of pesticides on and in food and animal feed, or establishes an exemption from the requirement for a tolerance, under the Federal Food, Drug and Cosmetic Act (FFDCA). The FDA under the FFDCA is responsible for ensuring the safety and proper labeling of all plant-derived foods and feeds, including those developed through modern biotechnology. To help sponsors of foods and feeds derived from genetically engineered crops comply with their obligations, the FDA encourages them to participate in its voluntary consultation process. Pioneer (2011) has indicated that a New Protein Consultation for the GAT4621 protein was submitted to FDA on January 31, 2007 and completed on October 7, 2009, and that a safety and nutritional assessment for food and feed derived from 73496 canola was submitted to FDA on February 25, 2011 (Natalie Weber, Pioneer, personal communication). A food safety consultation, which found no safety concerns for 73496 canola, was completed by the FDA (BNF No. 129) on May 1, 2012. A copy of the text of the letter responding to BNF 129, as well as a copy of the text of FDA's memorandum summarizing the information in BNF 129, is available for public review via the FDA Completed Consultations on Bioengineered Foods page at www.fda.gov/bioconinventory. Submission of a tolerance petition and supporting residue data to the U.S. EPA to amend the glyphosate tolerance to include *N*-acetylglyphosate for canola was submitted on February 18, 2011.

B. Development of 73496 Canola

Canola (*Brassica spp*) is an oil seed crop primarily cultivated in China, India, Europe, and Canada and is becoming popular in the United States, South America and Australia, where annual production has increased sharply over the last few years (OECD 1997). There are two types of *B. napus*: 1) oil-yielding oleiferous rape, of which one subset with specific quality characteristics is often referred to as "canola" (vernacular name), and 2) the tuber-bearing swede or rutabaga. At present, three species of *Brassica* (*B. napus, B. rapa and B. juncea*) have commercialized varieties with "double low" characteristics, i.e.

low erucic acid content (<2%) in the fatty acid profile and very low glucosinolate content (<30 μ moles/g) in the air-dried oil-free meal, characteristics desirable for high-quality vegetable oil and high-quality animal feed (CCC 2003). In North America these species are considered to be of "canola" quality. *Brassica napus* is grown as a winter annual in regions where winter conditions do not result in very low temperatures, which would kill the plants. These biotypes typically require vernalization before the onset of stem elongation, raceme development, flowering and seed set. In North America and northern parts of Europe, spring biotypes of *B. napus* that require no vernalization prior to flowering are grown. The spring biotypes are typically lower yielding than the winter annual types, but require considerably less time to complete their life cycle (OECD 1997).

73496 canola (*B. napus*), a spring biotype, has been genetically modified to express the glyphosate acetyltransferase 4621 (GAT4621) protein. The GAT4621 protein, encoded by the *gat4621* gene, confers resistance to glyphosate-containing herbicides by acetylating glyphosate, thereby rendering it non-phytotoxic (Castle et al. 2004). As detailed later in this document, this mechanism is different from the other glyphosate herbicide resistance mechanism involving 5-enolpyruvylshikimate-3-phosphate synthase (*epsps)* gene.

According to Pioneer, 73496 canola was developed with glyphosate resistance to provide an alternative to existing herbicide-resistant canola products on the market and to enable growers to proactively manage weed populations in canola crops (p. 15 in Pioneer 2011).

Description of the genetic modifications

As described in the petition (p. 16, Pioneer 2011) 73496 canola was developed through biolistic transformation of microspores (Chen and Tulsieram 2007) of a donor canola line 1822B. Gold particles coated with the PHP28181A DNA fragment encoding the *gat4621* gene expression cassette were used for biolistic transformation (Klein et al. 1987). Following bombardment, transformed embryogenic tissue was selected on medium containing glyphosate and regenerated into whole plants. The PHP28181A fragment was constructed with a *gat4621* cassette containing the *UBQ10* promoter, *gat4621* gene, and *pin*II terminator (Figure 3, p. 19 in Pioneer 2011) as described below (Table 2, p. 20 in Pioneer 2011):

- Polylinker region: Short segment (7 base pairs) of DNA containing restriction sites for cloning genetic elements.
- Promoter: From *Arabidopsis thaliana UBQ10* polyubiquitin gene (Norris et al. 1993).
- Polylinker region: Short segment (23 base pairs) of DNA containing restriction sites for cloning genetic elements.
- Gene: Synthetic glyphosate *N*-acetyltransferase gene (Castle et al. 2004; Siehl et al. 2007).
- Polylinker region: Short segment (17 base pairs) of DNA containing restriction sites for cloning genetic elements.

- Terminator: Proteinase inhibitor II (*pin*II) gene terminator from *Solanum tuberosum* (Keil et al. 1986; An et al. 1989).
- Polylinker region: Short segment (6 base pairs) of DNA containing restriction sites for cloning genetic elements.

Pioneer provided evidence demonstrating that,

- the final product does not contain any of the backbone sequences from the plasmid PHP28181outside of the *gat4621* gene cassette borders of the transformation vector fragment PHP28181A, which was removed by restriction digest (Section V-B3, pp. 22-23 & Figure 14, p. 33 in Pioneer 2011);
- the DNA inserted into the 73496 canola genome is present at a single locus and contains one functional copy of *gat4621* gene (Table 4, p. 25 & Figures 8-13, pp. 27-32 in Pioneer 2011); and,
- the inserted *gat4621* expression cassette DNA (as determined through gene-specific PCR analysis) was stably inherited across five generations (Figure 2, p. 17 & Figures 8-13, pp. 27-32 in Pioneer 2011); plants that contain the inserted DNA also express the glyphosate herbicide resistance phenotype (as determined through a glyphosate herbicide spray injury assay), and the inserted DNA and resistance trait both segregate according to Mendel's laws of segregation consistent with the finding of a single insertion locus for the *gat4621* gene expression cassette (as determined by Chi-square test for 3 segregating populations) (Table 5, p. 35 in Pioneer 2011).

C. Expression of the Gene Product, Enzymes and Changes to Plant Metabolism

USDA-APHIS assessed whether changes in plant metabolism or composition in 73496 canola is likely to alter its plant pest risk. The assessment encompasses a consideration of the expressed protein or enzyme and its effect on plant metabolism and an evaluation of whether the nutrients and antinutrient levels in harvested seed derived from 73496 canola are comparable to those in the conventional canola control 1822B or to other reference canola cultivars considered for the composition analysis. Forage is not considered, as harvested vegetative canola biomass is never used as forage. Host plant quality (including such components as carbon, nitrogen, amino acid sources, trace elements, and defensive metabolites) is known to affect herbivore performance and fecundity; and higher-trophic level interactions, such as the performance of predators and parasitoids, may also be affected (reviewed by Awmack and Leather 2002). Similarly a vast array of secondary metabolites in plants is known to provide defense against microbes (Dixon, 2001). Thus APHIS assessed whether changes in host plant quality have the potential to affect 73496 canola's performance against pest and disease incidences.

The *gat4621* gene encodes GAT4621 protein, a variant of *N*-acetyltransferase protein sequences derived from *Bacillus licheniformis*. GAT4621 is 147 amino acids in length and has a molecular weight of 16.5 kDa (Figure 15, p. 36 in Pioneer 2011). *Bacillus*

licheniformis is a widespread saprophytic bacterium that substantially contributes to nutrient cycling (p. 36 in Pioneer 2011). The bacterium was never reported to be pathogenic to plants and is extensively used for large-scale industrial production of enzymes (Schallmey et al. 2004). The variant form of the gat4621 gene was produced using a DNA shuffling process employing three native gat genes from *B. licheniformis* as parental templates (Castle et al. 2004). The resulting GAT4621 protein in 73496 canola is 75-78% identical and 90-91% similar (identical amino acids plus functionally similar amino acids) at the amino acid level to each of the three native GAT enzymes from which it was derived (Table 6, p. 38 in Pioneer 2011). The modified GAT4621 enzyme exhibited a 7000-fold increase in catalytic activity on glyphosate substrate relative to the native enzymes (Siehl et al. 2005). In addition, the modified gene was optimized for plant expression to improve protein production in 73946 canola. The GAT4621 protein was found to be expressed throughout the plant, including roots and seed (p. 39 in Pioneer 2011), during the lifecycle of 73496 canola plants grown from up to six field trial locations in Canada and the United States (p. 102, Appendix 3 in Pioneer 2011). This was to be expected since expression of the gat4621 gene in 73496 canola is driven by the constitutive UBQ10 promoter from Arabidopsis.

GAT protein confers herbicide resistance by detoxifying the herbicide glyphosate to the non-phytotoxic, stable form, *N*-acetylglyphosate (Figure 16, p.37 in Pioneer 2011). This herbicide-resistance mechanism is different from the one involving EPSPS proteins. EPSPS has been identified as the primary target of glyphosate in GE plants. In the EPSPS-based herbicide resistance mechanism, an insensitive form of EPSPS either from a microbe (*e.g.*, CP4 EPSPS from *Agrobacterium tumefaciens*) or from plants (*e.g.*, modified EPSPS from *Zea mays*), provides protection against the glyphosate-based herbicides (Kishore and Shah 1988; Funke et al. 2006). On the contrary, in the GAT protein based herbicide resistance mechanism GAT enzymes acetylate the secondary amine of glyphosate producing the non-phytotoxic form, *N*-acetylglyphosate (Figure 16, p. 37 in Pioneer 2011; Castle et al. 2004). Furthermore, the GAT protein variant used in 73496 canola is optimized for acetylation of glyphosate (Siehl et al. 2007) and has a narrow substrate range for other agrochemicals (Castle et al. 2004; Siehl et al. 2005; Siehl et al. 2007).

Pioneer carried out a compositional assessment of 73496 canola and control canola following the OECD consensus document on compositional considerations for canola (OECD 2001). Because seed oil and meal are the primary commercial products, all compositional assessments come from seed samples. The samples for compositional assessment were collected from five locations comprising both the U.S. and Canadian experimental field sites during the 2009 growing season. The five field sites were chosen to represent the typical spring canola growing regions of the United States and Canada (p. 54, Figure 18, p. 46 & Figure 19, p. 82 in Pioneer 2011). The quantified analytes included proximates, fiber, fatty acids, amino acids, vitamins, minerals, glucosinolates, antinutrients, and secondary metabolites such as phytosterols. For comparative analysis, levels in 73496 canola (treated with glyphosate at a rate of 0.367-0.414 pounds acid equivalent per acre) were compared to corresponding levels in the near-isoline control (untreated) and to statistical tolerance intervals generated from five non-genetically modified conventional commercial varieties (Table 1, p. 18 in Pioneer 2011) grown at

five field locations in canola-growing areas of the U.S. and Canada in 2008 and 2009–the latter group was included in the analysis to establish a spectrum of normal variation for the measured analytes in 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, p. 56 in Pioneer 2011). Being an oilseed crop, canola seed is rich in fatty acids such as palmitic, stearic, oleic, linoleic and α-linolenic acids. In addition to the major fatty acids of canola, Pioneer also measured six additional fatty acids (Table 16, p. 58 in Pioneer 2011) following the Codex Alimentarius Commission definition of canola oil (CODEX 2005). Except for oleic and linoleic acids, none of the measured fatty acids showed significant differences between 73496 canola and control canola (Table 16, p. 58 in Pioneer 2011). The small, but statistically significant differences between 73496 canola and the control canola observed for oleic and linoleic acids were within the natural variation observed for commercial canola varieties.

A total of 18 amino acids and 26 free amino acids were measured for 73496 canola and control canola lines. No statistically significant differences were observed between the two for 18 amino acids (Table 17, p. 61 in Pioneer 2011). For 26 free amino acids, however, any statistical significant differences observed were within the tolerance interval estimated for canola varieties (data not shown).

In addition to acetylation of glyphosate, the GAT4621 enzyme also exhibits measureable activity (albeit very low relative to glyphosate) with five of the 21 amino acid substrates tested–aspartate, glutamate, glycine, serine and threonine (Appendix 7, p. 116-117 in Pioneer 2011). Concentrations of the five acetylated amino acids (*N*-acetylaspartate (NAA), *N*-acetylglutamate (NAG), *N*-acetylglycine (NAGly) *N*-acetylserine (NAS), *N*-acetylthreonine (NAT) were measured in seed samples and whole plant samples of 73496 canola and control canola. In the seed samples, 73496 canola had higher mean values for all five of these acetylated amino acids, especially NAA and NAG. Only NaGly levels were not significantly different from the control canola, but only NAA and NAG were outside (higher than) the tolerance interval for the commercial varieties (Table 22, p. 73 in Pioneer 2011). Likewise, except for NAS, acetylated amino acids NAA, NAG, NAGly, and NAT were significantly different (higher) in whole plant samples of greenhouse grown 73496 canola compared to controls (but tolerance intervals for whole plants in commercial canola were not provided) (Table 23, p. 74 in Pioneer 2011). The significant changes observed for the above-mentioned acetylated amino acids are unlikely to make 73496 canola more susceptible to pests and diseases, because the disease, insect pest, and agronomic data presented for both 73496 canola (discussed later in this document) and previously deregulated DP-Ø9814Ø-6 corn (USDA 2009) and DP-356043-5 soybean (USDA 2008) events with the *gat* gene did not indicate any significant difference for the aforementioned observations.

No statistically significant differences between 73496 and the control canola were observed for mean values for vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B3 (niacin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), and vitamin B9 (Table 18, p. 64 in Pioneer 2011). 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 (Table 18, p. 65 in Pioneer 2011), however, the differences were small in magnitude, and the range of values was within the respective tolerance interval determined using commercial canola varieties.

Several minerals, both macro (calcium, phosphorus, magnesium, potassium, sodium) and micro (iron, copper, manganese, zinc), have been recognized as essential plant nutrients for normal growth and development. There was a statistically significant difference detected in seed magnesium concentration between 73496 and the control canola, but the means and ranges in values were within the tolerance interval determined for seed magnesium concentrations using commercial canola varieties (Table 19, p. 66 in Pioneer 2011).

Canola seed meal contains a few antinutrient compounds such as tannins, sinapine, and phytic acid (OECD 2001) and secondary metabolites such as phytosterols, which are cholesterol-like molecules found in all plant foods which inhibit the absorption of dietary cholesterol (Ostlund 2002). Although there is a significant difference between 73496 and control canola for cholesterol, the range of values for cholesterol was well within the established tolerance interval (Table 21, p. 71 in Pioneer 2011).

Another important undesirable component in canola are glucosinolates, which 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), 2-hydroxy-4-pentenyl glucosinolate (napoleiferin) and 4-pentenyl (glucobrassicanapin) (Srahidi and Gabon 1989). Although a statistically significant difference was observed in progoitrin concentration between 73496 canola and control canola samples, the range of concentrations of progoitrin in 73496 canola was within the tolerance intervals established using commercial canola varieties (Table 20, p. 68 in Pioneer 2011). The total glucosinolate concentration for 73496 canola was 5.66 μmoles/g dry weight (p. 67 in Pioneer 2011), which is within the acceptable definition for canola (maximum 30 μmoles/g dry weight; OECD 2001). Higher glucosinolinates in some Brassica species have also been reported to reduce the feeding rates of larvae of the pest species White cabbage butterflies (*Pieris rapae*) and the fecundity of the Cabbage aphid (*Brevicoryne brassicae*) (Awmack and Leather 2002). However, as noted later in this PPRA, pest damage ratings for both of these species were not different between the 73496 canola and control canola.

Previously GAT4621 protein was part of the deregulated soybean event DP-356Ø43-5 (USDA 2008) and corn event DP-Ø9814Ø-6 (USDA 2009). Although the two deregulated events are not currently under commercial cultivation, so far there are no reports of plant pest characteristics exhibited by them.

Based on all the above noted considerations, APHIS concludes that 73496 canola poses no more of a plant pest risk from new gene products, changes to plant metabolism or composition than conventional canola varieties.

D. Potential Impacts on Disease and Pest Susceptibilities

USDA-APHIS assessed whether 73496 canola is likely to have significantly increased disease and pest susceptibility because of the introduced *gat4621* gene compared to the control canola variety. This assessment encompasses a thorough consideration of introduced traits, their impact on agronomic traits (discussed later in the document) and plant composition (discussed earlier), and quantitative and/or observational data on pest and disease responses. Important changes are those which would (1) affect not only the new GE crop, but that would also result in significant introduction or spread of a damaging pest or disease to other plants; and/or (2) result in the introduction, spread, and/or creation of a new disease or (3) result in a significant exacerbation of a pest or disease for which APHIS has a control program. Important changes would not include changes in susceptibility to diseases and pests that are within the acceptable range of currently cultivated varieties.

Plant Protection and Quarantine (PPQ) is an APHIS program that safeguards agriculture and natural resources from the entry, establishment, and spread of animal and plant pests and noxious weeds into the United States of America; and supports trade and exports of U.S. agricultural products. PPQ responds to many new introductions of plant pests to eradicate, suppress, or contain them through various programs in cooperation with state departments of agriculture and other government agencies. These may be emergency or longer term domestic programs that target a specific pest. A variety of insect, plant disease, mollusk, or weed programs exist (USDA APHIS 2013); however none specifically target pests of canola.

As detailed in the petition (p. 44 in Pioneer 2011) for both agronomic and pest and disease observations, the trial locations (Figure 17 & Table 10, p. 45 in Pioneer 2011) were selected in order to provide data representative of the major spring annual 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. The genetic backgrounds selected for these trials are adapted for cultivation both in northern U.S. and in Canada. In addition, insect pests and diseases at the Canada locations are typical of those found in the U.S. 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, p. 46 in Pioneer 2011). 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 complex of insects that feed upon the Brassicas is one of the important factors limiting the production of commercial Brassica crops (Lamb 1989: Weiss et al. 2009). Brassicaceous plants produce a family of sulphur compounds called glucosinolates whose breakdown products are attractants and stimuli for feeding and oviposition but, on the other hand, act as deterrents or toxins for herbivores not adapted to plants of the Brassicaceae. Some of the more important insects are Flea beetles (*Phyllotreta* spp), Cabbage stem flea beetle (*Psylliodies chrysocephala*), Stem weevils (*Ceutorhynchus* spp), Aphid species (*Lipaphis erysimi, Brevicoryne brassicae, Myzus persicae*), Diamondback moth (*Plutella xylostella*)*, Cabbage white butterfly* (*Pieris brassicae*)*,* Pollen beetles (*Meligethes* species), Seed pod weevil (*Ceuthorhynchus assimilis*), and Pod midge (*Dasineura brassicae*). Likewise, Brassica crops are subject to a broad range of pathogens (APS 2001). Out of all the diseases affecting Brassica crops, the three most troublesome diseases are blackleg or stem canker (*Leptosphaeria maculans*); Sclerotinia stem rot (*Sclerotinia sclerotiorum*); and clubroot (*Plasmodiophora brassicae*).

The pest and disease incidences were observed on 73496 canola and control lines (near-isolines and/or conventional canola lines) from six locations for four growing seasons (2007-2010) in the U.S. and from 6-14 locations for two growing seasons (2008-2009) in Canada. During each growing season, at least once in every four weeks, insect pest and disease data were recorded as mild (<10% or very little disease or insect injury), moderate (10-30% or noticeable plant tissue damage), and severe (>30% or significant plant tissue damage). The observations were recorded on the following common insect pests of canola: Alfalfa looper (*Autographa californica),* Cutworm (*Euxoa ochrogaster),* Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*), Green peach aphid (*Myzus persicae*), Cabbage aphid (*Brevicoryne brassicae*), Imported cabbage moth (*Pieris brassicae*), Grasshopper (*Melanoplus sanguinipes),* Diamond back moth (*Plutella xylostella),* White cabbage butterflies (*Pieris rapae),* White cabbage moths (*Mamestra brassicae*), Lygus bug (*Lygus sp.*), and Thrips (*Thrips tabaci)* (Tables 5.1 and 5.3, pp. 111-113 in Pioneer 2011). Likewise data were also collected on the following common canola diseases: Downey mildew (*Peronospora parasitica*), Powdery mildew (*Erysiphe polygoni*), White mold/Sclerotinia (*Sclerotinia sclerotiorum),* Fusarium wilt (*Fusarium oxysporum*), and Alternaria (*Alternaria brassicae)* (Tables 5.2 and 5.4, pp. 112 & 114 in Pioneer 2011).

A majority of pest and disease responses were mild to moderate for both 73496 canola and control canola. In 2009, at two locations in Canada, there were mild to severe grasshopper and cutworm infestations, yet the extent of infestations were similar between 73496 canola and control canola (Table 5.3, p. 113 in Pioneer 2011).

Foliar disease incidence and insect damage were also assessed just during the pod elongation period for 73496 canola and the near-isoline control canola in agronomic field trials planted in one location in Ephrata, Washington and two locations in Saskatoon, SK Canada for the 2009 growing season (Map Sites 8-10 in Table 10, p. 45 in Pioneer 2011). Visual estimates of foliar disease incidence or insect damage were rated on a scale of 1-9, where 1 equals poor insect or disease resistance and 9 equals best disease or insect resistance (Table 13, p. 50 in Pioneer 2011). No statistically significant differences were

observed in the means across locations for disease incidence or insect damage between 73496 canola and control canola (Table 14, p. 51 in Pioneer 2011).

As discussed earlier there were no significant changes in 73496 canola compositions that would render 73496 canola more susceptible to pests and diseases over its control or reference canola varieties. As presented later in this document, the observed agronomic traits also did not reveal any significant changes that would indirectly indicate that 73496 canola is or could be relatively more susceptible to pests and diseases over control or reference canola varieties, e.g. there were no significant reductions in early growth and seed yield across seven sites in Canada in 2008 field trial experiments (Table 10, p. 45 and Table 12, p. 48 in Pioneer 2011). Thus 73496 canola is expected to be susceptible to the same plant pathogens and insect pests as conventional canola. The introduced genes did not significantly alter the observed insect pest infestation and disease occurrence or resulting damage on 73496 canola over the control line. For this reason, there is also unlikely to be any indirect plant pest effects on other agricultural products.

E. Potential Impacts on Nontarget Organisms, Including those Beneficial to Agriculture

73496 canola is not engineered for pest resistance, thus there are no 'target' species, and thus no 'nontarget' species either. However, APHIS assessed whether exposure or consumption of herbicide-resistant 73496 canola containing the GAT4621 protein or the acetylated products produced by the GAT4621 enzyme would have an adverse effect on beneficial species or wildlife associated with canola.

As discussed earlier, 73496 canola is similar in nutritional and compositional analysis to unmodified control variety 1822B. Except for the expression of the GAT4621 protein to confer herbicide resistance to glyphosate, the only compositional differences that fell outside the tolerance interval for the commercial canola varieties were for elevated levels of certain acetylated amino acids, especially *N*-acetylaspartate (NAA) and *N*-acetylglutamate (NAG). . Earlier, Pioneer completed the food and feed safety (allergenicity and toxicity) assessment of GAT4621 protein as part of New Protein Consultation (NPC) 005 on 7 October, 2009 (Rood 2007a; FDA 2009; Appendix 10, pp. 130-147 in Pioneer 2011). Additional information on 73496 canola, including human and livestock exposure assessments, was submitted to FDA on 25 February, 2011as part of the consultation process for bioengineered foods (personal communication with the applicant, Natalie Weber). Furthermore, the GAT4621 protein was previously assessed for the determination of non-regulated status for glyphosate-resistant corn event DP-Ø9814Ø-6 (USDA 2009). A GAT protein (GAT4601), which is 91% identical and 96% similar to the GAT4621 protein (Figure 24, p.54 in Pioneer petition 07-152-01p, Rood 2007b), was also assessed for the determination of non-regulated status for soybean event DP-356043-5 (USDA 2008).

GAT4621 protein produced in 73496 canola demonstrated physicochemical equivalence to the microbial-produced GAT4621 protein that was used in the protein safety studies (acute toxicity testing and digestibility studies) that were conducted for maize event DP-

Ø9814Ø-6 (Pioneer 2011, pp. 104-110). These bridging data also support the conclusion that the GAT4621 protein expressed in 73496 canola and in maize event DP-Ø9814Ø-6 are equivalent.

Pioneer provided the following information justifying the safety of 73496 canola (pp. 40-41 in Pioneer 2011). 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 nonallergenic (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. The GAT4621 protein is expressed in 73496 canola at similar levels in whole plant tissue and roots as it is in seeds (Table 7, p. 39 in Pioneer 2011). This GAT4621 protein is unlikely to raise a safety concern for beneficial organisms (e.g. honeybees or earthworm) or wildlife (e.g. birds) that consume tissues of 73496 canola, because GAT4621 is very similar to other *N*-acetyltransferases that are common to a variety of organisms.

APHIS analyzed additional information provided by Pioneer to justify the safety of the GAT4621 protein in 73496 canola for humans and wildlife (pp. 40-41 in Pioneer 2011).

i) *Bacillus licheniformis*, the donor organism for sequences used to generate the *gat4621* gene, is a common soil bacterium widely distributed in the environment; is known as a contaminant of food; has been safely used in the production of food enzymes, as a biocontrol agent, and as a probiotic; and is not associated with any adverse effects on humans or animals (Federal Register 1997; EU Commission 2000; FDA 2001; Alexopoulos et al. 2004a; Alexopoulos et al. 2004b; Kritas et al. 2006). Moreover, *B. licheniformis* enters the human digestive system several times a day without causing harm (Tatzel 1994; EPA 1997). There are strains of *B. licheniformis* capable of producing toxins (Salkinoja-Satonen et al. 1999; Mikkola et al. 2000), but these were not used as a donor in the development of the *gat4621*gene. The GAT4621 protein was never found to be allergenic or toxic to any organisms as described in the following paragraphs.

ii). The amino acid sequence of the GAT4621 protein did not exhibit any significant similarity with known and putative allergenic proteins. Pioneer's analysis revealed that there was a lack of both amino acid identity and immunologically relevant similarities between the GAT4621 protein and known protein allergens. Moreover, GAT4621 protein was found to be degraded rapidly within gastric and intestinal fluids (less than 30 seconds and less than 5 minutes, respectively). In addition, protein glycosylation, an indicator for allergenicity (Altman 2007), did not occur with GAT4621 protein produced in canola (Appendix 4, p. 104 in Pioneer 2011).

iii) 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, although members of this superfamily have a diversity of substrate specificities (Dyda et al. 2000). The GAT4621 protein has a high specificity for glyphosate compared to other substrates, as previously discussed (Castle et al. 2004; Siehl et al. 2005; Siehl et al. 2007).

iv) An updated sequence similarity search of the GAT4621 protein sequence against the NCBI (National Center for Biotechnology Information) Protein dataset for biologically meaningful similar proteins identified 577 proteins. None of the similar proteins 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.

v) There was no evidence of acute toxicity in mice at a target dose of 2000 mg of 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 the no observed adverse effect level for mice, and expression levels of the GAT4621 protein in 73496 canola toasted meal, there is a wide margin of safety for the GAT4621 protein for poultry livestock.

Additionally, Pioneer provided an analysis of the safety and history of consumption of acetylated amino acids (Appendix 8, pp. 118-121, in Pioneer 2011). In addition to glyphosate, the GAT4621 enzyme is known to acetylate five amino acids: aspartate, glutamate, threonine, serine, and glycine, and mean levels of all but the NAGly were found to be significantly increased compared to the mean found in control canola (Table 22, p. 73 in Pioneer 2011). Acetylated amino acids are ubiquitous in nature, are part of many biological systems in plants and animals, and 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, however most of the data cited by Pioneer was related to safety to humans, livestock or mammalian species (FDA 2009; Rood, 2007; Appendix 10). It is important to note that the free amino acids were not significantly different in the 73496 canola compared to control canola, and only NAA and NAG were elevated above the tolerance interval for the commercial canola varieties (Table 22, p. 73 in Pioneer 2011).

Pollen is the most important source of essential amino acids for honeybees, and oilseed rape pollen was shown to contain a greater proportion of the most essential amino acids required by honeybees (valine, leucine, and isoleucine) (Table 1 in Cook et al. 2003), and moreover, honeybees tend to show a preference for oilseed rape pollen (Cook et al. 2003; Keller et al. 2005). However, the amino acids that are acetylated by GAT4621 in canola are considered nonessential amino acids in pollen for honey bees (Cook et al. 2003).

Therefore, based on the food and feed safety analyses, including toxicity and allergenicity data, it can be inferred that 73496 canola is unlikely to cause any significant adverse effects on nontarget organisms (including beneficial species or wildlife associated with canola) compared to other commercial canola varieties.

F. Potential for Enhanced Weediness of 73496 Canola

APHIS assessed whether 73946 canola has attained characteristics as a result of genetic engineering that would enhance its weediness compared to the nontransgenic progenitor and whether the engineered trait affects methods of control for canola in situations where it is managed as a volunteer in subsequent crops or in feral populations.

Canola is a domesticated *Brassica* species. Canola is not identified as a noxious weed in the Federal Noxious Weed List nor does it appear in any state weed lists (USDA-NRCS 2012). However, canola does possess a few attributes commonly associated with weeds, such as a large seed crop and harvest yield loss (Thomas et al. 1991; Brown et al. 1995), prolonged seed dormancy of 2-5 years, and an ability to persist as feral populations in disturbed habitats (Table 25, pp. 78-80 in Pioneer 2011). Pioneer collected major agronomic data relevant to weedy traits such as early growth, plant height, days to flowering, final seed yield, and seed shattering etc. from field experiments in a total of 10 locations across the U.S. and Canada during 2008 and 2009 growing seasons (Table 10 & Figure 17, p. 45 in Pioneer 2011). In addition, Pioneer also collected seed germination data (as an indicator of seed dormancy) under the standard laboratory conditions (Table 9, p. 43 in Pioneer 2011).

Out of three different temperature regimes (warm, cold, and diurnal) in which seeds were tested for their germination success (Table 8, p. 42 in Pioneer 2011), only under the warm temperature regime was the mean germination rate for 73496 canola seeds lower compared to the control canola seeds (98.8% vs. 98.4%, respectively), yet the observed significant difference ($p<0.05$), was still within the natural variation (84-100%) observed amongst reference commercial canola varieties (Table 9, p. 43 in Pioneer 2011). Seed shattering or seed yield loss during harvest combined with extended seed dormancy have the potential to create volunteer and weed problems for subsequent crops. Indeed canola is known to shatter seeds with about 2-7% of the seed yield lost during seed harvest (Table 2 in Gan et al. 2008). Despite significant seed loss during harvest, a majority of fallen seeds in the soil tend to germinate (> 90%) in the first season after harvest, and the remaining seeds generally exhibit 1-2 years of dormancy (Gulden et al. 2003). A few research reports also noted canola seed dormancy periods extending beyond 3 years (Légère et al. 2001; Simard et al. 2002; D'Hertefeldt et al. 2008), yet it was also observed that very few canola volunteers emerge during and after the third year of the post-harvest (Simard et al. 2002). Nonetheless, volunteer canola plants have still been documented at low densities four and 5 years after production (Simard et al. 2002).

In the field evaluation of agronomic traits, 73496 canola was tested in diverse agroecological conditions representing the major spring canola growing regions in the U.S. and Canada (Appendix 6, p. 115 &, Figures 17-18, pp. 45-46 in Pioneer 2011).

Except for days to flower, a majority of traits that have potential to enhance weediness (*e.g.* early growth, plant height, days to maturity, seed shattering, seed yield etc.) showed no statistically significant differences between 73496 canola and control canola (Table 12, p. 48 and Table 14, pp. 51-52 in Pioneer 2011). 73496 canola on average took a day longer to flower over its control canola variety in Experiment A involving 7 locations (Table 12, p. 48 in Pioneer 2011), but was not significantly different in Experiment B involving 3 locations (Table 14, pp. 51-52 in Pioneer 2011). Although early flowering is one of the potential indicators of enhanced weediness (Campbell et al. 2009; Ridley and Ellstrand 2009), 73496 canola is a late flowering type and a single day difference in flowering is unlikely to facilitate any weediness potential to 73496 canola.

A significant body of research exists on the ability of canola to form feral populations (Simard et al. 2002 and references therein; Schafer et al. 2011) and there is a widespread concern that herbicide-resistant feral populations may become an unmanageable weed problem around field edges and minimally managed agroecosystems. Unlike highly domesticated crops such as corn and soybean, canola is a relatively newly domesticated crop plant and possesses a few traits (e.g. prolonged seed dormancy, large seed yield, seed shattering; also (see Table 25, p. 78-79 in Pioneer 2011) that facilitate canola to persist as feral populations (Crawley and Brown 1995; Pessel et al. 2001). On the contrary, a mere possession of the potential weedy traits (Baker 1965) does not appear to predispose a plant taxon to become a weed (Perrins et al. 1992; Sutherland 2004).

Despite possessing some of the weed traits, canola is unlikely to become an unmanageable weed with the introduced trait. Like other crop plants, canola has several domesticated traits such as high seed output under optimum agronomic practices, self-pollination, etc., that make canola less competitive in unmanaged or minimally managed ecosystems (Crawley et al. 1993; Crawley et al. 2001; Salisbury 2002). The agronomic characteristics and germination data discussed earlier in this section 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-resistance trait conferred by *gat4621* gene is unlikely to provide a selective advantage in unmanaged ecosystems, but rather only in settings where glyphosate is being applied for weed control.

As described in the Addendum to the petition (Pioneer 2011), herbicide-resistant canola is no more likely to form feral populations than unmodified canola, nor is it more likely to be more invasive or competitive or persistent in habitats where the target herbicide is not applied (Warwick et al. 2009a; Andersson and de Vicente 2010). Even in those areas where herbicide-resistant canola has been grown extensively in the last several years, there is no indication of altered weediness or invasiveness potential imparted to feral canola or volunteer canola populations (Hall et al. 2005).

Volunteer herbicide-resistant canola (73496 canola, as well as other commercially available glyphosate, glufosinate, and imidazolinone-resistant varieties) should be controlled before planting canola cultivars with different herbicide resistance traits to reduce the potential for gene flow to result in stacked herbicide resistance, as such stacked herbicide resistance in canola has already been documented (Hall et al. 2000; Shaefer et al. 2011) (discussed in more

detail in the following section). Although herbicide-resistant and nonresistant canola varieties are documented to persist as volunteers or establish feral populations, there are alternative herbicides that can be used to control them. Paraquat and diuron (alone or in various combinations, some including glyphosate) were shown to be effective herbicides for control prior to planting in conservation tillage systems in the Pacific Northwest (Rainbolt et al. 2004). Producers need to consider which crop will follow the canola when making herbicide selections. Canola best follows cereal grains or fallow in rotation, and is rarely planted within one or two years following canola and other crops highly susceptible to sclerotinia (a stem rot) e.g. sunflower, dry edible beans or crambe, and sugar beet (Berglund et al. 2007). Glyphosate-resistant canola is typically rotated with other crops, typically wheat in a two year rotation or with wheat and soybean in a three year rotation, and other rotation crops include oats, barley, and flax (Berglund et al. 2007; Brown et al. 2008; p. 81, Pioneer 2011). Several (26) herbicide formulation options provide at least good to excellent control of volunteer glyphosate-resistant canola, particularly at the 3-6 leaf stage, as described in the 2012 North Dakota Weed Control Guide, and several can be used in the most common rotation crops of canola (NDSU 2012, p. 115). Tillage can also be used. The specificity of the GAT enzyme for glyphosate is high: other common agrichemicals including herbicides such as phosphinothricin, atrazine, and sulfonylureas are not acetylated by GAT (Castle et al. 2004; Siehl et al. 2005). Therefore, 73496 canola is expected to be sensitive to the same herbicides as other glyphosate-resistant canola already commercialized.

Therefore, based on this characterization, 73496 canola is no more likely to establish feral populations than either existing transgenic herbicide-resistant canola varieties, and such populations can be controlled using current weed control practices.

G. Potential of 73496 Canola to Impact the Weediness of Other Plants with which It Can Interbreed

Gene flow is a natural biological process with significant evolutionary importance. A number of angiosperm taxa are believed to be derived from hybridization or introgression between closely related taxa (Grant 1981; Soltis and Soltis 1993; Rieseberg 1997; Hegde et al. 2006), and even in the existing floras, the occurrence of hybridization or introgression is reported to be widespread (Stace 1987; Rieseberg and Wendel 1993; Peterson et al. 2002). It has been a common practice by plant breeders to artificially introgress traits from wild relatives into crop plants to develop new cultivars. However, gene flow from crops to wild relatives is also thought of as having a potential to enhance the weediness of wild relatives, as observed in rice, sorghum, sunflower and few other crops (see Table 1 in Ellstrand et al. 1999).

APHIS considers two primary issues when assessing weediness of sexually compatible plants because of transgene flow: 1) the potential for gene flow and introgression and, 2) the potential impact of introgression.

Canola is predominantly self-pollinating, but outcrossing does occur via wind and insect pollination (William 1984; William et al. 1987). Depending on the size of the crop and

distance between plants or fields, a variety of outcrossing rates were observed for canola (See Table 1 in Beckie et al. 2003). Most outcrossing between fields generally occurs within the first 10-20 m of the recipient field, and rates decline with distance (Table 1 in Beckie et al. 2003; OGTR 2011). Pioneer provided a summary of crop and wild relatives of canola that occur in the U.S. (Table 26, p. 84 in Pioneer 2011) and the likelihood of hybridizations with canola based on information on the success rate of hand pollinations or spontaneous and natural hybridization, weediness, and presence of the species in winter or spring canola growing areas (pp. 81-91 in Pioneer 2011).

Canola is grown in very few places in the U.S. and a majority (~90%) of canola production occurs in North Dakota, while the remaining cultivation comes from Minnesota, Idaho, Washington, Montana, Oklahoma, and Oregon (USDA-NASS 2009; USDA-NASS 2010; USDA-ERS 2010). Spring and winter canola varieties are generally grown in different regions of the U.S. based on climate zones most suitable for the varieties (Figure 19, p. 82 in Pioneer 2011). Brassica crops involve a number of diploid and polyploidy species (Figure 20, p. 83 in Pioneer 2011) and the family Brassicaceae involves a number of major weed species (OECD 1997).

In a majority of crop species, gene flow is idiosyncratic depending upon biology and ecology of both crop and sexually compatible relatives (Gliddon et al. 1999; Ingram 2000; Warwick et al. 2009a). Accordingly, there are several important considerations for a successful gene flow and introgression between 73496 canola and sexually compatible crop and weedy relatives such as spatial proximity, overlapping phenology, F1 hybrid fertility, self-sustaining reproductively fertile hybrid derived (backcrossed) populations, and neutral or beneficial introgressed genes (Devos et al. 2009).

Genus *Brassica* and related genus *Raphanus* contain oil seed, vegetable, and forage crop species (Ellstrand 2003; FitzJohn et al. 2007; Table 26, p. 84 in Pioneer 2011) such as *B. napus* (oil seed, swede) and *B. rapa* (oilseed, turnip and Chinese cabbage), *B. oleraceae* (cauliflower, cabbage, and broccoli), *B. juncea* (Indian mustard), and *R. sativus* (radish). 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). 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 showing genome relationships among cultivated Brassicaceae (Nagaharu 1935) are *B. rapa, B.nigra,* and *B. oleracea*. As depicted in the Figure 20 (p. 83 in Pioneer 2011), *B. napus* and *B. rapa* outcross readily with each other, while *Brassica napus* and *B. juncea* share a common set of chromosomes, enhancing the likelihood of interspecific hybridization and gene flow (Myers 2006). 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 20, p. 83 in Pioneer 2011).

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. These *Brassica* crop species can also outcross, albeit rarely, with a wide range of wild and weedy species (summarized in OGTR 2002). As mentioned in the Table 26 (p. 84 in Pioneer 2011) some *Brassica* crops and their wild relatives will hybridize only under artificial conditions in laboratories or highly contrived field conditions; whereas others will hybridize at very low rates under natural conditions (Raybould, 1999; Barton and Dracup, 2000; Warwick et al. 2009b). Through an extensive literature survey, Warwick and colleagues compiled an exhaustive list of interspecific and intergeneric hybridization among the members of the tribe Brassiceae, including large-scale artificial intergeneric hybridizations between various members of the tribe (Table 1 in Warwick et al. 2009b) and reported very few natural hybrids. As noted earlier, several reproductive and ecological barriers between canola and its wild species prevent formation of successful introgressed, self-sustaining hybrid derived populations (see detail descriptions on pp. 87-91 in Pioneer 2011).

Feral canola is a quite common occurrence along canola field edges and transportation routes (Bagavathiannan and Van Acker 2008; see Table 1 in Devos et al. 2012). The major concern raised about herbicide-resistant GE canola is about the ability of feral canola populations to act as a sink for the accumulation (stacking) of multiple herbicide resistance genes from the four different herbicide-resistant (GE glufosinate-resistant, GE bromoxynil-resistant, GE glyphosate- resistant, non-GE imidazolinone-resistant) canola currently under cultivation in Canada (Hall et al. 2000). There are reports indicating that canola cultivars with different HR traits resulted in volunteers with multiple resistance at a field site in western Canada (Hall et al. 2000). Therefore, the stacking of HR traits has the potential to facilitate the evolution of invasive feral canola populations (Ellstrand 2003; Knispel et al. 2008; Warwick et al. 2009a).

In 1997 in northern Alberta, a field of glyphosate-resistant canola was grown adjacent to a field of glufosinate-resistant and imidazolinone-resistant canola. Volunteers were selected with glyphosate in 1998 (Hall et al. 2000). These volunteers flowered and produced seeds that contained individuals resistant to glyphosate and glufosinate; glyphosate and imazethapyr; and glyphosate, imazethapyr, and glufosinate. Two triple herbicide-resistant individuals were detected, with one plant located 550 m from the glyphosate resistant pollen source. More recently, a study at 11 sites in Saskatchewan, Canada, where glyphosate HR *B. napus* canola was grown adjacent to glufosinate HR *B. napus* canola, documented gene flow to the limits of the study areas—a maximum distance of 800 m—on the basis of occurrence of double-HR volunteers (Beckie et al. 2003). The results of both studies suggest that herbicide-resistant gene stacking is common in *B. napus* canola volunteers in western Canada. In a similar study Knispel et al (2008) surveyed for the presence of single and multiple herbicide-resistance traits and assessed the extent of gene flow within escaped canola populations. Seed was collected from 16 escaped canola populations along the verges of fields and roadways in four agricultural regions in southern Manitoba from 2004 to 2006. Glyphosate resistance was found in 14 (88%) of these populations, glufosinate resistance in 13 (81%) populations,

and imidazolinone resistance in five (31%) populations. Multiple herbicide resistance was observed at levels consistent with previously published canola outcrossing rates in 10 (62%) of the tested populations. These reports indicate that intraspecific gene flow results in stacking of herbicide resistance traits in individuals within escaped canola populations (Knipsel et al. 2008). Similar multiple herbicide-resistant canola feral populations were also reported from Japan around transportation routes, although Japan never cultivates GE canola varieties and only imports them for food and feed purposes (Aono et al. 2006; Kawata et al. 2009).

Gene flow from 73496 canola was evaluated thoroughly with respect to plant pest risk. The introduced *gat4621* gene in 73496 canola is not expected to change the ability of the plant to interbreed with other plant species. Furthermore, the APHIS-BRS evaluation of data provided by Pioneer (2011) 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. In addition, gene flow has been occurring between non-GE canola (both herbicide-resistant and other canola varieties) and sexually compatible species even before the advent of GE canola varieties. Therefore, the consequences of gene flow and introgression of the glyphosate-resistant trait from 73496 canola to the same or sexually compatible species is anticipated to be the same as for existing commercial glyphosate-resistant canola varieties.

Successful hybridization of canola and a wild/weedy relative is highly unlikely and even if those successful rare events occur, the herbicide-resistance 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 or treated rights of way). Any herbicide-resistant feral and hybrid-derived populations are likely to be controlled using other available chemical or mechanical means. Many herbicides that are effective for control of glyphosate-resistant canola are also effective for control of wild mustards (NDSU 2012, pp. 115, 117, 119, and 120). As described by Beckie and colleagues (see Beckie et al. 2004 and references therein) the following cultural or mechanical practices are recommended to growers to manage multiple-HR canola volunteers: (1) leaving seeds on or near the soil surface as long as possible after harvest because a high percentage will germinate in the fall and be killed by frost, whereas seeds incorporated into the soil may develop secondary dormancy that will increase persistence; (2) using tillage immediately before seeding; (3) silaging and green manuring to prevent seed set in volunteers; (4) isolating fields of canola with different herbicide resistance traits to reduce outcrossing; (5) following canola with a cereal crop and rotating canola in a 4-yr diverse cropping sequence to deplete volunteers from the seedbank over time (which also facilitates use of alternative herbicides with different modes of action) and growing competitive crops to minimize volunteer canola interference (by choice of species and manipulation of agronomic practices such as higher seeding rates and precision fertilizer placement); (6) scouting fields for volunteers not controlled by weed management treatments and preventing seed set; (7) using pedigreed seed to reduce the probability of the presence of off-types with different herbicide resistance traits; and (8) reducing seed loss during harvest by swathing at the correct crop development stage and properly adjusting

combine settings. Herbicide treatments such as metribuzin, 2,4-D, or MCPA, alone or in a mixture, can control single or multiple herbicide-resistant canola volunteers when densities warrant, either pre-seeding or in-crop where registered. Previous studies have shown no difference in fitness among non-herbicide, single herbicide-resistant, or multiple herbicide-resistant canola. These findings indicate that multiple herbicide-resistant canola can be controlled equally well as non-herbicide-resistant or single herbicide-resistant plants by alternative herbicides within an integrated weed management program (Beckie et al. 2004).

Large-scale cultivation of herbicide-resistant 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-resistance genes into cultivated or wild relatives of canola (Andersson and de Vicente 2010). The International Survey of Herbicide Resistant Weeds has no confirmed cases of glyphosate-resistant weeds that are wild or weedy relatives of canola (Heap 2012). The 73496 canola is not expected to expand the amount of acreage planted to canola or to glyphosate-resistant canola, but rather to provide an alternative to other varieties of glyphosate-resistant canola already commercially available (p. 15, Pioneer 2011).

Therefore, it is highly unlikely that canola plants in the United States will be found outside of an agricultural setting, except along roadsides along seed transportation routes. It is also highly unlikely that gene flow and introgression will occur between 73496 canola plants and wild or weedy species in a natural environment. Herbicides are available to control volunteer glyphosate-resistant canola and weedy relatives. USDA has therefore determined that any adverse consequences of gene flow from73496 canola to wild or weedy species in the United States are highly unlikely.

H. Potential Changes to Agriculture or Cultivation Practices

APHIS considered whether there are likely to be significant changes to agricultural practices associated with cultivation of 73496 canola, and if so are they likely to significantly exacerbate plant diseases or pests, especially those for which APHIS has a control program. Glyphosate-resistant 73496 canola is not a new type of GE crop, as three different types of herbicide-resistant canola varieties (conventionally derived imidazolinone-resistant (Clearfield); GE glyphosate-resistant (Roundup Ready); and GE glufosinate-resistant (InVigor)) are already available for cultivation in the U.S. (Brown et al. 2008). In addition, a significant acreage of genetically-modified, herbicide-resistant canola (predominantly glyphosate-resistant) has been planted commercially in the U.S. since 1999. For instance, in 2008, genetically engineered herbicide-resistant canola was estimated to be 95% of the U.S. canola crop (Brookes and Barfoot 2010). No changes in cultivation or management practices such as planting times, row spacing, irrigation, crop residue management, tillage or pesticide use are anticipated with the introduction of glyphosate-resistant 73496 canola, which is comparable to other currently available canola types in phenotypic, agronomic, ecological, and compositional characteristics as mentioned earlier in this document. According to Pioneer, 73496 canola is an alternative product for currently available glyphosate-resistant canola varieties, and because 73496

canola has similar agronomic characteristics to conventional canola and currently available GE herbicide-resistant varieties, no change in general production practices are anticipated. Moreover, glyphosate use is restricted to no more than two applications from emergence to bolting on herbicide-resistant canola (Berglund *et al.*, 2007) and no changes to the glyphosate label are expected (Natalie Weber, Pioneer, personal communication).

I. Potential for Transfer of Genetic Information to Organisms with which 73496 Canola Cannot Interbreed and Potential Effects of Such Transfer

The horizontal gene transfer (HGT) between unrelated organisms is one of the most intensively studied fields since 1940, and the issue gained extra attention with the release of transgenic plants into the environment (Dröge et al. 1998) and sequencing of large number of genomic sequences (Choi and Kim 2007). HGT contributed to major transitions in evolution of prokaryotic organisms (Woese 2002) and has been implicated as a major contributor to the spread of antibiotic resistance amongst pathogenic bacteria and the emergence of increased virulence in viruses, bacteria, and eukaryotes. Although, gene exchange has been documented for nearly all types of genes and between unrelated organisms at an evolutionary scale (Gogarten et al. 2002; Yoshida et al. 2010), the frequency of HGT among higher organisms are shown to be extremely rare and, consequently, such transfers did not play any major role in their evolution (Kurland et al. 2003).

APHIS examined the potential for the new genetic material inserted into 73496 canola to be horizontally transferred to other organisms without sexual reproduction and whether such an event could lead directly or indirectly to disease, damage, injury or harm to plants. The 73496 canola does not contain any coding sequence from plant pathogenic organisms. Furthermore, horizontal gene transfer and expression of DNA from a plant species to other fungal, bacterial, or parasitic species is unlikely to occur based on the following observations.

Although there are many opportunities for plants to directly interact with fungi, bacteria, and parasitic plants (e.g. as commensals, symbionts, parasites, pathogens, decomposers, or in the guts of herbivores), so far there are no reports of significant horizontal gene transfer between evolutionarily distant organisms (as reviewed in Kurland et al. 2003; Keese 2008). Accumulated evidence show that there are universal gene-transfer barriers, regardless of whether transfer occurs among closely or distantly related organisms (Koonin et al. 2001; Wood et al. 2001; Kaneko et al. 2002; Brown 2003; Sorek et al. 2007). Many genomes (or parts thereof) have been sequenced from bacteria that are closely associated with plants including *Agrobacterium* and *Rhizobium* (Kaneko et al. 2002; Wood et al. 2001). There is no evidence that these organisms contain genes derived from plants. In cases where review of sequence data implied that horizontal gene transfer occurred, these events are inferred to occur on an evolutionary time scale on the order of millions of years (Koonin et al. 2001; Brown 2003), so also the case with the recent report about HGT between sorghum and purple witchweed. According to authors (Yoshida et al. 2010), the incorporation of a specific genetic sequence occurred between

sorghum and purple witchweed before speciation of purple witchweed (*Striga hermonthica*) and related cowpea witchweed (*S. gesnerioides*), a parasitic plant of dicots, from their common ancestor. In other words, HGT is an extremely rare event, and a majority of those rare events occur over millions of years.

Transgene DNA promoters and coding sequences are optimized for plant expression, not prokaryotic bacterial expression. Thus even if horizontal gene transfer occurred, proteins corresponding to the transgenes are not likely to be produced. FDA has evaluated horizontal gene transfer from the use of antibiotic resistance marker genes and has concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of humans or animals, or in the environment, is remote (FDA 1998: http://www.fda.gov/Food/GuidanceCompliance RegulatoryInformation/GuidanceDocuments/Biotechnology/ucm096135.htm). Therefore APHIS concludes that horizontal gene transfer is unlikely to occur from 73496 canola to microorganisms, and thus no significant plant pest risk is expected from horizontal gene transfer.

J. Conclusion

APHIS has reviewed the information submitted by the petitioner and conducted a plant pest risk assessment on 73496 canola. APHIS concludes that 73496 canola is highly unlikely to pose a plant pest risk based on the following reasons:

a. The introduced synthetic *gat4621*gene encoding the glyphosate *N*-acetyltransferase enzyme GAT4621 in 73496 canola results in the detoxification of applied glyphosate herbicide; neither the introduced sequences nor the method of transformation has resulted in disease symptoms, pathogen infection, or expression of a pathogen in 73496 canola.
b. Changes in gene expression, enzymes or metabolism in 73496 canola are unlikely to pose a plant pest risk; minor differences in the compositional constituents of 73496 canola seed compared to the control canola that could directly or indirectly impact plant pests or plant health were well within the range of commercial reference varieties, and did not raise any plant pest risks.
c. The observed insect pests and diseases or resulting damage on 73496 canola compared to the control canola suggest that 73496 canola is unlikely to be more susceptible to pathogens and insect pests of conventional canola or to bring about indirect plant pest effects on other agricultural products.
d. Based on the toxicity and safety assessment of the GAT4621 enzyme and minor changes in canola seed composition, 73496 canola is unlikely to adversely impact wildlife or other organisms beneficial to agriculture any more than conventional canola varieties.
e. There were no meaningful observed differences in traits between 73496 canola and control canola that would (i) enhance weediness in canola, or (ii) enhance its gene flow potential to wild or weedy relatives and consequently increase weedy characteristics in wild or weedy relatives. Furthermore alternative herbicides are available to control glyphosate resistant canola and weedy relatives.

f. The glyphosate resistance trait and observed agronomic and pest response characteristics in 73496 canola are not expected to change agricultural or cultivation practices compared to those currently used in widely-cultivated glyphosate-resistant canola varieties, which experience has shown has not increased pests or diseases or impacted their control.
g. Genes encoding variants of the GAT4621 protein already exist in a widespread, non-pathogenic soil bacterium in the environment, and horizontal transfer of the inserted glyphosate resistance gene from 73496 canola to other organisms with which it cannot interbreed is highly unlikely, and thus should not pose a plant pest risk.

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