NUSEED Petition (17-236-01p) for Determination of Non-regulated Status of DHA Canola

OECD Unique Identifier: NS-B5ØØ27-4

Plant Pest Risk Assessment

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TABLE OF CONTENTS

A. Introduction

Nuseed Americas Inc. (hereafter referred to as Nuseed) 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) DHA canola (*Brassica napus*) event B50027-4, (hereafter referred to as DHA canola), expressing omega-3 long chain polyunsaturated fatty acids (ω3 LC-PUFA) is unlikely to pose a plant pest risk and, therefore, should no longer be a regulated article under APHIS' 7 Code of Federal Regulations (CFR) part 340 (Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There Is Reason To Believe Are Plant Pests) (7 CFR part 340). This petition was assigned the number 17-236-01p and is hereafter referenced as Nuseed 2017. APHIS administers 7 CFR part 340 under the authority of the plant pest provisions of the Plant Protection Act (PPA) of 2000 (7 USC 7701 *et seq.*)[1]. This plant pest risk assessment (PPRA) was conducted to determine if DHA 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 7 CFR part 340 when APHIS determines that it is unlikely to pose a plant pest risk. A GE organism is considered a regulated article under 7 CFR 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 meets the definition of plant pest, or is an unclassified organism and/or an organism whose classification is unknown, or any product which contains such an organism, or any other organism or product altered or produced through genetic engineering which the Administrator determines is a plant pest or has reason to believe is a plant pest[2]. DHA canola was produced by *Agrobacterium tumefaciens*-mediated transformation of cotyledonary petioles from germinating seedlings of canola cultivar AV Jade using the plasmid binary vector pJP3416_GA7-ModB (Nuseed 2017, pp. 23-25). Portions of the inserted genetic material were derived from plant pest organisms listed in 7 CFR 340.2 (i.e., coding sequences from *Streptomyces viridochromogenes* (genus is listed in 7 CFR 340.2; however the species *S. viridochromogenes* is not considered a plant pest), enhancer sequences from *Tobacco mosaic virus* 59; promoter sequences from *Cauliflower mosaic virus*, and terminator and T-DNA border sequences from *A. tumefaciens*) (Nuseed 2017, Table 5, pp. 27-28). Therefore, DHA canola is considered a regulated article under APHIS regulations at 7 CFR part 340. Nuseed conducted field releases of DHA canola in Australia and Canada, in part, to gather information to support

[1] Plant Protection Act in 7 USC 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."

[2] Limited exclusions or exemptions apply for certain engineered microorganisms and for interstate movement of some organisms, as in 7 CFR 340.1 and 340.2.(b).

that DHA canola is unlikely to pose a plant pest risk in the United States (Nuseed 2017, Table 54, page 122).

Potential impacts in this plant pest risk assessment are those that pertain to plant pest risk associated with DHA canola and its progeny and their use in the absence of confinement relative to the unmodified recipient line and/or other appropriate comparators. APHIS utilizes data and information submitted by the applicant, in addition to current literature, to determine if DHA 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 assess information submitted by the applicant about DHA canola related to: plant pest risk characteristics; expression of the gene product, new enzymes, or changes to plant metabolism; disease and pest susceptibilities and indirect plant pest effects on other agricultural products; effects of the regulated article on nontarget organisms; 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; 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 1986; 57 FR 22984 1992a). Under the Coordinated Framework, the oversight of biotechnology-derived plants rests with 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.

EPA regulates under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136 *et seq)* 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. 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) (21 U.S.C. Chapter 9). Prior to registration for a new use for a new or previously registered pesticide, EPA must determine through testing that the pesticide does not cause unreasonable adverse effects on humans, the environment, and nontarget species when used in accordance with label instructions. EPA must also approve the language used on the pesticide label in accordance with Data Requirements for Pesticides (40 CFR part 158). Other applicable EPA regulations include Pesticide Registration and Classification Procedures (40 CFR part 152) and Experimental Use Permits (40 CFR part 172). No EPA reviews are relevant to DHA canola since it was not engineered to produce any plant-incorporated protectants or be resistant to herbicides.

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 early food safety evaluation for new non-pesticidal proteins produced by new plant varieties intended to be used as food (FDA 2006) and a more comprehensive voluntary consultation process prior to commercial distribution of food or feed (57 FR 22984 1992b). Nuseed submitted a safety and nutritional assessment for food and feed derived from DHA canola to the FDA in March 2017.

B. Development of the DHA Canola

Canola (*Brassica spp.)* is an oil seed crop primarily cultivated in Canada, China, India, and European Union (E.U.). Worldwide production of canola is usually grouped with rapeseed production. Canada produces 20 percent of the world's canola (GE and spring). The E.U. and China are also predominant canola (winter) producing geographic areas. Canola has shown potential to be successfully grown in the United States, South American and Australia (CFIA 2012; Smith 2017).

In North America, cultivated canola can be one of three Brassica species: *B. napus*, *B. rapa* and *B. juncea*. The term 'canola' refers to cultivars of *B. napus, B. rapa* or *B. juncea* that meet specific standards for levels of erucic acid in the oil and levels of glucosinolates in the meal. Specifically, canola-quality cultivars must yield oil low in erucic acid (<2%) and air-dried, oil-free meal low in glucosinolates (<30μmol/g) (OECD 2011; Canola Council of Canada 2018a). Canola is primarily produced for its seeds, which contain 35-45% oil; the oil is mainly used as cooking oil. Canola meal is a by-product of oil extraction and is widely used as a high protein animal feed (Roth-Maier 2015; Yun et al. 2018).

Brassica napus is the predominant canola species grown in the United States (Brown et al. 2008). *B. napus* is of relatively recent origin resulting from the interspecific hybridization between two diploid species, *B. oleracea* and *B. rapa* (CFIA 2012; OECD 2012). There are two types of *B. napus*, the oil-yielding oleiferous rape, of which canola is a type having specific quality characteristics, and the tuber-bearing swede or rutabaga. The oleiferous type of *B. napus* can be subdivided into spring (annual) and winter (biennial) types. The primary difference is that the winter type typically requires vernalization to induce flowering and bolting. *B. napus* is also grown as a winter annual in regions where winter conditions do not result in very low temperatures, which would kill the plants (CFIA 2012).

B. napus is a cool-season crop, widely adapted in temperate climates. It is primarily self-pollinating, however outcrossing does occur, the frequency varying with the cultivar, weather and availability of insect pollinators (Oplinger et al. 1989; OGTR 2011).

Canola producers in the U.S. planted a record high 2.16 million acres in 2017. Canola production is concentrated in the Northern Plains with 80% of production in North Dakota as depicted in Table 1 and smaller amounts in Oklahoma, Montana, Washington, Kansas and Minnesota, respectively (USDA-ERS 2012; USDA-NASS 2017). Spring canola is grown mainly in the northern states, including North and South

Dakota, Minnesota, Montana, Idaho, and Washington. Winter canola that requires vernalization is grown in the Pacific Northwest, the Great Plains and Midwest regions of the U.S. (Brown et al. 2008). Winter canola produced in the southeast region of the U.S. is planted in the fall and does not require vernalization (Brown et al. 2008; Buntin et al. 2013).

Canola Area Planted and Harvested – States and United States: 2016 and 2017

State	Area planted		Area harvested	
	2016	2017	2016	2017 [1]
	(1,000 acres)	(1,000 acres)	(1,000 acres)	(1,000 acres)
Idaho	21.0	25.0	20.5	24.3
Kansas	25.0	50.0	23.0	45.0
Minnesota	29.0	30.0	27.5	28.5
Montana	62.0	130.0	60.0	126.0
North Dakota	1,460.0	1,700.0	1,445.0	1,690.0
Oklahoma	80.0	160.0	75.0	135.0
Oregon	4.0	6.0	3.7	5.5
Washington	33.0	60.0	31.0	57.0
United States	1,714.0	2,161.0	1,685.7	2,111.3

[1] Forecasted.

Table 1. Acreage (USDA-NASS 2017)

Nuseed genetically engineered DHA canola line to express high concentrations of DHA (docosahexaenoic acid) in canola seed. DHA, an omega-3 long chain (>C20) polyunsaturated fatty acid (ω3 LC-PUFA) with established health benefits, is the most important structural component of many human tissues, and crucial for brain development and function. Nuseed developed DHA canola to provide an alternative, direct source of ω3 LC-PUFAs to meet increased human consumption and demand (Nuseed 2017, pp. 15-16).

AV Jade, broadly adapted to the Australian cropping zone as an open pollinated variety, was the recipient canola line for production of DHA canola due to its transformation efficiency (Nuseed 2017, page 20).

AV Jade canola was transformed with the plasmid binary vector pJP3416_GA7-ModB and used for the induction of embryogenesis. Cotyledonary petioles isolated from seedlings germinated from sterilized seeds were used as explants and infected with disarmed *A. tumefaciens* strain AGL1. After co-cultivation on MS media then selection media, explants were transferred to shoot initiation media and healthy shoots were transferred to rooting media. A T0 plant with positive T-DNA was selected for the DHA canola breeding process. The herbicide-tolerant selectable marker, phosphinothricin N-acetyltransferase (PAT), was used in the initial transformation and tissue culture selection process; however according to the petitioner the herbicide resistant *pat* gene is not intended to be used in the breeding process nor is it intended to be marketed in DHA canola varieties (Nuseed 2017, pp. 24-25).

DHA canola is comprised of seven trans-membrane enzyme proteins which fall into 3 functional classes; yeast acyl-CoA type fatty acid desaturases, microalgae fatty acid elongases and front-end desaturases. The inserted target genes express the seven fatty acid desaturases and elongases that convert oleic acid to DHA as depicted in the DHA biosynthesis pathway engineered into DHA canola (Nuseed 2017, Figure 1, page 21).

- Two yeast acyl-CoA type fatty acid desaturases
    - Lackl-Δ12D from *Lachancea kluyveri*
    - Picpa-ω3D from *Pichia pastoris*
- Two microalgae fatty acid elongases
    - Pyrco-Δ5E from *Pyramimonas cordata*
    - Pyrco-Δ6E from *Pyramimonas cordata*
- Three algae front-end desaturases
    - Micpu-Δ6D from *Micromonas pusilla*
    - Pavsa-Δ5D from *Pavlova salina*
    - Pavsa-Δ4D from *Pavlova salina*

Nuseed's DHA canola accumulates a substantially higher concentration of DHA in the seed oil compared to other conventional canola varieties. Specifically, the DHA mean for Nuseed's canola was 8.38% substantially higher than 0.24/0.11% for the parental and reference varieties, respectively (Nuseed 2017, Table 45, page 95).

Nuseed evaluated the phenotypic and agronomic performance of DHA canola by comparing it with the non-GE parental line AV Jade and at least 6 non-GE commercial varieties at ten sites in major canola growing regions of Australia and Canada (Nuseed 2017, Table 3, page 23). Nuseed also used the non-GE commercial varieties as reference lines for a detailed nutrient composition assessment. According to the petitioner, the reference lines selected represent a diverse range of natural variability of cultivars grown in Australia. Field trials included eight (8) locations in Western Victoria, Australia in 2015 and two (2) locations in Canada, one each in Alberta and Saskatchewan, in 2016. Supplemental information submitted by the petitioner includes one (1) agronomic evaluation in 2016 in the Imperial Valley of California and an additional three (3) evaluations in each of Canada and the U.S. in 2017 (Nuseed Supplemental Document 1).

Based on canola biology (OGTR 2011; CFIA 2012; OECD 2012) and the data presented by Nuseed (2017), APHIS concludes that DHA canola was developed in a manner common to other GE crops using *Agrobacterium*-mediated transformation (USDA-APHIS-BRS 2018). APHIS believes the use of the non-GE parental line AV Jade and other reference varieties as comparators is sufficient to determine whether DHA canola does not differ from its non-GE parental line and non-GE conventional canola varieties (USDA-APHIS-BRS 2018).

C. Description of Inserted Genetic Material, Its Inheritance and Expression, Gene Products, and Changes to Plant Metabolism

To inform the potential hazards resulting from the genetic modification and potential routes of exposure related to the inserted DNA and its expression products, APHIS assessed data and information presented in the petition related to the transformation process; the source of the inserted genetic material and its function in both the donor organism and the GE crop event; and the integrity, stability and mode of inheritance of the inserted genetic material through sexual or asexual reproduction based on the location of the insertion (e.g., nucleus or organelle) and the number of loci inserted.

APHIS also assessed data presented in the petition on whether the genetic modification results in expression of new genes, proteins, or enzymes or changes in plant metabolism or composition in DHA canola relative to the nontransgenic counterpart and other canola comparator varieties. The assessment encompasses a consideration of the expressed fatty acids from seven yeast and microalgae genes, the expressed phosphinothricin N-acetyltransferase (PAT) protein encoded by *pat* gene from *S. viridochromogenes*, and any observed or anticipated effects on plant metabolism including, for example, any relevant changes in levels of metabolites, anti-nutrients, or nutrients in harvested grain or forage derived from DHA canola compared to those in the conventional counterpart and other comparators.

This information is used later in this risk assessment to inform whether there is any potential for plant pest vectors or sequences to cause disease or greater plant pest risks in the GE crop event; or for expression of inserted DNA, new proteins or enzymes, or changes in metabolism to affect plant pests or diseases, non-target beneficial organisms, weediness, agricultural practices that impact pests or diseases or their management, or plant pest risks through horizontal gene flow.

Description of the genetic modification and inheritance of inserted DNA

As described in the petition (Nuseed 2017, page 23), DHA canola was developed essentially as described in (Bhalla and Singh 2008) and (Belide et al. 2013). The pJP3416_GA7-ModB plasmid vector contained two parts: 1) seven fatty acid biosynthesis genes from yeast and microalgae and a *pat* gene as selectable marker, each incorporated into its own expression cassette that included seed-specific promoters, enhancers, and terminators, and 2) backbone sequences necessary for maintenance or selection of the plasmid vector in bacteria. Backbone sequences are not expected to be transferred to the transformed plant (Nuseed 2017, Figure 2, page 24).

The transcription of the fatty acid enzyme genes is initiated by seed-specific promoters that therefore confine the synthesis pathway to the seed. The 5′ UTR leader sequence from *tobacco mosaic virus* is present in all of the fatty acid cassettes and functions to enhance the translation of the mRNA transcribed from each of the seven genes thereby enhancing expression of each protein.

The matrix attachment region from the Rb7 gene of tobacco (Hall et al. 1991; Halweg et al. 2005) was used as a spacer between two of the genes (Pavsa-Δ5D and Lackl-Δ12D), which enhances expression and stability, and thereby assists in maximizing production of substrates in the pathway. DHA canola (derived from the T0 generation event B0050) (Nuseed 2017, Figure 2, page 22) was ultimately chosen as the lead event based on superior agronomic, biochemical, genetic and molecular characteristics (Nuseed 2017, page 21).

Binary Plasmid Vector pJP3416_GA7-ModB

The pJP3416_GA7-ModB binary vector is approximately 31.6 kb. It contains eight gene expression cassettes which are delineated by right border (RB) and left border (LB) sequences of T-DNA, as well as backbone vector sequences outside of the two T-DNA border sequences. Table 5 in (Nuseed 2017, pp. 27-28) lists all genetic elements in the binary plasmid vector pJP3416_GA7-ModB. Genes and regulatory elements within the T-DNA regions are shown in Figure 3 (Nuseed 2017, page 24). The first seven cassettes contain the genes of interest. The eighth cassette contains the selectable marker used during the transformation process.

T-DNA cassettes

1. Micpu-Δ6D cassette
    - Coding sequence of delta-6-desaturase from microalgae *M. pusilla* (Petrie et al. 2010)
2. Pyrco-Δ5E cassette
    - Coding sequence of delta-5-elongase from microalgae *P. cordata* (Petrie et al. 2010)
3. Pavsa-Δ5D cassette
    - Coding sequence of delta-5-desaturase from microalgae *P. salina* (Zhou et al. 2007)
4. Picpa- ω3D cassette
    - Coding sequence of delta-15-/omega-3-desaturase from yeast *P. pastoris* (Zhang et al. 2008)
5. Pavsa-Δ4D cassette
    - Coding sequence of delta-4-desaturase from microalgae *P. salina* (Zhou et al. 2007)
6. Lackl-Δ12D cassette
    - Coding sequence of delta-12-desaturase from yeast *L. kluyveri* (Petrie et al. 2012)
7. Pyrco-Δ6E cassette
    - Coding sequence of delta-6-elongase from microalgae *P. cordata* (Petrie et al. 2010)
8. PAT cassette
    - Coding sequence of phosphinothricin N-acetyltransferase from bacterium *S. viridochromogenes* (Dröge et al. 1992)

Characteristics, Stability, and Inheritance of the Introduced DNA

DHA canola was characterized with vector-targeted sequencing, whole-genome sequencing and polymerase chain reaction (PCR)-amplicon sequencing. Nuseed reports that the DHA canola contained no vector backbone, no binary vector bacterial selectable marker gene neomycin phosphotransferase II (NPT II) nor any *A. tumefaciens* genome sequence. Sequencing also indicated that DHA canola contained two T-DNA inserts, one on chromosome A05 and the other on chromosome A02. The full genomic DNA sequences of the two T-DNA inserts were verified by Nuseed and the sequence of each

T-DNA insert perfectly matched the reference of vector pJP3416_GA7-ModB. Both T-DNA inserts were required to accumulate the desired amount of DHA in seed oil.

Expression of inserted DNA, changes in gene expression, new proteins or metabolism, and toxicity and allergenicity

Fatty Acid Biosynthesis Proteins

In DHA canola, the seven fatty acid biosynthesis proteins convert the typical fatty acids in canola seeds into the desired ω-3 LC-PUFAs. Plant oils are primarily composed of triacylglycerols that in turn comprise three fatty acid chains usually 16 or 18 carbons long (Durrett et al. 2008). The most abundant plant fatty acids are linoleic acid and α-linolenic acid, produced by desaturation of oleic acid. Some plants do produce LC-PUFAs but not at levels that are commercially viable (Abedi and Sahari 2014). In DHA canola, the genetic modification results in the favoring of the ω-3 pathway from the outset. Expression of fatty acid intermediates is maximized by the use of genes with high native expression, and enhancers (TMV 5′ UTR and tobacco Rb7) to ensure the desired flux of substrates.

The seven fatty acid biosynthesis proteins are similar to other proteins in consumed food, food production, or in animal feeds. Each protein was quantified in multiple tissue types collected over a growing season. None of the proteins were detected from the non-seed tissues of DHA canola. All seven of the enzymatic proteins were detected in developing and/or mature seeds of DHA canola. Conventional canola does not express DHA, therefore the inserted DNA causes the expected changes in the fatty acid profile of DHA canola and no unintended effects.

Nuseed performed digestibility studies on each of the seven fatty acid biosynthesis proteins. Digestibility of the proteins was assessed with an *in vitro* stability assay using a standard protocol (Thomas et al. 2004), followed by LC-MS analysis. The approach to analyze digestibility in this study mimics the typical mammalian digestive system that exposes food proteins to both pepsin (stomach) and trypsin (intestine) enzymes in transit through the gut. Rapid digestion of full-length protein is one of many factors that indicate protein safety. Nuseed determined that each protein was readily digestible.

Nuseed evaluated the potential toxicity and allergenicity of DHA canola by comparing its sequence homology with known toxins and allergens, and showed that DHA and its intermediate proteins have no significant homology to known protein toxins and allergens (Nuseed 2017, Section VI, pp. 50-51). Furthermore, DHA canola comprises five fatty acid desaturases and two elongases. Each newly expressed protein is similar to proteins commonly consumed in food, food production, or in animal feeds, suggesting proteins in DHA canola have a history of prior exposure and a history of safe use (Nuseed 2017, pp. 50-51). Nuseed concludes that DHA canola lacks toxic and allergenic potential based on the broad weight of evidence.

PAT Protein

PAT protein is expressed in very low concentration in DHA canola. It was used in the initial transformation and tissue culture selection process as a selectable marker. PAT protein has been assessed by regulatory authorities around the world. Based on those studies, Nuseed concludes that PAT protein expressed in DHA canola does not have toxic or allergenic potential.

Potential new ORFs

In addition to the seven expressed proteins and PAT protein, Nuseed analyzed the potential new open reading frames (ORFs) that are likely to result from the insertion of T-DNA. Based on the bioinformatics analysis of the sequence data, Nuseed concluded that the 25 hypothetical ORFs did not match any near-full-length matches to known allergens and toxins even at E value = 0.1, although it was noted that 35% identity matches over 80 AA segments and eight-AA contiguous matches were found (FARRP 2016). It was concluded that the 25 putative polypeptides had no significant matches to, and were unlikely to contain, any cross-reactive immunoglobulin E (IgE)-binding epitopes with any proven or putative allergen and toxin proteins in the databases, even using the most stringent criteria suggested by Codex Alimentarius Commission (CODEX 2003).

The results from Nuseed studies demonstrated that the genes, gene sources and inserted DNA raise no safety concerns for DHA canola. The vector used to produce DHA canola contained expression cassettes of seven microalgae and yeast genes in the DHA biosynthetic pathway and a selection marker gene. These sources are from organisms that are commonly found in the environment and have a history of safe consumption and use in food/feed. DHA canola is a stable event as measured across seven generations of breeding by both genetic and phenotypic analysis. The analysis of potential ORFs did not reveal any similarities to known toxins or allergens.

Metabolism Composition Analysis

To assess any potential metabolite alteration as a result of the expression of the above inserted genes, Nuseed analyzed the metabolism composition of DHA canola grown at eight field sites in major canola growing regions in Australia, in comparison with the parental variety, AV Jade, and commercial reference varieties representing a range of the natural variability (Nuseed 2017 pp. 81-120). The compositional analysis included the following analytes: protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber, ash, carbohydrates, FAs, AAs, vitamins, minerals, phytosterols and key anti-nutrients.

The analytes for compositional assessment were selected considering the OECD revised consensus document (OECD 2011). Among the numerous compositional analyses that were carried out, concentrations of most analytes were not significantly different between DHA canola and control canola. Statistically significant differences were noted for

concentrations of oleic and linolenic fatty acids; delta- and total tocopherols; magnesium; the glucosinolate progoitrin; and cholesterol. The magnitudes of the differences were small, however, and in every case the ranges of values were 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.

Based on the OECD guidelines for compositional equivalence, Nuseed has concluded that DHA canola was compositionally comparable to conventional canola except for the intentional production of the ω3 fatty acids. There are no observed or anticipated unintended metabolic composition changes in the DHA canola that could impart any new plant pest or disease risk than non-GE canola varieties.

The expression of the inserted genetic material and the resulting phenotype in DHA canola are consistent with the stability/inheritance of the introduced genetic material. The ORF analysis showed no evidence of new ORFs or any unintended effects resulting from the insertion of the genetic material (Nuseed 2017). Based on the multi-location field test and evaluation of DHA canola as well as the previous citations and deregulated petitions for similar genes and gene products that have a history of safe use and have not been implicated in disease or pest issues, the gene products in DHA canola are not expected to incur any additional plant pest or increased disease risks.

D. Potential Plant Pest and Disease Impacts

APHIS assessed whether potential plant pest or disease impacts are likely to result from the transformation process, from DNA sequences from plant pests, or from any other expression products, new enzymes, proteins or changes in plant metabolism or composition in DHA canola that are known or anticipated to cause disease symptoms, or to affect plant pests or diseases or plant defense responses (as identified from the previous section). APHIS also assessed whether DHA canola is likely to have significantly increased disease and pest susceptibility based on data and observations from field trials on specific pest and disease damage or incidence and any agronomic data that might relate to such damage. Impacts or changes are assessed to determine if they would (1) affect the new GE crop and/or result in significant introduction or spread of a damaging pest or disease to other plants; (2) result in the introduction, spread, and/or creation of a new disease; and/or (3) result in a significant exacerbation of a pest or disease for which APHIS has a control program. Any increase in pest or disease susceptibility is evaluated with respect to the context of currently cultivated varieties, the ability to manage the pest or disease, and the potential impact on agriculture.

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, nematode or weed programs exist (USDA-APHIS 2018); however none of these programs specially target pests of DHA canola.

Canola is not a plant pest in the United States (7 CFR 340.2). The genetic modifications of DHA canola, including the genetic elements, expression of the gene products and their functions have been summarized above. *Agrobacterium*-mediated transformation is the most common method for Brassica transformation. This transformation method only delivers the modified T-DNA of the plasmid, thereby eliminating the random integration of vector sequences and the analysis of the transformed DNA is more straightforward (Bhalla and Singh 2008). As previously mentioned, DHA canola was transformed by *A. tumefaciens* strain AGL1. DHA canola was propagated by seed through seven generations and *A. tumefaciens* is not transmitted via seeds between generations. Nuseed found no evidence of the presence of *A.tumefaciens* after sequencing of DHA canola. In addition, the portions of the inserted genetic material derived from plant pests, such as promoter sequence from Cauliflower mosaic virus, enhancer sequence from tobacco mosaic virus, and terminator sequence from *A. tumefaciens*, do not result in the production of infectious agents or disease symptoms in plants. Thus, it is unlikely that DHA canola could pose a plant pest risk.

Canola is grown in a wide range of soils, climates and environments representing a wide variety of plant hardiness zones. As mentioned in the petition (Nuseed 2017, page 121), many similarities exist in agronomic practices used in canola production between Australia and North America including weed, insect and disease control practices. In Australia, canola is grown across the southern dryland cropping zone with winter-dominant rainfall environments similar to the plant hardiness zone of the southern U.S. Australian production is mostly from spring canola cultivars typically sown after the first major rainfall from April to May with yield determined by water availability during the growing season and water use efficiency of the cultivar, similar to conditions in dryer regions of Canada and U.S. In North America, spring canola is also grown in western Canadian provinces including Alberta and Saskatchewan and in the Pacific Northwest and other U.S. states that border Canada with cultivation ranging from plant hardiness zones 1-2 in Canada and 3-8 in the U.S. (Nuseed Supplemental Document 1).

DHA canola was field tested at ten field trial locations in representative canola growing regions of Australia over three years (2014-2016) and in Canada during the summer of 2016 (Nuseed 2017, Table 64, page 132) to provide data representative of the major spring type canola growing regions in Australia and Canada. The field trials were located across varying environments for soil type and rainfall, and agronomic management practices. These regions are typical of the areas in the Pacific Northwest states in particular North Dakota where most of the U.S. canola crop is grown. In 2015, field trials were planted across eight locations in Western Victoria, Australia (6 transgenic lines and 8 cultivars). In 2016, one field trial planted in Alberta and another in Saskatchewan, Canada (2 transgenic lines and 10 cultivars) (Nuseed 2017).

Nuseed also provided supplemental information to support similarities between Australia and the U.S. in canola growing environments, ecotyopes, and agronomic practices. In addition to the studies presented in the petition, Nuseed conducted six more studies in 2017 in North America (George et al. 2015; Nuseed Supplemental Document 1).

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 (*Leptosphaeria maculans and Leptosphaeria biglobosa*); Sclerotinia stem rot (*Sclerotinia sclerotiorum*); and clubroot (*Plasmodiophora brassicae*). However, blackleg disease is considered the most important disease of canola worldwide and can cause significant yield losses in susceptible varieties. Blackleg, also known as stem canker or phoma stem canker, is a disease complex attributed to two fungal species. *L. biglobosa* is a mild or weakly virulent species associated with upper stem lesions infecting canola late in the season. It rarely causes significant yield losses and is considered a minor problem. *L. maculans* is the virulent species infecting canola from the seedling stage onward. It progressively damages the crop throughout the season by girdling stems and restricting moisture and nutrient uptake, leading to significant yield loss (Ash 2010; Van De Wouw et al. 2016; Canola Council of Canada 2018b).

All locations were observed for naturally occurring disease and insect biotic stressors. The AV Jade parental control and at least six (6) reference varieties were compared to DHA canola at ten trial locations. Field observations to track the presence of insect and disease stressors and plant responses were recorded at these sites in 2015 and 2016 (Nuseed 2017) and included data from additional agronomic studies performed in Canada and the U.S. in 2017 (Nuseed Supplemental Document 1).

Insect pest and disease data were recorded as mild (<10% or very little disease or insect injury) or moderate (10-30% or noticeable plant tissue damage). The observations were recorded and included the following common insect pests of canola: Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*), Lucerne flea (*Sminthurus viridis*), Earth mites (*Halotydus destructor* or *Penthaleus spp*.), Cabbage aphid (*Brevicoryne brassicae*), Green peach aphid (*Myzus persicae*) and Turnip aphid (*Lipaphis erysimi*). Likewise, data were also collected on the blackleg (*Leptosphaeria maculans and Leptosphaeria biglobosa*) and blackspot (*Alternaria brassicae).*

As previously mentioned, AV Jade was selected as the recipient line for DHA Canola due to its multiple characteristics including blackleg resistance.The majority of visual estimates of blackleg and blackspot were mild and insect damage observed were at very low levels across all sites. Given the lack of stem cankering and breakage, the main cause of yield loss and basis for resistance rating in Australia (Sosnowski et al. 2004), all lines used in the study can be considered resistant to blackleg disease pressure.

No statistically significant differences were observed across locations for blackleg disease incidence between DHA canola and all the comparators (AV jade, other DHA lines, and reference varieties) (Nuseed 2017, Table 66, page 133; Nuseed Supplemental Document 1). Most notably, DHA canola remains resistant or tolerant to blackleg disease.

Based on field observation at all sites, the introduced genes did not significantly alter any observed insect pest infestation and disease occurrence or resulting damage on DHA canola over the parental control line. There were no significant changes in DHA canola composition that would render DHA canola more susceptible to pests and diseases over its control or reference canola varieties. The observed agronomic traits also did not reveal any significant changes that would indirectly indicate that DHA canola is or could be relatively more susceptible to pests and diseases over AV Jade parental control or reference canola varieties. Thus DHA canola is unlikely to be more susceptible to plant pathogens and insect pests than conventional canola. For this reason, DHA canola is unlikely to differ from conventional canola in its ability to harbor or transmit plant pathogens or pests and cause indirect plant pest effects on other agricultural products.

E. Potential Impacts on Nontarget Organisms Beneficial to Agriculture

DHA canola is not engineered for pest resistance, thus there are no 'target' species, and thus no 'nontarget' species either. APHIS assessed whether exposure or consumption of DHA canola would have a direct or indirect adverse impact on species beneficial to agriculture**.** Organisms considered were representatives of the species associated with production of the regulated crop in the agricultural environment. The assessment includes an analysis of data and information on DHA canola compared to the non-GE counterpart, AV Jade, and other reference varieties for any biologically relevant changes in the phenotype or substances produced **(**e.g. proteins, nutrients, antinutrients, analytes, etc.**)** which may be novel or expressed at significantly altered amounts that are associated with impacts on organisms beneficial to agriculture, and/or any observations of beneficial organisms associated with the plants.

As previously described, the vector used to produce DHA canola converted oleic acid to DHA in canola seed, and contained seven genes obtained from microalgae and yeast, (*L. kluyveri* Δ12-desaturase, Lackl-Δ12D; *P. pastoris* ω3-/Δ15-desaturase, Picpa-ω3D: *M. pusilla* Δ6-desaturase, Micpu-Δ6D; *P. cordata* Δ6-elongase, Pyrco-Δ6E; *P.salina* Δ5-desaturase, Pavsa-Δ5D; *P. cordata* Δ5-elongase, Pyrco-Δ5E; *P. salina* Δ4-desaturase Pavsa-Δ4D). DHA canola contains two T-DNA inserts that are required to accumulate the amount of DHA in seed oil to produce the desired trait. In addition, DHA canola contains the herbicide selection marker phosphinothricin N-acetyltransferase (PAT) gene

from *Streptomyces viridochromogenes* that confers glufosinate tolerance. The glufosinate tolerance was only used as a selection marker during the transformation stage and was not used for selection during the breeding process. The PAT protein is present in many commercial biotechnology-derived crops and has an extensive history of safe use. *S. viridochromogenes* is a widespread saprophytic, soil-borne bacteria with no known safety issues (FDA 2017).

The seven trans-membrane enzyme proteins that comprise DHA canola fall into 3 functional classes:
· Two yeast acyl-CoA type fatty acid desaturases (Lackl-Δ12D and Picpa-ω3D)
· Two microalgae fatty acid elongases (Pyrco-Δ5E and Pyrco-Δ6E)
· Three algae front-end desaturases (Micpu-Δ6D, Pavsa-Δ5D and Pavsa-Δ4D)

Each protein has been fully characterized and quantitated in DHA canola tissues collected over a growing season. Nuseed provided data to show that the enzymatic proteins that produce DHA using seed-specific promoters were detected in developing seed and mature seed at low levels (20-740 ng/mg total protein), while none were detected in the non-seed tissues of DHA canola. Based on the molecular mass, Pyrco-Δ5E was present in the lowest abundance while Pavsa-Δ4D was the highest (Nuseed 2017, Table 37, 38, 39, pp.79-10). Furthermore, the introduced genetic material is derived from organisms commonly found in the environment with a history of safe consumption and use in the food and feed supply. Nuseed demonstrated that the genetic material used to engineer DHA canola raises no new safety concerns.

The similarities of enzymes in each functional class is reflected by a summary of each protein and its characteristics (functional activity, molecular weight (MW) theoretical isoelectric point (pI), potential glycosylation sites and representative protein used to characterize digestibility (Nuseed 2017, Table 19, page 51).

Any new impacts on animals arising from the LC-PUFA synthesis pathway in DHA canola are expected to be confined to seed feeding insect pests. LC-PUFAs such as DHA and EPA are typically not present in terrestrial plants and animals, but they are present in marine and aquatic organisms (Hixson et al. 2015). Lepidopteran larvae fed on diets supplemented with LC-PUFAs at levels similar to those found in DHA seed achieved higher adult weights but had lower survival and a high rate of wing deformities relative to those on diets with similar lipid levels but without the LC-PUFAs (Fraenkel and Blewett 1946; Hixson et al. 2016). However, silkworm larvae fed on diets with lower levels of these compounds did not exhibit these phenotypes (Yu et al. 2018). LC-PUFA synthesis and storage is therefore expected to be confined to seed tissue, and any detrimental effects due to LC-PUFA consumption are expected to be confined to insects that exclusively feed on seeds, if they occur. Seed-feeding insects in U.S. canola production include the cabbage seedpod weevil, *Ceutorhynchus assimilis*; the Bertha armyworm, *Mamestra configurata*; and the diamondback moth, *Plutella xylostella* (Weiss et al. 2018). None of these species have been reported to be involved with ecosystem services beneficial to agriculture. Therefore, no agriculturally important organisms are likely to be affected by consumption of DHA canola.

- NuSeed assessed the digestibility/stability of one representative enzyme from each functional class; Picpa-ω3D, Pyrco-Δ5E and Pavsa-Δ4D with an *in vitro* stability assay using a standard protocol (Thomas et al. 2004), followed by LC-MS analysis. The safety of the enzymatic proteins is further supported by their quick digestion by digestive enzymes pepsin and/or trypsin.

- Nuseed compared the amino acid sequences encoded by the eight genes expressed in DHA canola against the curated AllergenOnline database version 16 and the NCBI Protein database using BLASTP (FARRP 2016). None of the results carry significant risks of allergy or toxicity compared to commonly consumed proteins from a diverse variety of food sources (Nuseed 2017, pp. 49-50).).

- DHA canola has two T-DNA inserts on two of its chromosomes. Nuseed analyzed all potential ORFs at the DNA junctions due to the two T-DNA insertions. None of the results from bioinformatics searches identified significant homologies to known toxins or allergens from the potential open reading frame (ORF) analysis of these two T-DNA inserts (Nuseed 2017).

- Nuseed conducted compositional analyses of DHA canola seed and meal in 2015 from samples taken at eight (8) field release sites in major canola growing regions of Australia. Analytes for evaluation were chosen based on the standard parameters outlined in the OECD Consensus document on compositional considerations for new varieties of low erucic acid rapeseed (canola; *Brassica napus*) (OECD 2011). None of the compositional analytes showed any biologically significant differences between DHA canola and the AV Jade parental control or commercial reference varieties, aside from the intended changes to the fatty acid pathway (e.g., high DHA).

- 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). Moreover, honeybees tend to show a preference for oilseed rape pollen (Cook et al. 2003; Keller et al. 2005). No statistically significant differences were identified for the most essential amino acids nor were any nutritional concerns raised from the slightly higher levels of alanine, aspartic acid, glycine, lysine, threonine and tyrosine present in DHA canola compared to AV Jade and commercial reference varieties (Nuseed 2017, Table 42, pp. 86-89).

Therefore, based on the above analysis on food and feed safety, including toxicity and allergenicity data, APHIS concludes that exposure to and/or consumption of the GE plant are unlikely to have any adverse impacts to organisms beneficial to agriculture.

F. Potential for Enhanced Weediness of DHA Canola

APHIS assessed whether the GE crop event is likely to become more weedy (i.e. more prevalent, competitive, damaging or difficult-to-control in situations where it is not

wanted) than the nontransgenic progenitor from which it was derived, or other varieties of the crop currently under cultivation. The assessment considers the basic biology of the crop, the situations in which crop volunteers or feral populations are considered weeds, and an evaluation of the GE crop event compared to the nontrangsenic progenitor or commercial reference varieties evaluated under field (and/or lab) conditions characteristic for the regions of the U.S. where the GE crop is intended to be grown for characteristics related to establishment, competiveness, reproduction, survival, persistence and/or spread that could influence weediness and the ability to manage the crop as a weed. For this crop, such characteristics include seed dormancy and germination, vigor, rate of growth and development, flowering and yield.

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 (Madsen 1962 ; Vaughan et al. 1976; Pekrun et al. 1997; Pioneer 2011). Nuseed collected major agronomic data relevant to weedy traits including early population and seedling vigor, plant height and seed shattering, days to flowering and final seed yield, and blackleg disease incidence (Nuseed 2017, Table 55, page 103). According to Nuseed, there are many similarities in agronomic practices used in canola production between Australia, Canada and the United States (supplemental material). Six DHA-expressing transgenic lines were compared to eight Australian varieties across 10 field trial locations representing diverse regions across Australia and Canada in 2015 and 2016 where canola is commercially cultivated. 2017 data from U.S.A. In addition, Nuseed collected data from seed germination evaluations to assess different storage conditions on the physiological quality of DHA canola seeds.

Seeds from AV Jade and two segregates, one with high DHA and one with medium DHA, were collected from glasshouse grown plants at 25, 30 and 35 days after flowering. Evaluations used as indicators of seed quality and vigor included the standard germination test (SGT), accelerated aging test (AAT), the electrical conductivity (EC) test and tetrazolium chloride test-viability (Nuseed 2017, pp. 126-127). Results from the SGT at 16 and 24°C resulted in equal germination of DHA canola and AV Jade seeds (Nuseed 2017, Table 58, page 128). In addition, no significant differences were observed by visual comparison of rate of radicle emergence between all three lines (Nuseed 2017, Figure 27, page 128). However, there were significant differences (P>0.05) in germination percentages for high DHA canola at lower and higher (10°C and 32°C) temperatures when compared to AV Jade. Overall, DHA canola showed similar germination and vigor under the multiple parameters. Under most conditions, it is expected that DHA canola will exhibit equivalent seed viability and vigor as conventional canola.

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. Canola is known to shatter seeds with about 2-7% of the seed yield lost during seed harvest (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. Nonetheless, volunteer canola plants have still been documented at low densities four and 5 years after production (Simard et al. 2002).

Nuseed collected data on agronomic characteristics that influence reproduction, crop survival and potential weediness. Data collected were used to evaluate specific aspects of altered plant pest potential. The assessment encompassed phenotypic growth and development; germination and dormancy; plant interactions with insects, diseases and abiotic stressors; and persistence in cultivated fields or areas outside of cultivation. Statistical analysis included a restricted estimated likelihood analysis using ARSeml (Gilmour et al. 2009) procedures in GenStat (Version 17). Results from these evaluations show no meaningful differences between DHA canola, AV Jade parental control and the commercial reference varieties for traits that could be associated with increased weed potential.

A significant body of research exists on the ability of canola to form feral populations (Simard et al. 2002; Schafer et al. 2011). 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) 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). The germination data and agronomic characteristics discussed earlier in this section provide evidence that the genetic modification resulting in DHA canola did not alter any major characteristics of the plant that would allow for development of weedy characteristics different from other canola varieties.

Based on the agronomic field data and literature survey concerning weediness potential of the crop, DHA canola is unlikely to persist as a troublesome weed or to have an impact on current weed management practices. These data suggest that DHA canola is no more likely to become a weed than conventional canola varieties.

G. Potential Impacts on the Weediness of Any Other Plants with which DHA Canola 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; Rieseberg and Wendel 1993; Soltis et al. 1993; 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; Preston 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 (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.

Potential for gene flow, hybridization and gene introgression

Canola is predominantly self-pollinating, but outcrossing does occur via wind and insect pollination (Williams 1984; Williams 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 (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 (Beckie et al. 2003; OGTR 2011). Nuseed provided a summary of crop and wild relative species of canola on the basis of overlapping geographic ranges with canola production and reports of successful hybridization (by hand pollination or in the field) with *B. napus* 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 (Nuseed 2017, pp. 138-139; Pioneer 2011).

In the United States, spring canola is grown over the summer in North Dakota and neighboring states; winter canola is grown in the Pacific Northwest, the Great Plains and northern Georgia (Brown et al. 2008). In southern Georgia, spring canola is grown over the winter (Buntin et al. 2013). Brassica crops involve a number of diploid and polyploidy species (Nagaharu 1935; Myers 2006) and the family Brassicaceae involves a number of major weed species (OECD 2012).

In a majority of crop species, gene flow is idiosyncratic depending upon biology and ecology of both crop and sexually compatible relatives (Ingram 2000; Warwick et al. 2009a). Accordingly, there are several important considerations for a successful gene flow and introgression between DHA canola and sexually compatible 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; Pioneer 2011) such as *B. napus* (oil seed, swede) and *B. rapa* (oilseed, turnip and Chinese cabbage), *B. oleraceae* (cauliflower, cabbage, 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 are *B. rapa, B.nigra,* and *B. oleracea (Nagaharu 1935).* Several sexually compatible Brassica spp., including *B. napus* share components of their genomes (OECD 2012). The three species grown as canola (*B. napus, B. juncea* and *B. rapa*) all share the A genome: *B. napus* is the AACC tetraploid; *B. juncea* is the AABB tetraploid and *B. rapa* is the AA diploid. Introgression between species is much more likely when they have one of the three genomes in common. Additionally, there is potential for gene flow from *B. napus* to *B. rapa* and *B. juncea* and thereby to the species with which they are sexually compatible. 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. However, (Scott and Wilkinson 1998) reported low rates of hybridization between *B. napus* and *B. rapa. Brassica napus* and *B. juncea* also share a common set of chromosomes, enhancing the likelihood of interspecific hybridization and gene flow (Myers 2006).

The Brassicaceae family contains a number of major weeds, including those in the genera *Sinapis*, *Capsella, Thlaspi, Erucastrium, Raphanus*, and others (OECD 2012). 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). 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 (Warwick et al. 2009b) 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.

Feral canola is a common occurrence along canola field edges and transportation routes (Bagavathiannan and Van Acker 2008; Devos et al. 2012). Ecologically *B. napus* is described as a cultivated crop where escaped plants become colonizers of waste places.

However, they are not invasive of natural habitats (OECD 2012). Where *B. napus* is found outside of agriculture, it is almost universally considered a casual escape (Hall et al. 2005; OGTR 2011; OECD 2012). In the United Kingdom, (Crawley and Brown 1995) found that along undisturbed roadways the persistence of *B. napus* is about 3 to 4 years and that the density of such feral populations is correlated with human activities (OECD 2012). Unless the habitats are disturbed on a regular basis, *B. napus* will be displaced (OECD 2012). In Canada, anecdotal evidence suggests that *B. napus* populations can occur where seed has been spilled, but these populations are short-lived and not invasive (Hall et al. 2005). Similarly, (Schafer et al. 2011) has documented escaped canola populations growing along numerous roadways in ND, and (Munier et al. 2012) noted similar roadside populations in CA. In general, these populations are considered casual rather than feral, dying out in 2-4 years unless reseeded by agricultural activity (Crawley and Brown 1995; Hall et al. 2005). Gulden et al. (Gulden et al. 2008), in a review of the weediness of both *B. napus* and *B. rapa*, notes that while both species occur in disturbed areas, only *B. rapa* produces naturalized, self-sustaining feral populations.

No literature was encountered that suggested that these roadside populations are capable of persistence without continuous reseeding from human activity. Similarly, no literature was encountered that suggested that *B. napus* is invasive or capable of colonizing natural habitats, and extensive literature can be found supporting the view that *B. napus* is not invasive of natural habitats (OGTR 2011; CFIA 2012; OECD 2012).

Potential for enhanced weediness of recipients after gene flow and/or introgression

Gene flow from DHA canola was evaluated thoroughly with respect to plant pest risk. The introduced genetic material in DHA canola is not expected to change the ability of the plant to interbreed with other plant species. Furthermore, APHIS evaluation of data provided by Nuseed (2017) of agronomic and phenotypic properties of DHA canola, including those characteristics associated with reproductive biology, indicated no unintended changes likely to affect the potential for gene flow from DHA canola to sexually compatible species. It is highly unlikely that canola plants in the United States will be found outside of an agricultural setting, except along roadsides and seed transportation routes. It is also highly unlikely that gene flow and introgression will occur between DHA canola plants and wild or weedy species in a natural environment. Thus, APHIS has determined that any adverse consequences of gene flow from DHA canola to wild or weedy species in the United States are highly unlikely.

Based on the information presented in the petition and in relevant literature, APHIS has reached the following conclusions. The genetic modification in DHA canola is not expected to increase the potential for gene flow, hybridization and/or introgression to occur to sexually compatible taxa compared to the nontransgenic recipient or other varieties of the crop commonly grown. Gene flow, hybridization and/or introgression of genes from DHA canola to other sexually compatible relatives, including wild, weedy, feral or cultivated species in the U.S. and its territories is not likely to occur. Therefore, DHA canola is not expected to increase the weed risk potential of other species with which it can interbreed in the U.S. and its territories.

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 DHA canola, and if so are they likely to significantly exacerbate plant diseases or pests, especially those for which APHIS has a control program.

As discussed throughout this document, DHA canola is similar to conventional canola in its agronomic, phenotypic, ecological and compositional characteristics and has levels of tolerance to insects and diseases comparable to conventional canola varieties. Furthermore, many similarities in agronomic practices of canola production exist between North America (USA and Canada) and Australia, including weed, insect and disease control practices. Therefore, no significant impacts on current cultivation and management practices for canola are expected following the introduction of DHA canola.

I. Potential Impacts from Transfer of Genetic Information to Organisms with which DHA Canola Cannot Interbreed

APHIS examined the potential for the new genetic material inserted into DHA canola to be horizontally transferred without sexual reproduction to other organisms and whether such an event could lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants. The horizontal gene transfer (HGT) between unrelated organisms is one of the most intensively studied fields in the biosciences since the late 1940s (Soucy et al. 2015), and the issue gained extra attention with the release of transgenic plants into the environment (Droge et al. 1998). Keese (Keese 2008) reviewed potential risks from stable HGT from genetically engineered organisms to another organism without reproduction or human intervention. Mechanisms of HGT include conjugation, transformation and transduction, and other diverse mechanisms of DNA and RNA uptake and recombination and rearrangement, most notably through viruses and mobile genetic elements (Keese 2008; Soucy et al. 2015). HGT has been a major contributor to the spread of antibiotic resistance amongst pathogenic bacteria; emergence of increased virulence in bacteria, eukaryotes and viruses; and, in the long run, to major transitions in evolution (Keese 2008).

Potential for horizontal gene transfer to bacteria, fungi, or invertebrates

DHA canola contains genetic elements from bacteria, i.e. the *pat* gene from *S. viridochromogenes* and non-coding regulatory sequences from *A. tumefaciens*. Horizontal gene transfer and expression of DNA from a plant species to bacterial, fungal or invertebrate species is unlikely to occur based on the following observations. Although there are many opportunities for plants to directly interact with fungi and bacteria (e.g. as commensals, symbionts, parasites, pathogens, decomposers, or in the guts of herbivores) and with invertebrates as plant pests, there are almost no evolutionary examples of HGT from eukaryotes to bacteria or from plants to fungi or invertebrates (van den Eede et al. 2004; Keeling and Palmer 2008; Keese 2008). Examples of HGT between eukaryotes and

fungi primarily involve gene acquisition or transfer by fungi to or from other distantly related fungi or bacteria (Keeling and Palmer 2008; Keese 2008) and HGT between plants and fungi is extremely rare (Richards et al. 2009). Examples of HGT between plants and invertebrates are also extremely rare, and most examples of HGT in insects involve acquisition of genes from their pathogens or endosymbionts (Keese 2008; Zhu et al. 2011; Acuna et al. 2012).

Horizontal transfer from and expression in bacteria of the foreign DNA inserted into the nuclear genome of the GE plant is unlikely to occur. First, many genomes (or parts thereof) have been sequenced from bacteria that are closely associated with plants including *Agrobacterium* and *Rhizobium* (Wood et al. 2001; Kaneko et al. 2002). There is no evidence that these organisms contain genes derived from plants. HGT from plants to bacteria is a very low frequency event, primarily because functional and selective barriers to HGT increase with genetic distance (Keeling and Palmer 2008; Keese 2008). Second, 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; EFSA 2009). Third, 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. Fourth, both the FDA (FDA 1998) and the European Food Safety Authority (EFSA 2009) have evaluated horizontal gene transfer from the use of antibiotic resistance marker genes and 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 very rare or remote.

Potential for horizontal gene transfer to viruses

DHA canola contains non-coding regulatory sequences from *tobacco mosaic virus* and *cauliflower mosaic virus*. APHIS also considered whether horizontal transfer of DNA from the GE plant to plant viruses was likely to occur and would lead to the creation or selection of plant viruses that are more virulent or have a broader host range. This issue has been considered before by other science review panels and government regulatory bodies (EPA-FIFRA-SAP 2006; Keese 2008). HGT is not unusual among plant viruses; however this is generally limited to exchange between viruses present in the same host organism in mixed infections, and most commonly involves homologous recombination, relying on sequence similarity at the point of crossover (Keese 2008). HGT from virus sequences engineered into plants has been demonstrated with infecting or challenge viruses, including both DNA viruses (e.g. geminiviruses which replicate in the nucleus) (Frischmuth and Stanley 1998) and RNA viruses which typically replicate in the cytoplasm; however most have been under conditions that favor recombination to restore a defective virus (Fuchs and Gonsalves 2007; Keese 2008; Thompson and Tepfer 2010). Populations of recombinants between virus transgenes expressed in transgenic plants and infected related viruses are similar to recombinants found in mixed infections of the same viruses in nontransgenic plants, indicating that there was no novel recombination mechanism in the transgenic plants and no increased risk is expected over what is

expected from mixed infections (Keese 2008; Turturo et al. 2008). Nonhomologous recombination in HGT among viruses or between virus transgenes and infecting viruses can occur, but frequently results in gene deletions which can result in nonviable viruses (Morroni et al. 2013). Depending on the particular virus and sequences involved, various hot-spots for recombination have been found in both coding and noncoding regions, and strategies implemented in design of transgenes to avoid recombination have been suggested. No recombinant or undesirable viruses with new properties have been detected for over at least 8-10 years in field tests or during commercial growth of deregulated virus resistant plum, squash, or papaya engineered with genes from viruses that have been deregulated in the U.S. (Fuchs and Gonsalves 2007). Furthermore, all plant virus-derived sequences present in DHA canola are non-coding, regulatory sequences of known function.

Potential for horizontal gene transfer to parasitic plants

Evidence for HGT from plants to other plants is limited to two specific scenarios: (1) exchange of genes between a parasitic plant and its host; and (2) exchange of genes between cells of two plants living in close proximity, such as in a graft junction. In both cases, this type of HGT requires physical contacts between the two plants. Most cases of HGT in plants involve transfer of mitochondrial genomes, which are primarily maternally inherited in plants (Barr et al. 2005), to other mitochondria genomes, and mostly involve parasitic plants and their hosts (Richardson and Palmer 2007). A comparative genomics analysis implicated HGT for the incorporation of a specific genetic sequence in the parasitic plant purple witchweed (*Striga hermonthica*) from its monocot host plant (Yoshida et al. 2010). According to this study, the incorporation of the specific genetic sequence (with an unknown function) occurred between sorghum and purple witchweed. However, this HGT occurred before speciation of purple witchweed and related cowpea witchweed (*S. gesnerioides*) from their common ancestor. Furthermore, *S. hermonthica* is not found in the U.S. and *S. asiatica*, another related parasite of cereal crops, is only present in North Carolina and South Carolina (USDA-NRCS 2018). More recent studies demonstrated that in a few parasitic species of the Rafflesiaceae family, out of several genetic sequences examined, about 2.1% of nuclear (Xi et al. 2012) and 24 - 41% of mitochondrial (Xi et al. 2013) gene transcripts appeared to be acquired from their obligate host species. However, all the above-mentioned instances of HGT between parasitic plants and their hosts were reported to be of ancient origins, on an evolutionary time scale spanning thousands to millions of years ago. Furthermore in DHA canola, the DNA sequences were inserted into the nuclear genome, not the mitochondrial genome (Nuseed 2017).

If DHA canola becomes infected by a parasitic plant or is naturally grafted to another plant, there is a very low probability that HGT could result in the other plant acquiring DNA from DHA canola. However, in both scenarios this newly introduced DNA would likely reside in somatic cells, and with little chance of reaching the germ cells, this introduced DNA could not persist in subsequent generations unless the recipient plant reproduced asexually from the affected cells.

Based on the above analysis APHIS therefore concludes that HGT of the new genetic material inserted into DHA canola to other organisms is highly unlikely, and is not expected to lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

J. Conclusion

APHIS has reviewed the information submitted in the petition, supporting documents, and other relevant information to assess the plant pest risk of DHA canola compared to the unmodified variety from which it was derived. APHIS concludes that DHA canola is unlikely to pose a greater plant pest risk than its unmodified parental variety, AV Jade, based on the following findings.

- No plant pest risk was identified from the transformation process or the insertion of new genetic material in DHA canola. Sequencing of the DHA canola lines found no evidence of the presence of *A. tumefaciens* used as the vector to transfer the genetic material. The other plant pest sequences in the inserted genetic material include leader sequences from *Tobacco mosaic virus* 59; promoter sequences from *Cauliflower mosaic virus*, and terminator and T-DNA border sequences from *A. tumefaciens.* The addition of non-coding genetic material did not confer any plant pest characteristics to DHA canola.
- No increase in plant pest risk was identified in DHA canola from expression of new proteins (five desaturases, two elongases and one herbicide-resistant) from the inserted genetic material, or changes in metabolism or composition because there were no significant changes in agronomic, ecological and compositional characteristics that would render DHA canola more susceptible to pests and diseases over its parental control or reference commercial varieties.
- Disease and pest incidence and/or damage were not observed to be significantly increased or atypical in DHA canola compared to the nontransgenic parental variety or other comparators in field trials conducted in growing regions representative of where DHA canola is expected to be grown. Observed agronomic traits also did not reveal any significant differences that would indirectly indicate that the GE crop event is more susceptible to pests or diseases**.** Therefore no plant pest effects are expected on these or other agricultural products and no impacts are expected to APHIS pest control programs.
- Exposure to and/or consumption of DHA canola are unlikely to have any adverse impacts on organisms beneficial to agriculture based on the analysis of compositional, and phenotypic data.
- DHA canola is no more likely to become a weed than conventional canola varieties based on its observed agronomic characteristics, weediness potential and the current management practices available to control DHA canola as a weed.
- DHA canola is not expected to increase the weed risk potential of other species with which it can interbreed in the U.S. or its territories. Furthermore, there are no meaningful observed differences in traits between DHA canola and control canola that would (i) enhance weediness in canola, or (ii) enhance its gene flow potential to

sexually compatible relatives and consequently increase weedy characteristics in sexually compatible relatives.

- Significant changes to agricultural or cultivation practices (e.g. pesticide applications, tillage, irrigation, harvesting, etc.) from adoption of DHA canola were not identified.
- Horizontal gene transfer of the new genetic material inserted into DHA canola to other organisms is highly unlikely, and is not expected to lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

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