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Petition for Determination of Nonregulated Status for DHA Canola

OECD Unique Identifier: NS-BØØ27-4

The undersigned submits this petition under 7 CFR §340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR Part 340

Submitting Company

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Summary

Omega-3 (ω3) long chain polyunsaturated fatty acids (LC-PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have established health benefits, and currently can be obtained from algae or from algae-eating ocean fish. There is a need for alternative, direct sources of ω3 LC-PUFAs to help meet increased human consumption and demand. In collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO), Nuseed has developed a genetically modified canola line, DHA canola (elite event B0050-027; Organisation for Economic Development (OECD) unique Identifier (ID) NS-B5ØØ27-4) that accumulates high concentrations of DHA in canola seed. DHA canola was produced through *Agrobacterium tumefaciens*-mediated transformation of canola cultivar AV Jade with binary vector pJP3416_GA7-ModB.

Nuseed Americas Inc. (herein referred to as "Nuseed"), a wholly owned subsidiary of Nufarm Limited, is submitting a Petition for Determination of Nonregulated Status for DHA canola. Nuseed requests a determination from the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) that canola transformation event NS-B5ØØ27-4 and any canola lines derived from crosses between NS-B5ØØ27-4 canola and conventional canola or biotechnology derived canola granted nonregulated status by APHIS no longer be considered regulated articles under 7 Code of Federal Regulations (CFR) Part 340.

AV Jade is an elite canola line, broadly adapted to the Australian canola cropping zone as an open pollinated variety. This line was selected as the recipient line for production of DHA canola because, as an elite line, it displayed good transformation efficiency. Over years, it was crossed and selected for multiple characteristics, including blackleg resistance.

DHA canola was characterized with vector targeted sequencing, whole genome sequencing (WGS) and polymerase chain reaction (PCR) amplicon sequencing. Sequencing indicated that the DHA canola contained no vector backbone, no binary vector bacterial selectable marker gene neomycin phosphotransferase II (*nptII*) or any *A. tumefaciens* genome sequence. Sequencing also indicated that DHA canola contained two (transfer-deoxyribonucleic acid) T-DNA inserts. The full genomic deoxyribonucleic acid (DNA) sequences of the two T-DNA inserts were verified and the sequence of each T-DNA insert perfectly matched the reference of the vector. Both T-DNA inserts were required to accumulate the desired amount of DHA in seed oil.

The safety of the introduced proteins is supported by the history of safe use of proteins similar to those in DHA canola that have been routinely consumed for many years, their quick digestion in pepsin and/or trypsin and their lack of similarity to known allergens of toxins using *in silico* analysis. Each protein has been fully characterized and quantitated in DHA canola. The enzymatic proteins that drive the production of DHA using seed-specific promoters were only detected in developing seed and mature seed at low levels, while none of the DHA biosynthesis pathway enzymes were detected in the non-seed tissues of the transgenic canola, irrespective of the sampling time or the tissues tested.

Characterization of the DHA canola event demonstrated that there are no safety concerns. The open reading frames created by insertion of the DNA will not express a toxin or allergen, the expressed DHA pathway enzymes are very low in concentration and are only expressed in the seed, and the agronomic properties of the event are no different than AV Jade.

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 acid (OA), α-linolenic acid (ALA) and linoleic acid (LA); Δ 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 OECD guidelines for compositional equivalence, we have concluded that DHA canola was compositionally comparable to conventional canola except for the intentional production of the ω3 LC-PUFAs.

DHA canola was field tested at ten sites in major canola growing regions of Australia and Canada. All field tests were conducted under field permits in Australia and Canada. Agronomic performance assessments were conducted in multi-site field studies to measure characteristics such as emergence, seedling vigor, plant height, lodging, and yield. All field trials were also observed for opportunistic disease or insect stressors as well as normal phenotypic characteristics. Based on field observation at these sites, pathogen susceptibility or resistance characteristics of the DHA canola were unchanged when compared to those of the non-transformed cultivar line AV Jade. The DHA canola remained resistant or tolerant to blackleg. There were no meaningful differences observed between DHA canola and AV Jade for plant pest characteristics and no indication of a selective advantage that could result in increased weediness potential of DHA canola. It was observed that DHA canola did not exhibit a reduction of yield or agronomic performance.

Results of these studies demonstrated that, aside from fatty acid (FA) profile, DHA canola is agronomically, phenotypically, and phenologically equivalent to AV Jade and other conventional canola varieties. Further, these studies showed no different biological effect of DHA canola compared to AV Jade with respect to interaction with various pathogens or insects. Therefore, these results reinforce the conclusion that the cultivation of DHA canola poses no different plant pest or weediness potential and will have no different environmental impact than the cultivation of commercial canola varieties.

The growing practices for DHA canola will be the same as the current industry standard practices (pesticides, fertilizers, etc.) with the addition of identity preservation in the harvesting and processing practices, to capture the benefit of the oil profile. There are already industry standard

practices which preserve the identity of harvested and processed canola seed. A current example is Nuseed's Monola® (high OA, low ALA canola), which has been bred traditionally to have a stable oil profile at high temperatures and can be used as a "healthier" oil for deep-frying food. It is grown under contract and identity preserved to capture the benefit of the oil profile with grain traders and canola seed crushing plants. A similar practice will be undertaken with DHA canola to capture the benefits of its unique oil profile.

DHA canola will provide a sustainable source of ω3 LC-PUFAs, which promote numerous health benefits. The expanded market place will enable greater consumer access to these important FAs. Growers and the canola industry would have access to a higher value crop and improved varieties containing the ω3 trait. Animal feedlots and aquaculture feed manufacturers would have a sustainable and financially viable source of ω3 FAs. Benefits to industry in general include numerous market opportunities where this ω3 source could be utilized when not a limited commodity. Multiple niche opportunities may open for small to medium sized enterprises, along with larger businesses.

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List of Abbreviations and Definitions

AA	Amino acid
AAT	Accelerated aging test
ALA	α-Linolenic acid; C18:3n3, $18:3^{\Delta 9,12,15}$
ANOVA	Analysis of variance
APHIS	Animal and Plant Health Inspection Service
BBCH Scale	Bayer, BASF, Ciba-Geigy and Hoechst growth scale for canola
BLASTP	Basic local alignment search tool for proteins
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol medium
BRS	Biotechnology Regulatory Services
CBI	Confidential business information
CFIA	Canadian Food Inspection Agency
CFR	U.S. Code of Federal Regulations
$\chi 2$	Chi-squared
CI	Confidence interval
CSIRO	Commonwealth Scientific and Industrial Research Organization
CV	Coefficient of variation
ddPCR	Digital droplet PCR
Δ	Delta, double bond created at a fixed position from the carboxyl group
DHA	Docosahexaenoic acid, 22:6n-3; $22:6^{\Delta 4,7,10,13,16,19}$
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid; C22:5n-3; $22:5^{\Delta 7,10,13,16,19}$
DTA	Docosatetraenoic acid; C22:4n-$6^{\Delta 7,10,13,16}$
EC	Electrical conductivity
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid; C20:5n-3; $20:5^{\Delta 5,8,11,14,17}$
EST	Expressed sequence tags
ETA	Eicosatetraenoic acid; C20:4n-3; $20:5^{\Delta 8,11,14,17}$
FA(s)	Fatty acid(s)
FAMEs	Fatty acid methyl esters
FARRP	Food and Allergy Research and Resource Program
FDA	United States Food and Drug Administration
F pr	F distribution probability of significant difference
FOIA	Freedom of Information Act
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GLA	Gamma linolenic acid; C18:3 n-6; $18:3^{\Delta 6,9,12}$
GRAS	Generally recognized as safe
Ha(s)	Hectare
HPP	Hypothetical gene
ID	Identifier
IgE	Immunoglobulin E
KASP	Kompetitive allele-specific PCR

LA	Linoleic acid; C18:2 n-6; $18:2^{\Delta 9,12}$
Lackl-Δ12D	*Lachancea kluyveri* Δ12 desturase
LB	Left border
LC	Liquid chromatography
LC-PUFA(s)	Long-chain (≥C20) polyunsaturated fatty acid(s)
LOD	Limit of detection
LOQ	Limit of quantitation
LSD	Least significant difference
Micpu-Δ6D	*Micromonas pusilla* Δ6 desaturase
MQ	Milli-Q water, water purified through resin and reverse osmosis filters
MRM	Multiple reaction monitoring
MS	Mass spectrometry or mass spectrometer
MUFA	Monounsaturated fatty acid
MW	Molecular weight
NCBI	National Center for Biotechnology Information
ND	Non-detectable
NMR	Nuclear magnetic resonance
nptII	Neomycin phosphotransferase II
OA	Oleic acid; C18:1 n-9; $18:1^{\Delta 9}$
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
ω	Omega: double bond created between the third and fourth carbons from the methyl end of a fatty acid
ω3	Omega-3
ω6	Omega-6
ORFs	Open reading frames
pI	Theoretical isoelectric point
PAT	Phosphinothricin *N*-acetyltransferase
Pavsa-Δ4D	*Pavlova salina* Δ4 desaturase
Pavsa-Δ5D	*Pavlova salina* Δ5 desaturase
PCR	Polymerase chain reaction
Picpa-ω3D	*Pichia pastoris* ω3-/Δ15 desaturase
PPT	Phosphinothricin
pti	pto-interacting gene
PUFA(s)	Polyunsaturated fatty acid(s)
Pyrco-Δ5E	*Pyramimonas cordata* Δ5 elongase
Pyrco-Δ6E	*Pyramimonas cordata* Δ6 elongase
qPCR	Quantitative polymerase chain reaction
RB	Right border
SD	Standard deviation
SDA	Stearidonic acid; C18:4n-3; $18:4^{\Delta 6,9,12,15}$
SE	Standard error
SGF	Simulated gastric fluid
SGT	Standard germination test
SP	Signal peptide
T-DNA	Transfer DNA

TZ	Tetrazolium chloride
USDA	United States Department of Agriculture
USDA ERS	United States Department of Agriculture Economic Research Service
USDA FAS	United States Department of Agriculture Foreign Agricultural Service
VAR	Variance
WGS	Whole genome sequencing
wt	Wild type, alleles representative of non-modified comparators
YPD	Yeast extract-peptone-dextrose

Release of Information

Nuseed Americas Inc. is submitting the information in this assessment for review by the USDA as part of the regulatory process. By submitting this information, Nuseed does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C. § 552; USDA complies with the provisions of FOIA and USDA's implementation regulations (7 CFR Part 1.4); and this information is responsive to the specific request. Nuseed expects that, in advance of the release of the document(s), USDA will, pursuant to Executive Order 12600, the Privacy Act and other applicable authorities, provide Nuseed with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds, e.g. responsiveness, confidentiality, and/or competitive concerns. Nuseed understands that a confidential business information (CBI)-deleted copy of this information may be made available to the public in a reading room and upon individual request as part of a public comment period. Nuseed also understands that when deemed complete, a copy of the petition may be posted to the USDA-APHIS Biotechnology Regulatory Services (BRS) website or other U.S. government websites (e.g. www.regulations.gov).

Certification

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

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I. Rationale for the Development of DHA canola

1. Basis for the Request for a Determination of Non-regulated Status

ω3 LC-PUFAs, such as DHA and EPA, have established health benefits, and currently can be obtained from algae or from algae-eating ocean fish. There is a need for alternative, direct sources of ω3 LC-PUFAs to help meet increased human consumption and demand. In collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO), Nuseed has developed a genetically modified canola line, DHA canola (elite event B0050-027; OECD unique ID NS-B5ØØ27-4) that accumulates high concentrations of DHA in canola seed. DHA canola was produced through *Agrobacterium tumefaciens*-mediated transformation of canola cultivar AV Jade with binary vector pJP3416_GA7-ModB.

USDA APHIS has responsibility, under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S. APHIS regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Nuseed is submitting this request to APHIS for a determination of non-regulated status for the new biotechnology-derived canola product, DHA canola (*Brassica napus* L.), any progeny derived from crosses between DHA canola and conventional canola, and any progeny derived from crosses of DHA canola with biotechnology-derived canola that have previously been granted non-regulated status under 7 CFR Part 340.

2. Benefits of DHA Canola

Production of canola (also known as low erucic acid rapeseed) has grown rapidly over the past 40 years, rising from the sixth largest to the second largest oil crop in the world. Canola seed is not usually sold to consumers, but is crushed at processing facilities into oil and meal. In 2015/2016, approximately 67 million metric tons of oilseed were produced, representing 10% of the world's oilseed production. In 2015, Australia and Canada harvested 2.4 million hectares (ha) and 8.1 million ha, respectively (USDA-FAS, 2015). Although the U.S. share of world production remains relatively small (0.7 million ha in 2015), canola is an increasingly important crop in regional economies of the Northern Plains (USDA-ERS, 2017).

FAs are carboxylic acids with long-chain hydrocarbon side groups, typically found in esterified form as the major component of lipids. Lipids and FAs are sources of energy, integral in cell membranes, and indispensable for processing biological and biochemical information. ω3 FAs are essential, i.e. required for human health and obtained primarily from diet. ω3 FAs are a group of PUFAs that are important for several bodily functions, including muscle activity, blood clotting,

digestion, fertility, cell division and growth, and reducing inflammation (Gogus and Smith, 2010). ω3 PUFA play a critical role in the development and function of the central nervous system (Dyall, 2015). Indeed, approximately 20% of the dry weight of the brain is made up of PUFAs, and one out of every three FAs in the central nervous system is a PUFA (Logan, 2004). The three principal ω3 FAs are α-linolenic acid (ALA), EPA and DHA, with DHA being the most important structural component of many human tissues, and crucial for brain development and function (Logan 2004; Dyall 2015). This product provides a sustainable and cost-effective source of ω3 LC-PUFAs to help meet the increasing dietary demand for these important nutrients.

The safety of DHA and EPA, the predominant FAs in DHA canola, is well established. In fact, the European Food Safety Authority (EFSA) recently reviewed both compounds and found no studies that raised any safety concern for the addition of these PUFAs to a wide variety of foods (EFSA, 2014).

Not only are PUFAs safe, robust scientific literature supports multiple health benefits derived from dietary PUFAs (Yurko-Mauro et al., 2015). Although country recommendations differ regarding how much EPA and DHA should be consumed daily, it is widely accepted that intake in most populations should be increased (Stark et al., 2016).

There is a finite supply of EPA and DHA available from both wild capture fisheries and rendering of aquaculture fish that are fed a diet containing wild fish ingredients (Nichols et al., 2010). Additional sources of EPA and DHA have become available from single cell fermentation systems of microalgae and yeasts; however, these only produce very low volumes. Sustainable and affordable technologies are needed to ensure an adequate supply of EPA and DHA for existing and expanding markets. Canola combined with biotechnology provides a solution to help meet the needs of the growing fish oil market.

Nuseed's DHA oil, extracted from harvested DHA canola grain, contains the FAs listed in Table 1.

Table 1. FAs in DHA canola

Common Name	Lipid Name	Δ^x Name	Chemical Name	Amount (%)*
OA	C18:1 n-9	$18:1^{\Delta 9}$	9-octadecenoic acid	1 – 30
LA	C18:2 n-6	$18:2^{\Delta 9,12}$	9,12-octadecadienoic acid	4 – 35
Gamma linolenic acid (GLA)	C18:3 n-6	$18:3^{\Delta 6,9,12}$	6,9,12-octadecatrienoic acid	0 – 7
ALA	C18:3 n-3	$18:3^{\Delta 9,12,15}$	9,12,15-octadecatrienoic acid	4 – 40
Stearidonic acid (SDA)	C18:4 n-3	$18:4^{\Delta 6,9,12,15}$	6,9,12,15-octadecatetraenoic acid	0 – 10
Eicosatetraenoic acid (ETA)	C20:4 n-3	$20:4^{\Delta 8,11,14,17}$	8,11,14,17-eicosatetraenoic acid	0 – 6
EPA	C20:5 n-3	$20:5^{\Delta 5,8,11,14,17}$	5,8,11,14,17-eicosapentaenoic acid	7 – 35
Docosapentaenoic acid (DPA)	C22:5 n-3	$22:6^{\Delta 7,10,13,16,19}$	7,10,13,16,19-docosapentaenoic acid	0 – 10
DHA	C22:6 n-3	$22:6^{\Delta 4,7,10,13,16,19}$	4,7,10,13,16,19-docosahexaenoic acid	7 – 35
Total monounsaturated FA (MUFAs)				4 – 40
Total PUFAs				20 – 75

*0 indicates < LOQ

The DHA oil will be refined for use as an alternate source of ω3 FAs used in established ω3 FA markets. Possible product examples include:

- Dairy products currently enriched with fish oil: milk (flavored or plain), cream cheese products, yogurts, custard desserts and dairy alternatives (soy milk, soy cheese).
- Bread and cereals currently enriched with fish oil or ω3 FAs: muesli, breakfast cereal, cereal bars, white bread, and multigrain bread.
- Spreads, condiments and sauces containing ω3 FAs: margarine (or margarine blends), salad dressings, mayonnaise, dips (i.e. hummus).
- Canned fish in oil: canned tuna chunks, canned tuna sandwich filling (plain or flavored); canned bean mix.

The inclusion level of DHA oil (or enriched DHA oil) in these foods will depend on the food matrix, oil content of the food, and the degree of substitution of the current fish oil or ω3 FA ingredient. Many of these foods already have ω3 FA content or health-related claims associated with the inclusion of fish oil or other ω3 FA ingredients. Substitution with DHA oil would retain these claims. A target of 30-60 milligram (mg) DHA/serving would meet the various United States, European Union, and Australian criteria for content and health-related claims in the food. The

required amounts of DHA for food content and health related claims differ in jurisdictions around the world.

The consumption of DHA in these foods is expected to remain on current trends when DHA canola oil replaces a fish oil ingredient. Therefore, the introduction of DHA canola oil will not change consumption rates of these foods, so contemporary dietary modeling programs are considered adequate.

The market share of DHA canola oil in the fish oil food ingredient market is likely to be low initially, increasing over time and with market acceptance to as high as ~20% after 10 years. Market forces, consumer choices (enriched food vs. nutraceutical consumption) and demand for fish oil in all markets (i.e. in feed, food, and nutraceuticals) will determine the rate of adoption and market share of DHA canola oil in food.

Future food opportunities for DHA canola oil could arise with new processing technologies such as micro-encapsulation/micro-emulsion. These possibilities include foods like frozen/chilled meals, juice/smoothies or soups. Fish oil is also used in infant formula/infant diets, but Nuseed is not pursuing these applications at this time, as we acknowledge additional regulatory approval may be needed in various jurisdictions for these uses.

As noted, refined DHA canola oil can also be used in dietary supplements and nutraceutical products. Oils used for this application are typically encapsulated into a soft gel form for easier consumption; but flavored liquids, oil-water emulsions, and chewable pastels are becoming increasingly available. In some cases, the natural levels of ω3 PUFAs may be enriched to increase the relative amounts of EPA and DHA. These products are almost exclusively sold as liquid formulations or soft gel forms. Nuseed acknowledges it may need additional regulatory approvals should it pursue such applications.

Both canola and fish oils are widely used today in feed for cattle, swine, poultry, and some aquaculture species. LC-PUFAs are essential for the growth and health of many species, especially for aquaculture species. LC ω3 canola oil from DHA canola may be used as an ingredient for animal and aquaculture feed as an alternative to fish oil and other sources of ω3. Animal feed supplemented with ω3 oils yields increased amounts of ω3 FAs in foods such as salmon or eggs. Nuseed is considering these types of opportunities that contribute to the human diet.

The meal component of DHA canola is compositionally similar to other commodity canola meal and will be used in a manner similar to conventional canola meal, including use in animal feeds.

3. Submissions to Other Regulatory Agencies

Under the Coordinated Framework for Regulation of Biotechnology, responsibility for regulatory oversight of biotechnology-derived crops falls primarily on three U.S. agencies: U.S. Food and Drug Administration (FDA); the USDA; and, in the case of plant incorporated protectants, the Environmental Protection Agency. In March 2017, Nuseed submitted a safety and nutritional

assessment for food and feed derived from DHA canola to the FDA. As DHA canola does not contain a plant incorporated protectant, no submission will be made to the Environmental Protection Agency (confirmed by personal correspondence).

An application for commercial release was made to the Office of the Gene Technology Regulator (OGTR) for a license for dealings involving intentional release of genetically modified plants into the environment. Additionally, an application was made to Food Standards Australia New Zealand to amend the *Food Standards Code* Standard 1.5.2 - *Food Produced Using Gene Technology*. Submissions for food, feed, and environmental approval will be made to Health Canada and the Canadian Food Inspection Agency (CFIA). Submissions will also be made for import approvals in Mexico, Japan, South Korea, China, European Union, and other countries as required. Nuseed is committed to robust product stewardship prior to and continuing after all relevant authorizations are granted.

II. The History, Biology and Use of Canola

1. Overview of Canola Biology

Traditional rapeseed (*B. napus* L.) was considered unsuitable as a food source for either humans or animals due to naturally occurring erucic acid and glucosinolate content. The development of an edible version called 'canola', which has been grown and consumed since the 1980s, is described in the CFIA document "The Biology of *Brassica napus* L. (Canola/Rapeseed)" (CFIA, 1994). The international regulatory standard for the definition of canola is as follows:

> "Seeds of the genus *Brassica* (*Brassica napus*, *Brassica rapa* or *Brassica juncea*) from which the oil shall contain less than 2% erucic acid in its FA profile and the solid meal component shall contain less than 30 micromoles of any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy- 4-pentenyl glucosinolate per gram of air-dry, oil-free solid." (Canola Council, 2017).

Reference documents for *B. napus* L. are available from other national and international organizations (OGTR, 2017; OECD, 2012), and provide additional background on its biology, including:

- information on use of canola as a crop plant
- taxonomic status of *Brassica*
- identification methods
- reproductive biology
- centers of origin and diversity
- crosses, including intra- and inter-specific/genus crosses and gene flow
- agro-ecology, including information about cultivation, volunteers and weediness, soil ecology, and canola-insect interactions

Canola seed is processed into two main fractions: oil and meal. There is no special processing requirement to render the fractions safe for consumption as food or feed. Canola oil and meal have a history of safe use in human food and animal feed (OECD, 2011) and are Generally Recognized As Safe (GRAS) as a food or feed component (https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=184.1555). Canola oil is commonly used for cooking/frying and in food products such as margarines and salad dressings, along with a vast array of ready-to-eat and processed foods. Canola oil is also used as industrial oil and lubricant as well as a source of biodiesel. Canola meal is widely used as an animal feed for cattle, swine and poultry, with potential for use in aquaculture feed.

2. Description of the Recipient Canola Line

AV Jade is an elite canola line, broadly adapted to the Australian canola cropping zone as an open pollinated variety. This line was selected as the recipient line for production of DHA canola because, as an elite line, it displayed good transformation efficiency. It was developed through multiple crosses starting from line 'RR013' by the Department of Primary Industries Victoria (Oilseeds Breeding Programs, Grains Innovation Park, Horsham, Victoria), and the Grains Research and Development Corporation, as part of the National Brassica Improvement Program. 'RR013' was derived in 1998 from a cross between two Victorian breeding lines, 'RM30' and 'RM17'. Over years, it was crossed and selected for multiple characteristics, including blackleg resistance. Following transformation, AV Jade was used for product testing, safety assessment studies, and bulking up seed for oil research.

III. Development of DHA Canola

1. Description of the Transformation Event

Nuseed has developed a genetically modified canola variety, DHA canola event NS-B5ØØ27-4, which accumulates a substantially higher concentration of DHA in the seed oil compared with its recipient line, AV Jade, as well as other conventional canola varieties. DHA canola expresses seven FA desaturases and elongases that convert OA to DHA. The pathway is shown below in Figure 1.

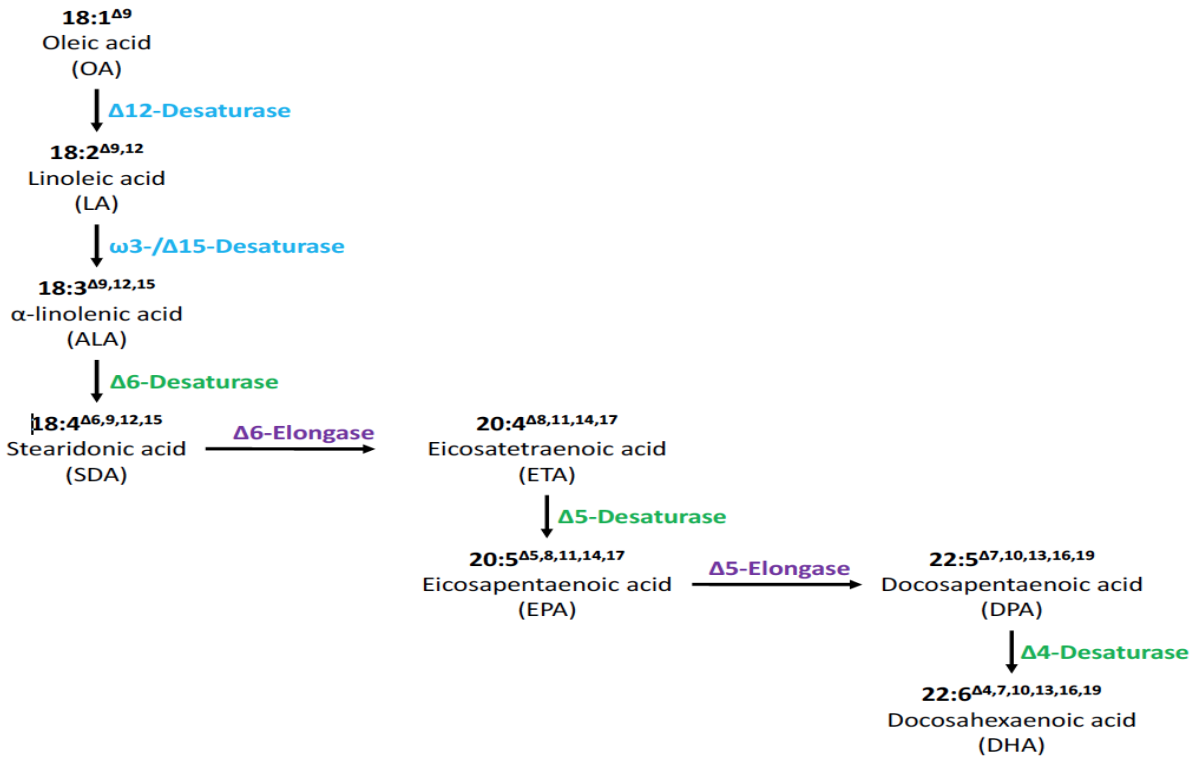

Figure 1. DHA biosynthesis pathway engineered into DHA canola

A single vector encoding seven enzymes was transformed into canola to convert OA to final product DHA. These enzymes were grouped into three classes: two FA desaturases from yeast (blue), two elongases from microalgae (purple), and three front-end desaturases from microalgae (green).

Canola cultivar AV Jade was transformed with *A. tumefaciens* strain AGL1 as described by Bhalla and Singh (2008) and Belide et al. (2013) using the plasmid binary vector pJP3416_GA7-ModB. (Refer to Section IV.1 for a detailed description of the plasmid containing the expression cassettes of the seven microalgae and yeast genes in the DHA biosynthetic pathway and an herbicide tolerance gene used for initial selection). Briefly, young seedling explants were inoculated with *Agrobacterium*. Explants were grown for one week in the absence of a selective agent before being transferred to a selective medium to recover transgenic shoots. The transgenic shoots were subjected to an additional round of selection on medium containing higher levels of the selective agent and a low-carbohydrate source, helping eliminate false-positive plants. Six transgenic lines were selected from T0 plantlets following a breeding re-selection program at the Nuseed Innovation Centre, in Horsham, Victoria, Australia.

The T1 seeds from T0 plant B0050-27 were advanced to T7 generation through single-seed descent and molecular, biochemical, genetic and agronomical evaluations (Figure 2). The selection and advancement of the candidate event was based on the following characteristics:

a) copy number and integrity of the T-DNA insert;

b) segregation and homozygosity of transgenic insert monitored by digital droplet PCR (ddPCR) and zygosity testing;
c) segregation pattern and production of DHA or LC-PUFA in the seed measured by gas chromatography (GC)-flame ionization detector (FID) (GC-FID) and nuclear magnetic resonance (NMR) spectrometer;
d) phosphinothricin (PPT) resistance;
e) genetic stability of the event in different generations and genetic backgrounds;
f) expression of ω3 FA biosynthesis genes monitored by quantitative PCR (qPCR), ddPCR, and Enzyme-linked immunosorbent assay (ELISA);
g) suitable agronomic traits (grain yield, oil content, blackleg resistance, plant emergence and vigor, flowering time and duration, maturity, plant survival, height and lodging at harvest, seed shattering, etc.) for crop production through field testing at different locations over winter and summer.

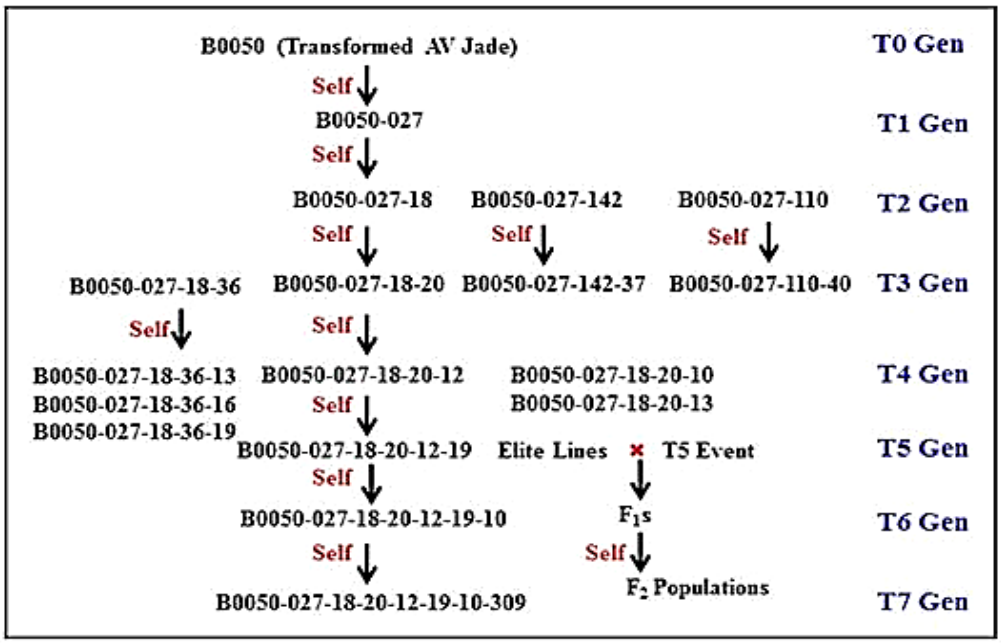

Figure 2. Breeding diagram of DHA canola

2. Selection of Comparators

Control canola lines were used as comparators for DHA canola (Table 2). All analyses used AV Jade as the untransformed near-isoline control, having the same genetic background as DHA canola. In addition, non-transgenic commercial canola reference lines (Table 3) were used for both agronomic comparisons and as reference lines for nutrient composition assessment, primarily to establish the range of natural variation of current commercial canola products with respect to key nutrients and anti-nutrients.

The commercial entries used for comparison represent an agronomically diverse (plant habit, phenology) range of well-adapted (high but varying yield potentials) and cultivars grown widely

in the Australian cropping zone. These cultivars are all open pollinated, evaluated extensively, and individually described in the Australian National Variety Testing Program and Regional annual crop reports (http://www.nvtonline.com.au). Variation for plant disease resistance is well described for blackleg in Australia (Van De Wouw et al., 2016). Other diseases occurring in Australia are not well described in terms of differential cultivar reactions and are considered sporadic and minor.

Table 2. Experimental entries

Line name	Elite event
NS-B5ØØ27-4	B0050-027*
AV Jade	Non transgenic
AV Zircon	Non-transgenic
AV Garnet	Non transgenic
ATR Bonito	Non transgenic
ATR Gem	Non transgenic
ATR Wahoo	Non transgenic
ATR Stingray	Non transgenic
Monola 515TT	Non transgenic
*T3 transgenic event generation	

Table 3. Commercial comparators and characteristics

Line name	Flowering	Maturity	Height
NS-B5ØØ27-4	Mid Season	Mid	Tall
AV Jade	Mid Season	Mid	Tall
AV Zircon	Mid Season	Mid	Tall
AV Garnet	Mid Season	Mid	Tall
ATR Bonito	Early to Mid Season	Early to Mid	Medium
ATR Gem	Early to Mid Season	Mid	Medium
ATR Wahoo	Late Season	Mid to Late	Medium
ATR Stingray	Early Season	Early	Short
Monola 515TT	Late Season	Mid to late	Medium
DK 74-44 BL	Early Season	Early to Mid	Short
L130	Mid Season	Mid	Medium

IV. Description of the Genetic Modification

1. Plasmid Vector Used for Transformation

This section describes the plasmid vector, the donor genes, and the regulatory elements used in the development of DHA canola and the amino acid (AA) sequence of the proteins produced. Expression cassettes are comprised of sequences to be transcribed and the regulatory elements necessary for the expression of those sequences.

Canola cultivar AV Jade was transformed with *A. tumefaciens* strain AGL1 essentially as described by Bhalla and Singh (2008) and Belide et al. (2013) using the plasmid binary vector

pJP3416_GA7-ModB (Figure 3). An herbicide-tolerant selectable marker, phosphinothricin *N*-acetyltransferase (PAT), was used in the initial transformation and tissue culture selection process, but was not used in the breeding process nor is it intended to be marketed in DHA canola varieties that will be grown commercially for food or feed.

The binary vector pJP3416_GA7-ModB was a variant of construct pJP3416_GA7 (Petrie et al., 2012). The seven FA biosynthesis genes were initially synthesized (GeneArt AG, Regensburg, Germany) as a single 19,750 bp fragment with flanking *Not*I sites, which was cloned into vector pJP3416_GA7 (Petrie et al., 2012). The vector pJP3416_GA7-ModB was built by re-synthesizing two T-DNA regions of the existing pJP3416_GA7. The first fragment with *Pyramimonas cordata* delta-6 elongase (Pyrco-Δ6E) was cloned into the *Sbf*I sites of pJP3416_GA7 to yield pJP3416_GA7-ModA. The second fragment with *Micromonas pusilla* delta-6 desaturase (Micpu-Δ6D) was cloned into the *Pme*I-*Asc*I sites of pJP3416_GA7-ModA to yield the final vector, pJP3416_GA7-ModB. The transformation vector was specifically designed to convert OA to DHA in canola seed. The 31,564 bp contained 8,052 bp vector backbone and 23,512 bp T-DNA. Each FA biosynthesis gene in the T-DNA was incorporated into its own expression cassette that included seed-specific promoters, enhancers, and terminators (Figure 3).

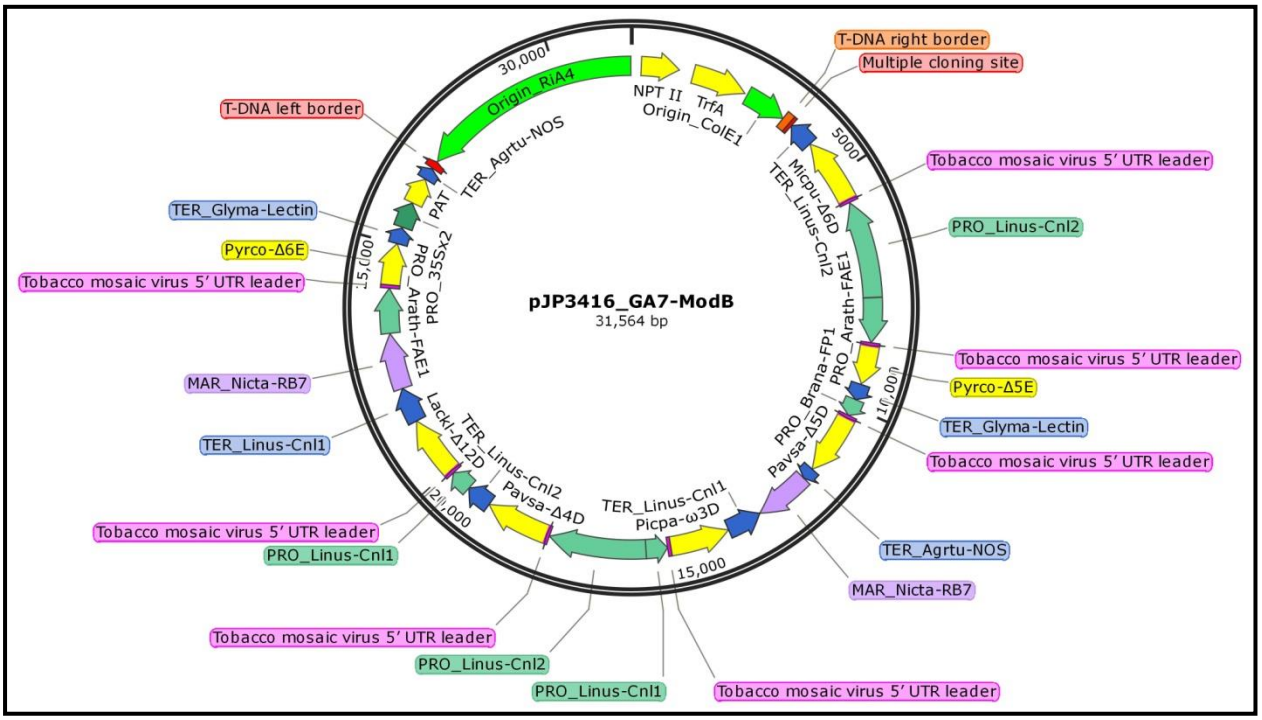

Figure 3. Annotation of binary vector pJP3416_GA7-ModB

The binary vector was transferred to *A. tumefaciens* strain AGL1. AGL1 carries attenuated tumor-inducing plasmid pTiBo542 from which native T-DNA sequences have been deleted, allowing transgene insertion and optimal DNA transformation in many dicotyledonous plants (Lazo et al., 1991). The vector sequence was confirmed through sequencing before *Agrobacterium*-mediated transformation of canola. A list of the abbreviations used for the introduced genes referred to in

this paragraph and throughout the petition can be found in the subsection below entitled "Donor genes and regulatory sequences."

The *A. tumefaciens* strain AGL1 was transformed with the binary vector pJP3416_GA7-ModB and used for the induction of embryogenesis. Briefly, seeds of AV Jade were sterilized using chlorine gas (Kereszt et al., 2007). Sterilized seeds were germinated on 1/2 strength shoot elongation media with 0.8% agar (pH 5.8) and grown at 24 ºC under fluorescent lighting. Cotyledonary petioles with 2-4 mm stalks were isolated aseptically from the seedling and used as explants. Freshly isolated cotyledonary petioles were infected with 20 mL of *A. tumefaciens* culture AGL1 with the binary vector for 6 minutes. The infected cotyledonary petioles were blotted on sterile filter paper to remove the excess *A. tumefaciens* and transferred to co-cultivation MS media with 1 mg/L thidiazuron (TDZ), 0.1 mg/L α-naphthaleneacetic acid (NAA), 100 µM acetosyringone, 50 mg/L L-cysteine, 15 mg/L ascorbic acid and 250 mg/L MES. The co-cultivated explants were cultured on selection media for 4 weeks at 24 °C with 16 h/8 h photoperiod with a biweekly subculture onto the same media. Explants with green calli were transferred to shoot initiation (hormone free) MS media (MS media + 1 mg/L kinetin + 3 mg/L $AgNO_3$ + 250 mg/L cefotaxime + 50 mg/L timentin + 5 mg/L PPT) and cultured for another 2-3 weeks. Healthy shoots with one or two leaves were selected and transferred to rooting media and cultured for 2-3 weeks. Leaves of these resistant shoots were used for testing of T-DNA presence by PCR amplification of FA biosynthesis genes and selection marker. The positive shoots with roots (T0 plants) were transferred to pots with seedling raising mix and grown to maturity for T1 seeds in glasshouse. A T0 plant with positive T-DNA was selected for breeding of DHA canola.

2. Donor Genes and Regulatory Sequences

The gene abbreviations used throughout were selected based on the donor gene source species, utilizing the first three letters of the genus name, the first two letter of the specific epithet, a dash followed by a delta or omega symbol, its position from the carboxyl group, and the letter D or E indicating a desaturase or elongase, respectively (Table 4).

Table 4. Listing of enzymes produced and their sources

Enzyme name	Gene abbreviation	Cloned from	Notes
Δ12 desaturase	Lackl-Δ12D	Previously cloned from the yeast *L. kluyveri* (Watanabe et al., 2004)	*L. kluyveri* was formerly known as *Saccharomyces kluyveri*, then reclassified (Kurtzman, 2003)
ω3/Δ15 desaturase	Picpa-ω3D	Previously cloned from yeast *P. pastoris* (Zhang et al., 2008)	*P. pastoris* was formerly known as *Komagataella pastoris*
Δ6 desaturase	Micpu-Δ6D	Previously cloned from microalga *M. pusilla* (Petrie et al., 2010b)	
Δ6 elongase	Pyrco-Δ6E	Previously cloned from microalga *P. cordata* (Petrie et al., 2010a)	
Δ5 desaturase	Pavsa-Δ5D	Previously cloned from microalga *P. salina* (Zhou et al., 2007)	
Δ5 elongase	Pyrco-Δ5E	Previously cloned from microalga *P. cordata* (Petrie et al., 2010a)	
Δ4 desaturase	Pavsa-Δ4D	Previously cloned from the microalga *P. salina* (Zhou et al., 2007)	

The genetic material introduced into DHA canola is the same chemical material (DNA) that humans and animals consume on a routine basis in food and feed. These seven genes are derived from organisms that are prevalent in the environment. The selectable marker gene is from a bacterium also common in the environment. Additionally, regulatory sequences (seed-specific promoter, enhancer, and terminator) were derived from plants, soil bacteria or plant viruses that are also widespread in the environment. Furthermore, none of the sources of these genes are known to create any food safety concerns since humans and animals have been routinely consuming these sources (e.g. yeast, algae) as well as the elongases and desaturases that they encode.

A. tumefaciens provided a vector for introducing transgenes in the transformation process. The DHA canola plants were not clonally propagated and *A. tumefaciens* is not transmitted from one generation to the next via seed. After seven generations of seed production, molecular analysis and sequencing of the DHA canola line genome detected no evidence of residual *A. tumefaciens*.

3. Identity and Source of Genetic Material

A description of the identity, function, origin, reference and location of each gene in plasmid pJP3416_GA7-ModB is depicted in Figure 3, above, and detailed in Table 5. The transgenic cassettes present in each independent locus in DHA canola are shown in Tables 6 and 7.

Table 5. Genetic elements in vector pJP3416_GA7-ModB

Genetic Element	Location in Vector	Length in bp	Function and Source
Vector Backbone (1 - 3,397 bp)			
nptII	213 - 1007	795	Coding sequence of neomycin-kanamycin phosphotransferase type II
TrfA	1306 - 2454	1149	Coding sequence of plasmid replication initiator protein TrfA derived from plasmid PK2
Origin_ColE1	2512 - 3392	881	Origin of replication (Col E1) for maintenance of high copy number of plasmid in *Escherichia coli*
T-DNA (3,398 - 26,909 bp)			
T-DNA right border	3398 - 3560	163	*A. tumefaciens* right border sequence used for transfer of T-DNA
Multiple cloning site	3561 - 3628	68	Multiple cloning site for vector construction
TER_Linus-Cnl2	3629 - 4166	538	Terminator of *Linum usitatissimum* conlinin2 (Chaudhary et al., 2001)
Micpu-Δ6D	4175 - 5569	1395	Coding sequence of Δ6-desaturase from microalgae *M. pusilla* (Petrie et al., 2010b)
Tobacco mosaic virus (TMV) 5' UTR leader	5573 - 5637	65	Enhancer from TMV 59 (Gallie et al., 1987)
PRO_Linus-Cnl2	5646 - 7678	2033	Promoter of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
PRO_Arath-FAE1	7685 - 8618	934	Promoter of *Arabidopsis thaliana* FA elongase1 (Rossak et al., 2001)
Tobacco mosaic virus 5' UTR leader	8619 - 8683	65	Enhancer from TMV 59 (Gallie et al., 1987)
Pyrco-Δ5E	8687 - 9493	807	Coding sequence of Δ5-elongase from microalgae *P. cordata* (Petrie et al., 2010a)
TER_Glyma-Lectin	9509 - 9842	334	Terminator of *Glycine max* lectin (Vodkin et al., 1983; Choi et al., 1995)
PRO_Brana-FP1	9843 - 10200	358	Promoter of *B. napus* napin (Stalberg et al., 1993)
TMV 5' UTR leader	10201 - 10265	65	Enhancer from TMV 59 (Gallie et al., 1987)
Pavsa-Δ5D	10269 - 11549	1281	Coding sequence of Δ5-desaturase from microalgae *P. salina* (Zhou et al., 2007)
TER_Agrtu-NOS	11550 - 11804	255	Terminator of *A. tumefaciens* nopaline synthase (Bevan 1984; Rogers et al., 1985; Sanders et al., 1987)
MAR_Nicta-RB7	11805 - 12972	1168	Rb7 matrix attachment regions of *Nicotiana tabacum* (Hall et al., 1991; Halweg et al., 2005)
TER_Linus-Cnl1	12973 - 13706	734	Terminator of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
Picpa-ω3D	13707 - 14957	1251	Coding sequence of Δ15−/ω3-desaturase form yeast *P. pastoris* (Zhang et al., 2008)
TMV 5' UTR leader	14961 - 15025	65	Enhancer from TMV 59 (Gallie et al., 1987)
PRO_Linus-Cnl1	15026 - 15475	450	Promoter of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)

Genetic Element	Location in Vector	Length in bp	Function and Source
PRO_Linus-Cnl2	15476 - 17508	2033	Promoter of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
TMV 5' UTR leader	17509 - 17573	65	Enhancer from TMV 59 (Gallie et al., 1987)
Pavsa-Δ4D	17577 - 18923	1347	Coding sequence of Δ4-desaturase from microalgae *P. salina* (Zhou et al., 2007)
TER_Linus-Cnl2	18924 - 19461	538	Terminator of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
PRO_Linus-Cnl1	19462 - 19911	450	Promoter of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
TMV 5' UTR leader	19912 - 19976	65	Enhancer from TMV 59 (Gallie et al., 1987)
Lackl-Δ12D	19980 - 21233	1254	Coding sequence of Δ12-desaturase from yeast *L. kluyveri* (Petrie et al., 2012)
TER_Linus-Cnl1	21234 - 21967	734	Terminator of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
MAR_Nicta-RB7	21968 - 23135	1168	Rb7 matrix attachment regions of *N. tabacum* (Hall et al., 1991; Halweg et al., 2005)
PRO_Arath-FAE1	23144 - 24077	934	Promoter of *A. thaliana* FA elongase1 (Rossak et al., 2001)
TMV 5' UTR leader	24078 - 24142	65	Enhancer from TMV 59 (Gallie et al., 1987)
Pyrco-Δ6E	24146 - 25015	870	Coding sequence of Δ6-elongase from microalgae *P. cordata* (Petrie et al., 2010a)
TER_Glyma-Lectin	25016 - 25349	334	Terminator of *G. max* lectin (Vodkin et al., 1983; Choi et al., 1995)
PRO_35S×2	25372 - 25909	538	Promoter of cauliflower mosaic virus gene encoding the 35S RNA (Kay et al., 1987; Coutu et al., 2007)
PAT	25919 - 26470	552	Coding sequence of PAT from bacterium *Streptomyces viridochromogenes* (Dröge et al., 1992)
TER_Agrtu-NOS	26479 - 26731	253	Terminator of *A. tumefaciens* nopaline synthase (Bevan 1984; Rogers et al., 1985; Sanders et al., 1987)
T-DNA left border	26749 - 26909	161	*A. tumefaciens* left border sequence used for transfer of T-DNA
Vector Backbone (26,910 - 31,564 bp)			
Origin_RiA4	26924 - 31559	4636	Origin of replication (RepA) for maintenance of plasmid in *Agrobacterium*

Table 6. Genetic elements of the A02 T-DNA insert and its flanking canola regions

Genetic Element	Location in Vector	Length in bp	Feature and Source
***B. napus* 5' flanking sequence (1 - 2,089 bp)**			
B. napus A02 upstream	1 - 352	352	*B. napus* A02 upstream
Hypothetical (HPP) gene 5' UTR	353 - 552	220	HPP gene 5' UTR
HPP gene exon 1	553 - 822	270	HPP gene exon 1
HPP gene intron 1	823 - 1686	864	HPP gene intron 1
HPP gene exon 2	1687 - 1860	174	HPP gene exon 2
HPP gene 3' UTR	1861 - 2089	229	HPP gene 3' UTR
T-DNA (2,090 - 14,199 bp)			
T-DNA right border (partial)	2090 - 2132	43	*A. tumefaciens* right border sequence used for transfer of T-DNA
Multiple cloning site	2133 - 2200	68	Multiple cloning site for vector construction
TER_Linus-Cnl2	2201 - 2738	538	Terminator of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
Micpu-Δ6D	2747 - 4141	1395	Coding sequence of Δ6-desaturase from microalgae *M. pusilla* (Petrie et al., 2010b)
TMV 5' UTR leader	4145 - 4209	65	Enhancer from TMV 59 (Gallie et al., 1987)
PRO_Linus-Cnl2	4218 - 6250	2033	Promoter of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
PRO_Arath-FAE1	6257 - 7190	934	Promoter of *A. thaliana* FA elongase1 (Rossak et al., 2001)
TMV 5' UTR leader	7191 - 7255	65	Enhancer from TMV 59 (Gallie et al., 1987)
Pyrco-Δ5E	7259 - 8065	807	Coding sequence of Δ5-elongase from microalgae *P. cordata* (Petrie et al., 2010a)
TER_Glyma-Lectin	8081 - 8414	334	Terminator of *G. max* lectin (Vodkin et al., 1983; Choi et al., 1995)
PRO_Brana-FP1	8415 - 8772	358	Promoter of *B. napus* napin (Stalberg et al., 1993)
TMV 5' UTR leader	8773 - 8837	65	Enhancer from TMV 59 (Gallie et al., 1987)
Pavsa-Δ5D	8841 - 10121	1281	Coding sequence of Δ5-desaturase from microalgae *P. salina* (Zhou et al., 2007)

Genetic Element	Location in Vector	Length in bp	Feature and Source
TER_Agrtu-NOS	10122 - 10376	255	Terminator of *A. tumefaciens* nopaline synthase (Bevan 1984; Rogers et al. 1985; Sanders et al., 1987)
MAR_Nicta-RB7	10377 - 11544	1168	Rb7 matrix attachment regions of *N. tabacum* (Hall et al., 1991; Halweg et al., 2005)
TER_Linus-Cnl1	11545 - 12278	734	Terminator of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
Picpa-ω3D	12279 - 13529	1251	Coding sequence of Δ15−/ω3-desaturase form yeast *P. pastoris* (Zhang et al., 2008)
TMV 5' UTR leader	13533 - 13597	65	Enhancer from TMV 59 (Gallie et al. 1987)
PRO_Linus-Cnl1	13598 - 14047	450	Promoter of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
PRO_Linus-Cnl2 (partial)	14048 - 14199	152	Promoter of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
***B. napus* 3' flanking sequence (14,200 - 15,004 bp)**			
HPP gene 3' UTR	14200 - 14223	24	HPP gene 3' UTR
B. napus A02 downstream	14224 - 15004	781	*B. napus* A02 downstream

Table 7. Genetic elements of the A05 T-DNA insert and its flanking canola regions

Genetic Element	Location in Vector	Length in bp	Feature and Source
***B. napus* 5' flanking sequence (1 - 1,159 bp)**			
B. napus A05 upstream	1 - 266	266	*B. napus* A05 upstream
PTI gene 5' UTR	267 -541	275	PTI gene 5' UTR
PTI gene intron 1	542 - 969	428	PTI gene intron 1
PTI gene 5' UTR	970 - 993	24	PTI gene 5' UTR
PTI gene exon 1	994 - 1075	82	PTI gene exon 1
PTI gene intron 2	1076 - 1157	82	PTI gene intron 2
PTI gene exon 2	1158 - 1159	2	PTI gene exon 2
T-DNA (upstream eight-gene cassette; 1,160 - 24,465 bp)			
T-DNA right border (partial)	1160 - 1199	40	*A. tumefaciens* right border sequence used for transfer of T-DNA
Multiple cloning site	1200 - 1267	68	Multiple cloning site for vector construction
TER_Linus-Cnl2	1268 - 1805	538	Terminator of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
Micpu-Δ6D	1814 - 3208	1395	Coding sequence of Δ6-desaturase from microalgae *M. pusilla* (Petrie et al., 2010b)
TMV 5' UTR leader	3212 - 3276	65	Enhancer from TMV 59 (Gallie et al., 1987)
PRO_Linus-Cnl2	3285 - 5317	2033	Promoter of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
PRO_Arath-FAE1	5324 - 6257	934	Promoter of *A. thaliana* FA elongase1 (Rossak et al., 2001)
TMV 5' UTR leader	6258 - 6322	65	Enhancer from TMV 59 (Gallie et al., 1987)
Pyrco-Δ5E	6326 - 7132	807	Coding sequence of Δ5-elongase from microalgae *P. cordata* (Petrie et al. 2010a)
TER_Glyma-Lectin	7148 - 7481	334	Terminator of *G. max* lectin (Vodkin et al., 1983; Choi et al., 1995)
PRO_Brana-FP1	7482 - 7839	358	Promoter of *B. napus* napin (Stalberg et al., 1993)
TMV 5' UTR leader	7840 - 7904	65	Enhancer from TMV 59 (Gallie et al. 1987)
Pavsa-Δ5D	7908 - 9188	1281	Coding sequence of Δ5-desaturase from microalgae *P. salina* (Zhou et al., 2007)
TER_Agrtu-NOS	9189 - 9443	255	Terminator of *A. tumefaciens* nopaline synthase (Bevan 1984; Rogers et al., 1985; Sanders et al., 1987)

Genetic Element	Location in Vector	Length in bp	Feature and Source
MAR_Nicta-RB7	9444 - 10611	1168	Rb7 matrix attachment regions of *N. tabacum* (Hall et al., 1991; Halweg et al., 2005)
TER_Linus-Cnl1	10612 - 11345	734	Terminator of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
Picpa-ω3D	11346 - 12596	1251	Coding sequence of Δ15−/ω3-desaturase form yeast *P. pastoris* (Zhang et al., 2008)
TMV 5' UTR leader	12600 - 12664	65	Enhancer from TMV 59 (Gallie et al., 1987)
PRO_Linus-Cnl1	12665 - 13114	450	Promoter of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
PRO_Linus-Cnl2	13115 - 15147	2033	Promoter of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
TMV 5' UTR leader	15148 - 15212	65	Enhancer from TMV 59 (Gallie et al., 1987)
Pavsa-Δ4D	15216 - 16562	1347	Coding sequence of Δ4-desaturase from microalgae *P. salina* (Zhou et al., 2007)
TER_Linus-Cnl2	16563 - 17100	538	Terminator of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
PRO_Linus-Cnl1	17101 - 17550	450	Promoter of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
TMV 5' UTR leader	17551 - 17615	65	Enhancer from TMV 59 (Gallie et al., 1987)
Lackl-Δ12D	17619 - 18872	1254	Coding sequence of Δ12-desaturase from yeast *L. kluyveri* (Petrie et al., 2012)
TER_Linus-Cnl1	18873 - 19606	734	Terminator of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
MAR_Nicta-RB7	19607 - 20774	1168	Rb7 matrix attachment regions of *N. tabacum* (Hall et al., 1991; Halweg et al. 2005)
PRO_Arath-FAE1	20783 - 21716	934	Promoter of *A. thaliana* FA elongase1 (Rossak et al., 2001)
TMV 5' UTR leader	21717 - 21781	65	Enhancer from TMV 59 (Gallie et al., 1987)
Pyrco-Δ6E	21785 - 22654	870	Coding sequence of Δ6-elongase from microalgae *P. cordata* (Petrie et al. 2010a)
TER_Glyma-Lectin	22655 - 22988	334	Terminator of *G. max* lectin (Vodkin et al., 1983; Choi et al., 1995)
PRO_35S×2	23011 - 23548	538	Promoter of cauliflower mosaic virus gene encoding the 35S RNA (Kay et al., 1987; Coutu et al., 2007)
PAT	23558 - 24109	552	Coding sequence of PAT from bacterium *S. viridochromogenes* (Dröge et al., 1992)

Genetic Element	Location in Vector	Length in bp	Feature and Source
TER_Agrtu-NOS	24118 - 24370	253	Terminator of *A. tumefaciens* nopaline synthase (Bevan 1984; Rogers et al., 1985; Sanders et al., 1987)
T-DNA left border (partial)	24388 - 24465	78	*A. tumefaciens* left border sequence used for transfer of T-DNA
T-DNA (downstream eight-gene cassette; 24,467 - 47,773 bp)			
T-DNA left border (partial)	24466 - 24543	78	*A. tumefaciens* left border sequence used for transfer of T-DNA
TER_Agrtu-NOS	24561 - 24813	253	Terminator of *A. tumefaciens* nopaline synthase (Bevan, 1984; Rogers et al., 1985; Sanders et al., 1987)
PAT	24822 - 25373	552	Coding sequence of PAT from bactrium *S. viridochromogenes* (Dröge et al., 1992)
PRO_35S×2	25383 - 25920	538	Promoter of cauliflower mosaic virus gene encoding the 35S RNA (Kay et al., 1987; Coutu et al., 2007)
TER_Glyma-Lectin	25943 - 26276	334	Terminator of *G. max* lectin (Vodkin et al. 1983; Choi et al., 1995)
Pyrco-Δ6E	26277 - 27146	870	Coding sequence of Δ6-elongase from microalgae *P. cordata* (Petrie et al., 2010a)
TMV 5' UTR leader	27150 - 27214	65	Enhancer from TMV 59 (Gallie et al., 1987)
PRO_Arath-FAE1	27215 - 28148	934	Promoter of *A. thaliana* FA elongase1 (Rossak et al., 2001)
MAR_Nicta-RB7	28157 - 29324	1168	Rb7 matrix attachment regions of *N. tabacum* (Hall et al., 1991; Halweg et al., 2005)
TER_Linus-Cnl1	29325 - 30058	734	Terminator of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
Lackl-Δ12D	30059 - 31312	1254	Coding sequence of Δ12-desaturase from yeast *L. kluyveri* (Petrie et al., 2012)
TMV 5' UTR leader	31316 - 31380	65	Enhancer from TMV 59 (Gallie et al., 1987)
PRO_Linus-Cnl1	31381 - 31830	450	Promoter of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
TER_Linus-Cnl2	31831 - 32368	538	Terminator of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
Pavsa-Δ4D	32369 - 33715	1347	Coding sequence of Δ4-desaturase from microalgae *P. salina* (Zhou et al., 2007)
TMV 5' UTR leader	33719 - 33783	65	Enhancer from TMV 59 (Gallie et al., 1987)

Genetic Element	Location in Vector	Length in bp	Feature and Source
PRO_Linus-Cnl2	33784 - 35816	2033	Promoter of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
PRO_Linus-Cnl1	35817 - 36266	450	Promoter of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
TMV 5' UTR leader	36267 - 36331	65	Enhancer from TMV 59 (Gallie et al., 1987)
Picpa-ω3D	36335 - 37585	1251	Coding sequence of Δ15−/ω3-desaturase form yeast *P. pastoris* (Zhang et al., 2008)
TER_Linus-Cnl1	37586 - 38319	734	Terminator of *L. usitatissimum* conlinin1 (Chaudhary et al., 2001)
MAR_Nicta-RB7	38320 - 39487	1168	Rb7 matrix attachment regions of *N. tabacum* (Hall et al., 1991; Halweg et al., 2005)
TER_Agrtu-NOS	39488 - 39742	255	Terminator of *A. tumefaciens* nopaline synthase (Bevan 1984; Rogers et al., 1985; Sanders et al., 1987)
Pavsa-Δ5D	39743 - 41023	1281	Coding sequence of Δ5-desaturase from microalgae *P. salina* (Zhou et al., 2007)
TMV 5' UTR leader	41027 - 41091	65	Enhancer from TMV 59 (Gallie et al., 1987)
PRO_Brana-FP1	41092 - 41449	358	Promoter of *B. napus* napin (Stalberg et al., 1993)
TER_Glyma-Lectin	41450 - 41783	334	Terminator of *G. max* lectin (Vodkin et al., 1983; Choi et al., 1995)
Pyrco-Δ5E	41799 - 42605	807	Coding sequence of Δ5-elongase from microalgae *P. cordata* (Petrie et al., 2010a)
TMV 5' UTR leader	42609 - 42673	65	Enhancer from TMV 59 (Gallie et al., 1987)
PRO_Arath-FAE1	42674 - 43607	934	Promoter of *A. thaliana* FA elongase1 (Rossak et al., 2001)
PRO_Linus-Cnl2	43614 - 45646	2033	Promoter of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
TMV 5' UTR leader	45655 - 45719	65	Enhancer from TMV 59 (Gallie et al., 1987)
Micpu-Δ6D	45723 - 47117	1395	Coding sequence of Δ6-desaturase from microalgae *M. pusilla* (Petrie et al., 2010b)
TER_Linus-Cnl2	47126 - 47663	538	Terminator of *L. usitatissimum* conlinin2 (Chaudhary et al., 2001)
Multiple cloning site	47664 - 47731	68	Multiple cloning site for vector construction
T-DNA right border (partial)	47732 - 47773	42	*A. tumefaciens* right border sequence used for transfer of T-DNA
***B. napus* 3' flanking sequence (47,774 - 49,789 bp)**			

Genetic Element	Location in Vector	Length in bp	Feature and Source
PTI gene exon 2	47774 - 48002	229	PTI gene exon 2
PTI gene intron 3	48003 - 48080	78	PTI gene intron 3
PTI gene exon 3	48081 - 48207	127	PTI gene exon 3
PTI gene intron 4	48208 - 48297	90	PTI gene intron 4
PTI gene exon 4	48298 - 48571	274	PTI gene exon 4
PTI gene intron 5	48572 - 48793	222	PTI gene intron 5
PTI gene exon 5	48794 - 48926	133	PTI gene exon 5
PTI gene intron 6	48927 - 49071	145	PTI gene intron 6
PTI gene exon 6	49072 - 49158	87	PTI gene exon 6
PTI gene intron 7	49159 - 49397	239	PTI gene intron 7
PTI gene exon 7	49398 - 49538	141	PTI gene exon 7
PTI gene 3' UTR	49539 - 49789	251	PTI gene 3' UTR

V. Characterization of the Genetic Modification

1. Overview

DHA canola was characterized with vector-targeted sequencing, whole-genome sequencing (WGS) and PCR amplicon sequencing. Sequencing indicated that the DHA canola contained no vector backbone, no binary vector bacterial selectable marker gene neomycin phosphotransferase II (*nptII*) or 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 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.

2. Analysis of the Insert and its Genetic Element

A. Structure of the insert and genetic elements

A total of 49,789 bp sequence, including 46,614 bp T-DNA and 3,175 bp (1,159 bp upstream and 2,016 bp downstream) flanking canola sequences, was characterized for the A05 T-DNA insert by genomic DNA sequencing. The A05 T-DNA insert was found to contain two eight-gene sets from binary vector pJP3416_GA7-ModB. The two eight-gene sets were linked by 156 bp of palindromic left border sequence and flanked by 40 bp of right border (RB) sequence upstream and 42 bp of RB sequence downstream, which formed a palindromic structure with RB-LeftBorder (LB):LB-RB orientation (Figure 4, Table 7). The sequence of the A05 T-DNA perfectly matched the reference sequence of the transformation vector, and thus no AA variations were observed in the protein sequences of the eight genes compared to their references. The integration of the T-DNA

insert with canola genomic sequence did not disrupt the expression of the eight genes since the two sets of eight genes still had their own complete expression cassettes. The palindromic structure did not affect the expression of the transgenes at this locus. The A05 T-DNA insert replaced a 20 bp of *B. napus* genomic DNA sequence from the second exon of the pto-interacting (*pti*) gene, and disrupted the expression of *pti* gene, which encodes a serine-threonine kinase involved in the hypersensitive response-mediated signaling and disease resistance located on chromosome A05 of *B. napus* reference genome. The knockout of *pti* gene did not have deleterious effects on canola plant based on the data from the breeding program and field trial observations. Annotation of the A05 T-DNA insert is shown in Figure 4.

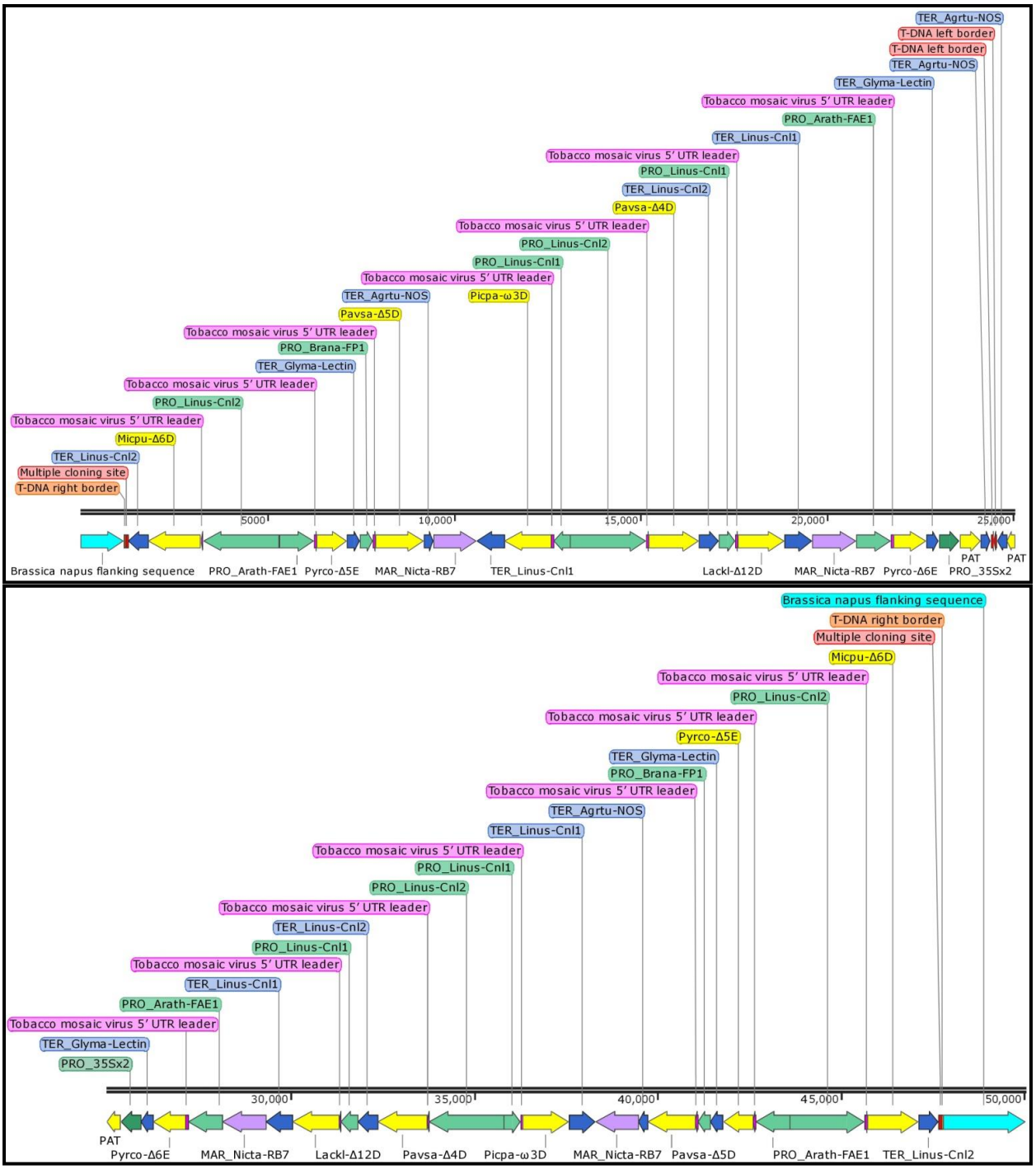

Figure 4. Annotation of the A05 T-DNA insert in DHA canola

A 15,004 bp sequence, which included 12,110 bp T-DNA and 2,894 bp (2,089 bp upstream and805 bp downstream) flanking canola sequences, was characterized for the A02 T-DNA insert by genomic DNA sequencing. WGS and PCR amplicon sequencing confirmed that the A02 T-DNA insert had a partial insert of the eight-gene cassette from binary vector pJP3416_GA7-ModB. The

A02 T-DNA insert was found to contain complete gene expression cassettes (including promoter, coding sequence, and terminator) for Micpu-Δ6D, Pyrco-Δ5E, Pavsa-Δ5D and Picpa-ω3D but did not contain gene expression cassettes for Pavsa-Δ4D, Lackl-Δ12D and Pyrco-Δ6E and PAT (Figure 5; Table 6). The sequence of the A02 T-DNA perfectly matched the reference of vector pJP3416_GA7-ModB; thus, no AA variations were observed in the protein sequences of the four genes compared to their references. The integration of the T-DNA insert with the canola genome did not disrupt the expression of the four genes, since the four genes in T-DNA still had their own intact expression cassettes. Annotation of the A02 T-DNA insert is shown in Figure 5. A 43 bp residual right border sequence was observed upstream of the T-DNA. The sequence of the A02 T-DNA insert perfectly matched the reference of vector pJP3416_GA7-ModB; thus, no AA variations were observed in the protein sequences of the four genes compared to their references.

The A02 T-DNA insert replaced 15 bp of the *B. napus* genomic DNA sequence (GTAGCACGACAAGTT) from the 3' UTR of the HPP gene. The HPP gene is located on chrUn_random of *B. napus* reference genome (Darmor) at position 118589903 - 118591677 and on chromosome A02 of the *B. rapa* reference genome (Chiifu) at position 18569298 - 18571066. According to the National Center for Biotechnology Information (NCBI) *B. napus* Annotation Release 100, the HPP gene has not been characterized and its function is unknown. The 15 bp deletion may or may not affect the expression and function of the HPP gene. However, no deleterious or adverse effects on any growth parameter, yield or oil composition were observed with DHA canola.

Eight *B. napus* expressed sequence tags (EST) which had 99% - 100% similarity with HPP gene, were identified from NCBI EST database for HPP gene annotation. The EST-based annotation indicated that HPP gene had two exons, one intron and 147 AAs. The A02 T-DNA insert didn't interrupt the expression of the HPP gene.

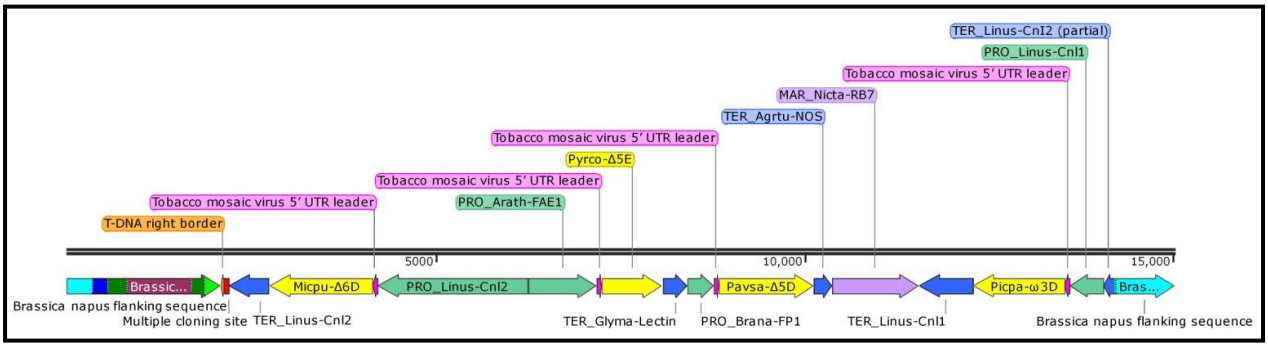

Figure 5. Annotation of the A02 T-DNA insert in DHA canola

B. Absence of plasmid backbone sequences

Two DHA canola T3 lines and six DHA canola T4 lines were sequenced to characterize the presence/absence of the vector backbone and *A. tumefaciens* sequences, insert copy number and T-DNA integration sites in DHA canola. The T-DNA region in eight DHA canola lines were enriched through PCR amplifications with vector-specific primers, and sequenced with Illumina MiSeq Platform (Illumina, San Diego, CA, U.S.A.).

QC-passed sequence reads ranged from 2,018,824 in line B0050-027-18-36-13 to 2,828,125 in line B0050-027-18-20-13. The percentage of sequence reads that aligned to the vector reference sequence of pJP3416_GA7-ModB varied from 19.7% (511,092 reads) in line B0050-027-18-36-16 to 38.9% (618,072 reads) in B0050-027-110-40; this indicated the T-DNA were highly enriched through vector-captured technology. The sequence reads aligned to the vector reference were selected for further analysis, while the remaining reads represented off-target amplification and were discarded.

The vector-targeted sequence reads from the eight DHA canola lines (T3 and T4) were all mapped to pJP3416_GA7-ModB at region from 3,518 to 26,827 bp where the T-DNA (3,398 - 26,909 bp; Table 5) was located; no sequence reads from the eight DHA canola lines were mapped to the vector backbone region (1 - 3,397 bp and 26,910 - 31,564 bp; Table 5). This demonstrated that DHA canola had at least one set of eight genes from binary vector pJP3416_GA7-ModB and DHA canola had no vector backbone sequence or antibiotic marker gene (*nptII*). It also demonstrated the high stability and purity in different generations and different lines.

Agrobacterium-mediated gene transformation only transfers the T-DNA region in a binary vector and doesn't transfer the bacterium genomic sequence. Also, the DHA canola has been propagated by seed through seven generations and *Agrobacterium* is not normally transmitted from one generation to the next via seeds. Further, sequencing of the DHA canola lines has found no evidence of the presence of *Agrobacterium* genome in the DHA canola lines.

3. Stability of the Insert across Generations

The DHA canola event has been advanced seven generations through single seed descent. Stability of the DHA canola insert was shown to be stable based on both phenotypic and genetic analysis. As shown in Figure 2, and further explored in Experiments 1 and 2, below, the transgenic loci were stably inherited from T0 to T7 generation, and the seed consistently produced the modified oil phenotypic trait with the desired amounts of DHA in T2 to T7 seeds in different environments. Chi-squared (χ^2) test and genetic analyses also demonstrated that the T-DNA inserts were stably inherited in different backgrounds (elite canola lines) and populations per Mendelian principles. DHA canola lines with both T-DNA inserts stably produced significant amounts of DHA in different genetic backgrounds and different environments.

Additionally, junction-specific PCR and Kompetitive Allele-Specific PCR (KASP) genotyping was used for genetic stability testing, trait purity testing and introgression to elite lines (LGC,

Boston, MA, U.S.A.; http://www.lgcgroup.com/products/kasp-genotyping-chemistry/). Four molecular markers were developed targeting the junctions of the two T-DNA inserts. Analyses of more than 593 seeds from generations T3 to T7, sequencing of twelve transgenic lines from generations T3 to T5, oil profiling, and inheritance analysis of F2 populations demonstrated that the genetic stability and trait purity of DHA canola were well maintained from one generation to the next, and from AV Jade to different genetic backgrounds in different years and locations.

As noted above, the ω3 FA trait is controlled by two independent transgenic loci: one on chromosome A05 (designated A = A05; a = wt, or wild type) and one on chromosome A02 (designated B = A02; b = wt, or wild type). For descriptive purposes, the A05 and A02 T-DNA inserts were denoted "Locus A" and "Locus B" and isoline or wildtype counterparts were denoted "a" and "b", respectively. Various crosses were made between DHA canola and non-GE varieties. For example, six BC_1F_2 populations were developed from crosses between six elite, non-GE lines and event B0050-027-18-20 T3. After the initial hybridization, resulting F_1 seed was then backcrossed with each elite non-GE line (Recurrent Parent) to produce BC_1F_1, and the BC_1F_1 with heterozygous Locus A and Locus B T-DNA inserts (AaBb) were used to generate BC_1F_2 populations through selfing (Experiment 1, Figure 6; Tables 10 and 11).

Additionally, six F_2 populations were generated from crosses between six elite, non-GE lines and DHA canola (event B0050-027-18-20-12-19 T5); F_1 seed was then used to generate F_2 populations through selfing. Twelve F_2 populations were produced (six recurrent parents by each of the two loci) and were expected to be segregating for both Locus A and Locus B T-DNA inserts (Experiment 2, Figure 8; Table 12).

Progeny plant tissue from each experiment was screened using digital PCR to estimate the locus copy number value based on specific genes (i.e. *pat*, Δ6 desaturase) and markers (KASP assay) specific to each locus insert.

Further, progeny seed FA content was determined using solvent extraction, followed by simultaneous saponification and methylation and analysis by GC-FID. This involved using an in-house method whereby seed samples were crushed and the oil was extracted from a crushed seed subsample into solvent. Samples were heated at 40 °C to speed up the reaction and then injected on GC-FID using a BPX-70 column for FA determination. Fatty acids were calculated as % composition of the oil where the area of each FA peak was determined as a percentage of the sum of all the FA peaks in the chromatogram. The % LC-PUFA was calculated as EPA% + DPA% + DHA%.

As noted above, for descriptive purposes the T-DNA inserts were denoted as "A" and "B" and wild-type counterparts were denoted "a" and "b", respectively. χ^2 test was used to check whether the segregation of the Locus A and Locus B T-DNA inserts in the F_2 populations fit Mendelian inheritance patterns, as shown below in Tables 8 and 9. F_1 progeny expected from an initial transgenic x wild-type cross include the following:

- 1 (AABB) : 1 (AAbb) : 2 (AABb) : 2 (AaBB) : 4 (AaBb) : 2 (Aabb) : 2 (aaBb) : 1 (aaBB) : 1 (aabb) for both T-DNA inserts;
- 1 (AA) : 2 (Aa) : and 1 (aa) for the Locus A T-DNA insert ; and
- 1 (BB) : 2 (Bb) : 1 (bb) for the Locus B T-DNA insert .

For identification purposes, colors were used for zygosity of lines illustrated in Tables 8 and 9:

- Red = wild type
- Orange = heterozygous for Locus B; wild type for Locus A
- Yellow = homozygous for Locus B; wild type for Locus A
- Green = other remaining variants heterozygous/homozygous for Locus A and/or Locus B
- Blue = homozygous for Locus A; homozygous for Locus B

Table 8. Expected two loci segregation of BC1F2 progeny

	AB	**Ab**	**aB**	**ab**
AB	AABB	AABb	AaBB	AaBb
Ab	AABb	AAbb	AaBb	Aabb
aB	AaBB	AaBb	aaBB	aaBb
ab	AaBb	Aabb	aaBb	aabb

Table 9. χ2 test for phenotype

Genotype	**Locus A05**	**Locus A02**	**Expected % of progeny**	**Expected Observable % LC PUFA**	**Expected number of progeny**	**Observed number of progeny**
aabb	wt	wt	0.0625	0	7.4	15
aaBB	wt	Hom	0.0625	<1	7.4	7
aaBb	wt	Het	0.125	<1	14.9	15
Aabb	Het	wt	0.125	>1 -7.5	81.8	75
AaBb	Het	Het	0.25	>1 -7.5		
AaBB	Het	Hom	0.125	>1 -7.5		
AAbb	Hom	wt	0.0625	>1 -7.5		
AABb	Hom	Het	0.125	>1 -7.5		
AABB	Hom	Hom	0.0625	> 7.5	7.4	7
$\chi 2 = 8.31$; $P<0.05$; df: 4 = 9.49; Chi test = 0.08						

Experiment Number 1

As introduced above, DHA canola was backcrossed to six recurrent parents (non-GE lines), and the F_1 progeny were crossed to the recurrent parents, then selfed to produce BC_1F_2 generation progeny (Figure 6). Mendelian inheritance was measured by both the % LC-PUFA phenotype (five phenotypes expected) and genotype (nine genotypes expected).

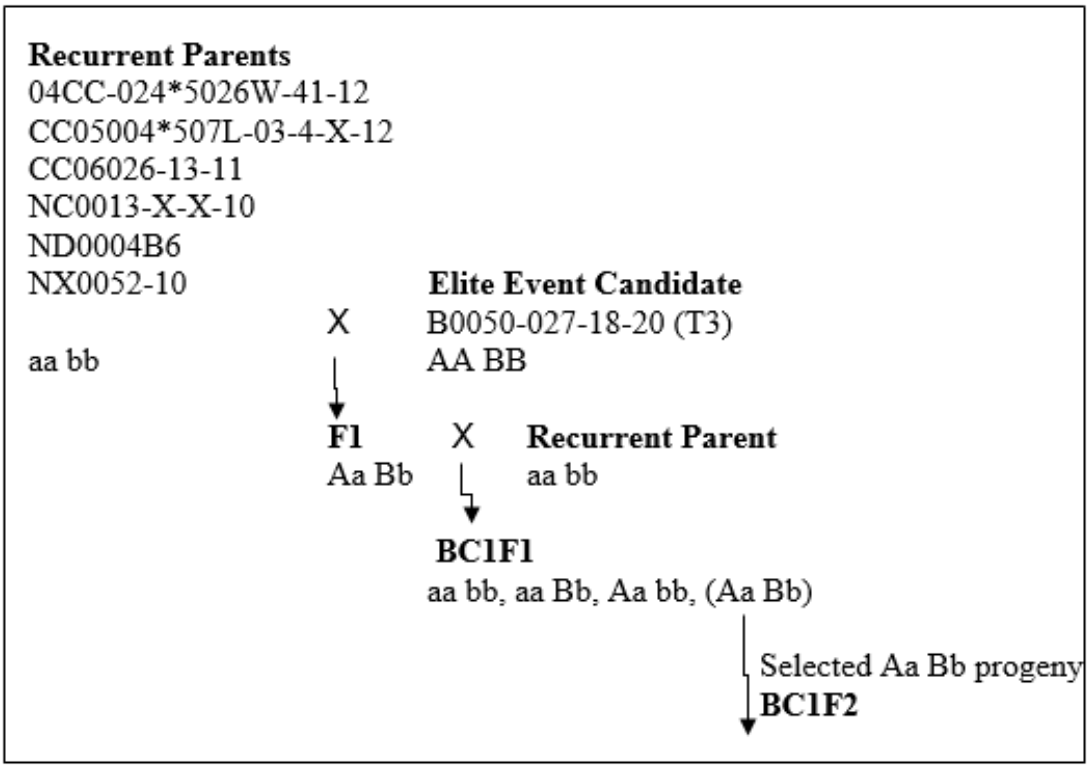

Figure 6. Schematic of crosses to produce BC1F2 progeny (Experiment 1)

The genotype and phenotype results for Experiment 1 are presented below. The segregation observed is the expected Mendelian inheritance pattern, with the expected/observed number of progeny. The expected % LC-PUFA are presented in Table 10 ($\chi 2 = 14.04$; P (0.05) df: 8 = 15.51; Chi test = 0.08), and the mean and standard deviation (SD) of the % LC-PUFA for the nine phenotypes are presented in Table 11. In combination, the two loci produce an increase of LC-PUFA. A Chi test of 0.08 was observed in both analyses (Tables 10 and 11).

Table 10. $\chi 2$ test for genotype

Genotype	Locus A05	Locus A02	Expected % of progeny	Expected % LC PUFA	Expected progeny	Observed progeny
aabb	wt	wt	0.0625	0	7.1	12
aaBB	wt	Hom	0.0625	<1	7.1	7
aaBb	wt	Het	0.125	<1	14.1	15
Aabb	Het	wt	0.125	>1 -7.5	14.1	17
AaBb	Het	Het	0.25	>1 -7.5	28.3	35
AaBB	Het	Hom	0.125	>1 -7.5	14.1	14
AAbb	Hom	wt	0.0625	>1 -7.5	7.1	3
AABb	Hom	Het	0.125	>1 -7.5	14.1	6
AABB	Hom	Hom	0.0625	> 7.5	7.1	4

Table 11. Observed mean and SD for % LC-PUFA for BC1F2 segregants

			Across Family	Across Family
			Mean	SD
Genotype	Locus A05	Locus A02	% LC PUFA	% LC PUFA
aabb	wt	wt	0.00	0.00
aaBb	wt	Het	0.23	0.11
aaBB	wt	Hom	0.45	0.14
Aabb	Het	wt	2.66	1.54
AAbb	Hom	wt	3.42	1.68
AaBb	Het	Het	4.20	1.40
AaBB	Het	Hom	5.95	0.96
AABb	Hom	Het	7.04	2.29
AABB	Hom	Hom	7.74	0.92

In addition, seed FA content was analyzed and the observed mean and SD for the production of LC-PUFA according to each genotype of the BC1F2 progeny was gathered, and is presented in Figure 7, below.

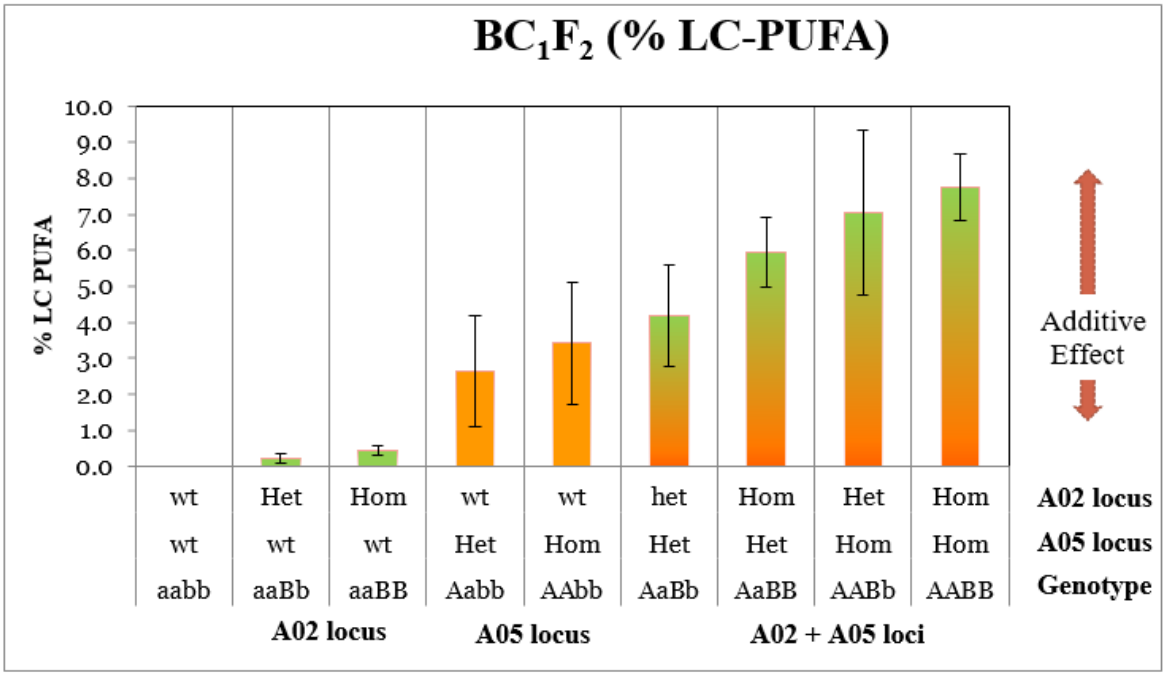

Figure 7. Observed mean and SD for the % LC-PUFA for each BC1F2 genotype

Experiment Number 2

In another set of breeding experiments, the elite event was crossed with a number of non-GE recurrent parents. The F_1 progeny were selfed to produce F_2 generation progeny (Figure 8).

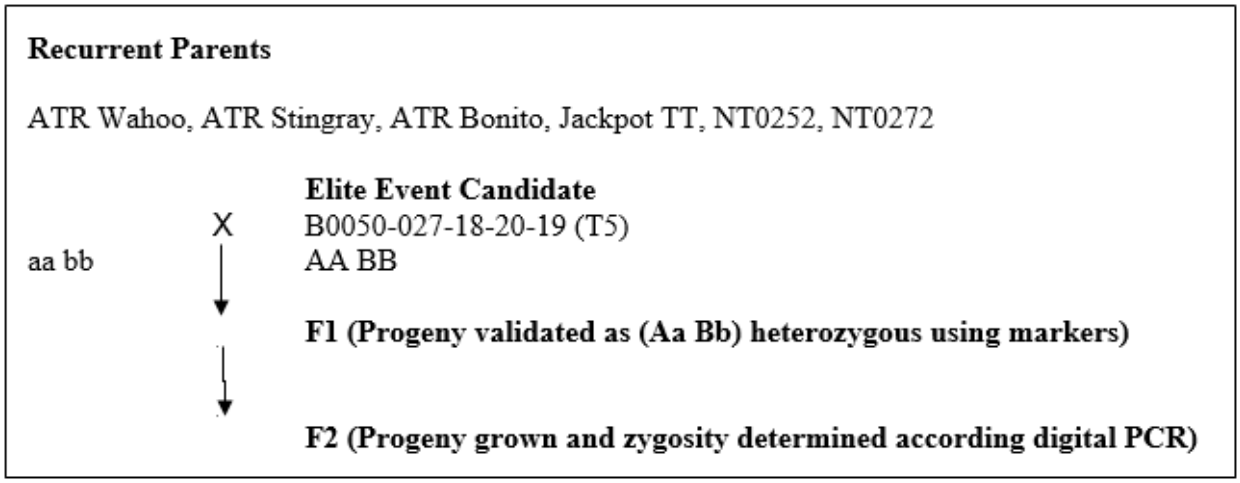

Figure 8. Schematic of crosses to produce F2 progeny (Experiment 2)

Expected inheritance is reflected in Table 12. Mendelian inheritance was determined for each locus separately by genotype.

Table 12. Expected single locus segregation for F2 progeny (1:2:1)

	A	a		B	b
A	AA	Aa	**B**	BB	Bb
a	Aa	aa	**b**	Bb	bb

Twelve F_2 progeny lines were developed. The genotype results for two recurrent parents (ATR Stingray and ATR Wahoo) are presented for Locus A in Table 13 (ATR Stingray) and Table 14 (ATR Wahoo) (χ^2 = 4.32; P (0.05) df: 2 = 5.99; Chi test = 0.12), and for Locus B in Table 15 (ATR Stingray) and Table 16 (ATR Wahoo). These four results were representative of all the F_2 progeny lines. (The results for the remaining eight F_2 progeny lines are not presented.) The segregation observed fits the expected Mendelian inheritance pattern, with a χ^2value of 2.25 and 4.32 for Locus A and 2.39 and 4.42 for Locus B.

Table 13. ATR Stingray locus A genotype χ2 test

Pedigree	**Zygosity**	**F_2 Locus A Observed**	**F_2 Locus A Expected 1:2:1**
ATR Stingray / B0050-027-18-20-12-19	Hom	13	16
	Het	38	32
	wt	13	16
		64	64
$\chi2$ = 2.25; *P*<0.05; df: 2 = 5.99; Chi test = 0.32			

Table 14. ATR Wahoo locus A genotype χ2 test

Pedigree	Zygosity	F_2 Locus A Observed	F_2 Locus A Expected 1:2:1
ATR Wahoo / B0050-027-18-20-12-19	Hom	9	15.5
	Het	38	31.0
	wt	15	15.5
		62	62.0
χ2 = 4.32; P (0.05) df: 2 = 5.99; Chi test = 0.12			

Table 15. ATR Stingray locus B genotype χ2 test

Pedigree	Zygosity	F_2 Locus B Observed	F_2 Locus B Expected 1:2:1
ATR Stingray / B0050-027-18-20-12-19	Hom	13	16
	Het	38	32
	wt	13	16
		64	64
χ^2 = 2.39; P (0.05) df: 2 = 5.99; Chi test = 0.30			

Table 16. ATR Wahoo locus B genotype χ2 test

Pedigree	Zygosity	F_2 Locus B Observed	F_2 Locus B Expected 1:2:1
ATR Wahoo / B0050-027-18-20-12-19	Hom	10	15.5
	Het	39	31.0
	wt	13	15.5
		62	62
χ2 = 4.42; P (0.05); df: 2 = 5.99; Chi test = 0.11			

The homozygous F_2 progeny for Locus A and Locus B were grown out and F_3 pooled seed data is presented in Table 17, showing a consistent high level of % LC-PUFA.

Table 17. % LC-PUFA in F2 derived F3 pooled seed for homozygous genotypes

F_2 derived: F_3 pooled Seed Pedigree	Locus A05 insert	Locus A02 insert	% LC-PUFA
ATR Bonito / B0050-027-18-20-12-19	Hom	Hom	17.6
ATR Stingray / B0050-027-18-20-12-19	Hom	Hom	8.9
ATR Wahoo / B0050-027-18-20-12-19	Hom	Hom	9.5
Jackpot TT / B0050-027-18-20-12-19	Hom	Hom	8.2
NT0252 / B0050-027-18-20-12-19	Hom	Hom	12.6
NT0272 / B0050-027-18-20-12-19	Hom	Hom	7.6

Experiment Number 3

Various homozygous materials covering T_3 through T_7 generations from glasshouses and field trials were analyzed for the percentage of DHA in canola seed oil to confirm stability of the trait.

Observations of the percentage DHA in seed oil expressed over seven generations of selfing of DHA canola, (Table 18) confirm stability of this trait for a range of growing environments. The candidate line is shown to be fixed and stable from T_3 – T_7 (five generations).

Table 18. Seed oil % DHA expression over seven generations

	Line	Seed % DHA	Seed Sample	Experiment Location	Geographical Location
T1	**B0050-027**	5.7	Single Plant Pooled Seed	Glasshouse	Horsham
T2	**B0050-027-18**	9.5	Single Plant Pooled Seed	Glasshouse	Horsham
T3	**B0050-027-18-20**	12.6	Single Plant Pooled Seed	Glasshouse	Horsham
T3-X	**B0050-027-18-20-X**	8.9	Bulk	Field	Nurrabiel: Winter 2014
T3-X-X	**B0050-027-18-20-X-X**	8.4	Bulk	Field	Colac: Summer 2014-15
T3-X-X-X	**B0050-027-18-20-X-X-X**	9.0	Bulk	Field	St Helens Plains Winter
T4	**B0050-027-18-20-12**	11.9	Single Plant Pooled Seed	Glasshouse	Horsham
T5	**B0050-027-18-20-12-19**	13.4	Single Plant Pooled Seed	Glasshouse	Horsham
T5-X	**B0050-027-18-20-12-19-X**	12.7	Bulk	Field	Nurrabiel: Winter 2015 (Tent 1)
T5-X-X	**B0050-027-18-20-12-19-X-X**	9.8	Bulk	Field	Colac: Summer 2015-16
T6	**B0050-027-18-20-12-19-10**	12.9	Single Plant Pooled Seed	Glasshouse	Horsham
T6-X	**B0050-027-18-20-12-19-10-X**	17.3	Bulk	Field	Colac: Summer 2015-16 (Tent 1)
T7	**B0050-027-18-20-12-19-10-309**	13.8	Single Plant Pooled Seed	Glasshouse	Horsham

Genetic stability was further confirmed in four assays (EAD02DJ517, EA05DJ380, EA02UJ284 and EA05UJ200) designed to amplify the junction regions of the two T-DNA inserts of DHA canola in T3 - T7 seeds (Figure 9). To facilitate the post-PCR multiplexing for electrophoresis, the four assays were designed with optimized amplicon size distribution (200, 284, 380 and 517 bp, respectively), and all spanned the novel joints of *B. napus* genome and the T-DNA. Note that a fifth junction occurs in the middle of the palindromic structure, but since there is no endogenous canola DNA at that junction it cannot be used for testing purposes.

A total of 100 seeds (20 seeds / generation) from T3 to T7 generation were used for genetic stability testing of DHA canola from one generation to the next. Each DNA sample was PCR amplified individually with one of the four assays. After PCR amplification, the PCR amplicons of the four assays were mixed, and used for electrophoresis. After electrophoresis, amplicons of the same expected sizes were observed in all 100 DHA canola seeds from T3 to T7 generation for each assay (Figure 9). This indicated the high generational stability of DHA canola.

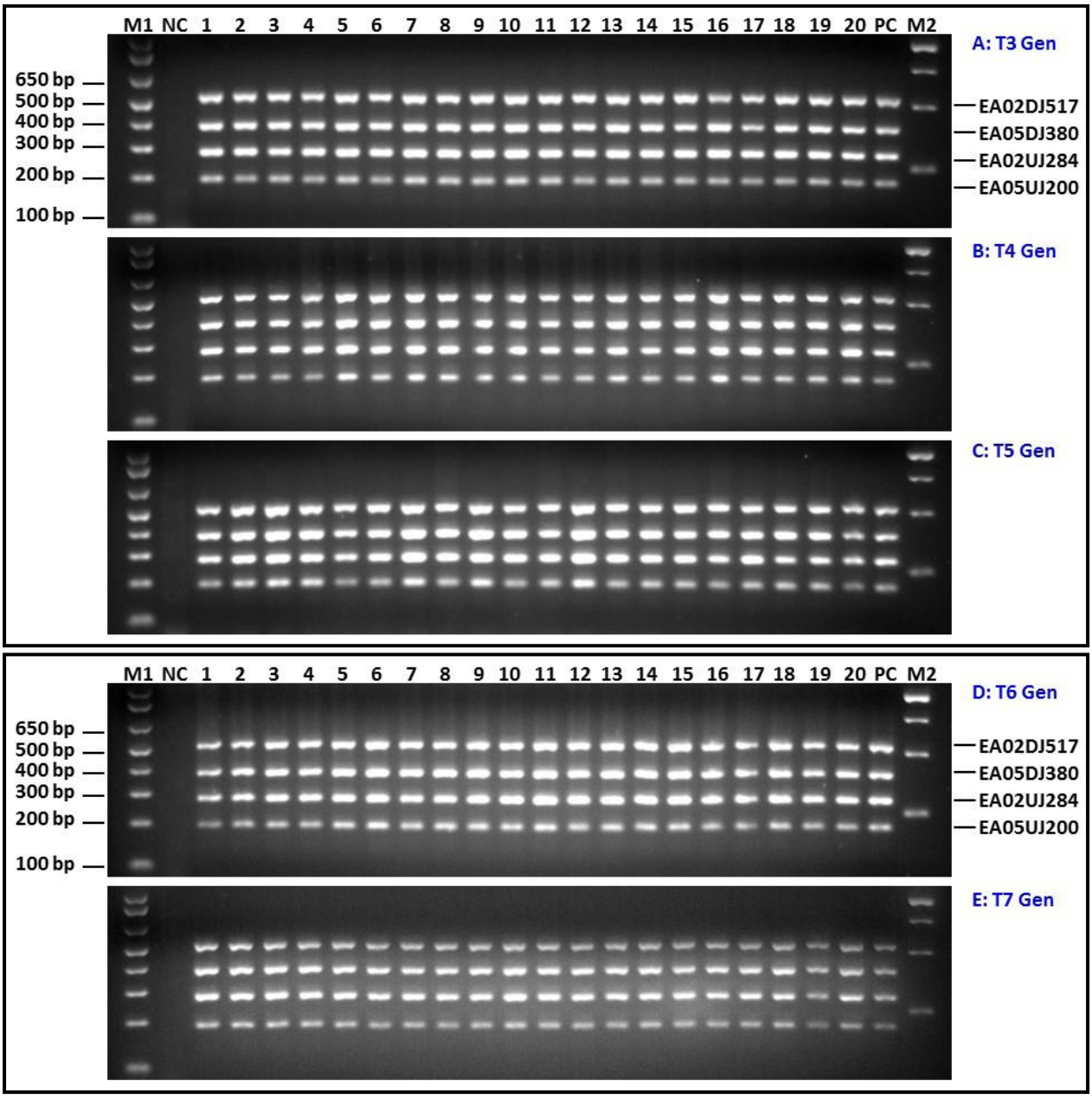

Figure 9. PCR of all four borders of five generations of DHA canola

PCR amplicon sizes of DHA canola T3 (**A**), T4 (**B**), T5 (**C**), T6 (**D**) and T7 (**E**) seeds genotyped with four assays (EAD02DJ517, EA05DJ380, EA02UJ284 and EA05UJ200). Each assay targeted one junction region of the two T-DNA inserts. All 100 seeds from T3 to T7 generations (20 seeds / generation) had the same expected amplicon sizes of 517 bp, 380 bp, 284 bp and 200 bp for EAD02DJ517, EA05DJ380 EA02UJ284 and EA05UJ200, respectively. M1: 1 Kb+ DNA Ladder (Invitrogen): M2: GeneRuler 1 Kb DNA Ladder (Thermo Scientific); NC: negative (non-GMO) control AV Jade; PC: DHA canola positive control (T5 seed B0005-027-18-20-12-19). Numbers 1 to 20 indicated 20 DHA canola seeds from each of T3 to T7 generations.

4. Analysis of potential new open reading frames

Bioinformatics searches were conducted to compare the AA sequence of each expressed protein in DHA canola against the curated AllergenOnline.org database version 16 (January 2016), which is maintained at the University of Nebraska, as well as searches against the National Center for Biotechnology Information (NCBI) protein databases using its basic local alignment search tool for proteins (BLASTP) with keyword limits for allergy and toxicity. Search criteria were set to identify matches of known allergens to predict potential cross reactivity. These criteria represent minimal risks when comparisons are made over stretches of 80 AA that do not show >35% identity to potential allergens, did not share a sequence of ≥ 8 AA consecutive identical AA with a potential allergen, as well as comparisons with sequences in NCBI Protein database for sequence matches of 50% or more with keyword limits (allergen, allergy, toxic) for risks of allergy and toxicity.

Translation of the five junction sequences with all six reading frames identified 25 hypothetical Stop-to-Stop open reading frames (ORFs) with 7 to 126 AAs, which spanned the five novel junctions created by the integration of the two T-DNA inserts within the DHA canola genome. While the fifth junction crosses the palindromic junction, with no endogenous canola DNA, ORF analysis was still conducted. Bioinformatics search of NCBI protein databases and Food and Allergen Research and Resource Program (FARRP) Allergen Protein Database (Version 16) with the 25 hypothetical ORFs did not identify any significant near-full-length matches even at E value = 0.1, 35% identity matches over 80 AA segments and eight-AA contiguous matches (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 (2003).

Summary

Two independent locus inserts are associated with the production of ω3 LC-PUFA in the seed. The results confirm that the LC-PUFA trait in seed oil follows a Mendelian inheritance pattern as expected for two independent loci contributing to expression. The study also confirms that the two loci denoting this trait together increase the expression of the percentage of LC-PUFA in DHA canola seed oil. Pooled seed samples from selfing of the DHA canola over seven generations of individual plants and bulk seed lots show that the seed oil percentage of DHA is highly stable for a wide range of growing environments.

Junction-specific PCR and KASP genotyping was used for genetic stability testing, trait purity testing and DHA canola introgression to elite lines. Four molecular markers were developed targeting the four junctions of the two T-DNA inserts. Analyses of more than 593 seeds from generations T3 to T7, sequencing of 12 transgenic lines from generations T3 to T5, oil profiling, and inheritance analysis of F2 populations demonstrated that the genetic stability and trait purity of DHA canola were well maintained from one generation to the next, and from AV Jade to different genetic backgrounds in different years and locations.

The results from these 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. The DHA canola is a stable event as measured across seven generations by both genetic and phenotypic analysis. The analysis of potential ORF did not reveal any similarities to known toxins or allergens.

VI. Characterization and Safety Assessment of the Introduced Proteins

1. Identities and functions of the proteins

Characterizing introduced transgenic proteins in genetically engineered crops remains important for establishing food, feed, and environmental safety. As described previously, DHA canola contains expression cassettes that enable seed-specific production of DHA and DPA. The protein content of each transgenic protein was quantified in DHA canola tissues collected over a growing season. This section summarizes: (1) the identity and function of the proteins produced in DHA canola; (2) assessment of equivalence between the plant-produced and *P. pastoris*-produced proteins; (3) the level of the proteins in plant tissues from DHA canola; (4) assessment of the potential allergenicity of the proteins produced in DHA canola; and (5) the food and feed safety assessment of the proteins produced. The data support a conclusion that the proteins produced in DHA canola are safe for human and animal consumption based on several lines of evidence summarized below.

DHA canola comprises seven FA desaturases and elongases, members of three different FA classes, introduced in a single vector to provide a biosynthetic pathway that converts OA to DHA (Figure 1). This section characterizes each of these enzymes in the order in which they occur within the introduced FA biosynthesis pathway. The pathway described in Figure 1, and the specific enzymes in Table 19, reflect the similarities of enzymes in each functional class. In addition, Table 19 also summarizes each protein and their characteristics (functional activity, molecular weight (MW), theoretical isoelectric point (pI), potential glycosylation sites and representative protein used to characterize digestibility).

Further information is also provided to support the AA sequence identity of these proteins to similar proteins commonly consumed in food, food production or in animal feeds. Each newly expressed protein is compared to others using their AA sequence. For example, the Lackl-Δ12D protein as expressed in DHA canola is listed in row 1 of Table 20. Each column across row 1 describes the level of AA sequence identity of Lackl-Δ12D with each of the other proteins listed in the Table 20. Column number 5, in row 1, identifies the sequence identity Lackl-Δ12D with Zygba-Δ12D as 69.0%. Likewise, column number 9, in row 1, compares Lackl-Δ12D with Orysa-Δ12D as 34.6%. From these tables, all proteins listed within each table can be compared to each of the other proteins, not just to the newly introduced protein. Using Table 20 as an example, the

AA sequence identity of Zygro-Δ12D as compared to Sesin-Δ12D is 36.1 (a cross of row 6 and column 13). These tables (see Table 20, 22, 25, 27, 29, 31, 33) also include the NCBI Accession numbers and sources of each protein used in the comparison.

The two yeast acyl-CoA-type desaturases, Lackl-Δ12D and Picpa–ω3D enzymes, have very similar functions, molecular weights (~48kDa) and pIs (~7.7). The Picpa–ω3D enzyme was used as a representative for yeast acyl-CoA desaturases. The two microalgae type elongases, Pyrco-Δ6E and Pyrco-Δ5E, have very similar functions, molecular weights (~32kDa) and pIs (~9.2). The Pyrco-Δ5E enzyme was used as a representative for microalgae type elongases. Finally, the three front-end desaturases, Micpu-Δ6D, Pavsa-Δ5D and Pavsa-Δ4D, have very similar functions, molecular weights (~50kDa) and pIs (~8.2-9.1). The Pavsa-Δ4D enzyme was used as a representative for the front-end desaturases.

Table 19. Summary of enzymes transformed to produce DHA canola

Protein / Protein Abbreviation	Catalyzes	MW (kDa)	pI	Potential Gly* sites	Digestibility
L. kluyveri Δ12-desaturase / Lackl-Δ12D	$18{:}1^{\Delta 9} \rightarrow 18{:}2^{\Delta 9,12}$	48.2	7.84	1	Picpa–ω3D as representative of yeast acyl-CoA type desaturase
P. pastoris ω3-desaturase / Picpa-ω3D	$18{:}2^{\Delta 9,12} \rightarrow 18{:}3^{\Delta 9,12,15}$	47.8	7.67	2	
M. pusilla Δ6-desaturase / Micpu-Δ6D	$18{:}3^{\Delta 9,12,15} \rightarrow 18{:}4^{\Delta 6,9,12,15}$	52.9	9.00	0	See Pavsa-Δ4D
P. cordata Δ6-elongase / Pyrco-Δ6E	$18{:}4^{\Delta 6,9,12,15} \rightarrow 20{:}4^{\Delta 8,11,14,17}$	33.1	9.09	0	See Pyrco-Δ5E
P. salina Δ5-desaturase / Pavsa-Δ5D	$20{:}4^{\Delta 8,11,14,17} \rightarrow 20{:}5^{\Delta 5,8,11,14,17}$	48.2	8.18	2	See Pavsa-Δ4D
P. cordata Δ5-elongase / Pyrco-Δ5E	$20{:}5^{\Delta 5,8,11,14,17} \rightarrow 22{:}5^{\Delta 7.10.13.16.19}$	31.3	9.33	1	Pyrco-Δ5E as rep of 2 microalgae elongases
P. salina Δ4-desaturase / Pavsa-Δ4D	$22{:}5^{\Delta 7,10,13,16,19} \rightarrow 22{:}6^{\Delta 4,7,10,13,16,19}$	49.3	8.66	0	Pavsa-Δ4D as rep of 3 front-end desaturases
* Potential glycosylation sites					

Several classes of glycans exist, which are widely distributed in nature, including *N*-linked glycans, glycolipids, *O*-GlcNac, and glycosaminoglycans. *N*-linked glycosylation occurs when glycans are attached to asparagine residues on the protein, which is an exclusive feature of plant allergens and could be implied in allergenic sensitization. *O*-linked glycans are most commonly attached to serine or threonine residues through the *N*-acetylgalactosamine residue. N-linked glycans are the most common in plants, and typically, can only be found as a linkage to an asparagine residue (N) where it is flanked on the C-terminal side by X-S or X-T. The number of potential glycosylation sites are identified for each FA pathway enzyme.

2. Digestibility / Stability of the proteins

The allergenic potential of a protein is determined by weight of evidence approach because no single method can predict the allergenicity of a protein. Protein digestibility is one aspect of the overall allergenicity assessment that is conducted for transgenic proteins expressed in GE crops.

We determined the digestibility / stability of three representative enzymes from each enzyme class: Picpa-ω3D, Pyrco-Δ5E and Pavsa-Δ4D. In the absence of functioning antibodies against these integral membrane proteins, as typically used in traditional Western blot analysis, a sensitive LC-MS analytical method was developed. Digestibility / stability of these three proteins were assessed with an *in vitro* stability assay using a standard protocol (Thomas et al., 2004), followed by LC-MS analysis. Two test systems, pepsin digestion (representing simulated gastric fluid, SGF) and a combined pepsin-trypsin digestion, were utilized independently to test the stability of each of the three proteins. SGF contained the proteolytic enzyme pepsin in a buffer adjusted to an acidic pH 1.2, using a highly purified form of pepsin, formulated at an enzyme:protein reaction ratio of 3:1. In parallel, each protein was tested in combination with a novel pepsin-trypsin assay, employing mass spectrometry to monitor the precise degradation products. The extent of protein digestion was evaluated by the appearance of peptic peptide products and the disappearance of tryptic peptide products (the latter as a proxy for intact protein).

The complete digestion of a protein by a single enzyme is difficult to judge, especially when employing a non-specific enzyme such as pepsin. Although it is possible to judge the disappearance of the intact protein on a gel or by Western blotting techniques, the protein may be hydrolyzed once (cleaved at a single site) or multiple times often resulting in small and overlapping fragments. Allergenic reactions require that a protein or protein fragment simultaneously bind to two IgE molecules to induce mast cell degranulation. This IgE binding places theoretical limits on the peptide size of between 1500 and 3500 daltons. Gel analysis with various staining or antibody techniques is typically able to detect peptides down to approximately 3,000 daltons. When employing gel analysis solely, to judge the completeness of digestion, a high level of purity is required. When employing antibodies, the hydrolysis of a protein by a proteolytic enzyme may result in cleavage of the antigenic site (epitope) thus rendering antibody-based detection methods unsuitable. Likewise, cleavage of a protein at a single site in a protein may yield two protein fragments, in which one may contain the epitope (recognized by a monoclonal antibody) while the other does not. In this instance, large protein fragments may evade detection.

By using LC-MS/MS analysis, the peptide products resulting from both pepsin and trypsin digestions were first determined qualitatively and subsequently a quantitative LC-MS/MS method was developed (See protein Appendix A for further method details). LC-MS analysis is capable of simultaneously monitoring peptides spanning the entire protein sequence that are generated by proteolytic digestion. 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.

3. Theoretical prediction of digestion curves

Upon digestion with pepsin alone, there are a number of scenarios that may occur (Figure 10A). The simplest one is when the protein is rapidly digested to produce fully peptic fragments wherein the response rapidly increases reaching a maximum and creating a plateau (filled circle). The second one involves the slow digestion that does not reach a plateau within the experimental duration (filled triangles). This scenario is difficult to judge for completeness as LC-MS monitors the peptide response (peptide peak intensity or area). The third one involves a rapid, but incomplete digestion that may appear to be complete as judged by the plateau in peptide response (empty circles). Lastly, slow and incomplete digestion may be observed (empty triangles).

By employing trypsin post-pepsin (Figure 10B), it is possible to judge the completeness of the digestion by comparison to an experimental control (time 0, no pepsin added) wherein the tryptic peptides liberated appear at the maximum value (in this instance as the multiple reaction monitoring (MRM) peak area). If the protein is not digested, then no decrease in peptide response will be observed (circles, dashed line). If the protein is partially digested, a partial decrease in the peptide response will be observed (squares, dotted line). If the protein is completely digested, the peptide response will drop to zero within the experiment duration (triangles, solid line).

Thus, by examining the pepsin proteolytic fragments, the breakdown of a protein could be monitored, but note the difficulty in determining whether degradation has reached completion. To overcome this challenge, the tryptic peptide products were used as a proxy for intact protein, wherein in the absence of pepsin, the amount of tryptic peptide was equivalent to 100% protein. In the presence of pepsin (at varying time points during digestion), the level of tryptic peptides would be expected to decrease for peptides that contained a pepsin cleavage site. In this way, the complete degradation of the protein could be monitored.

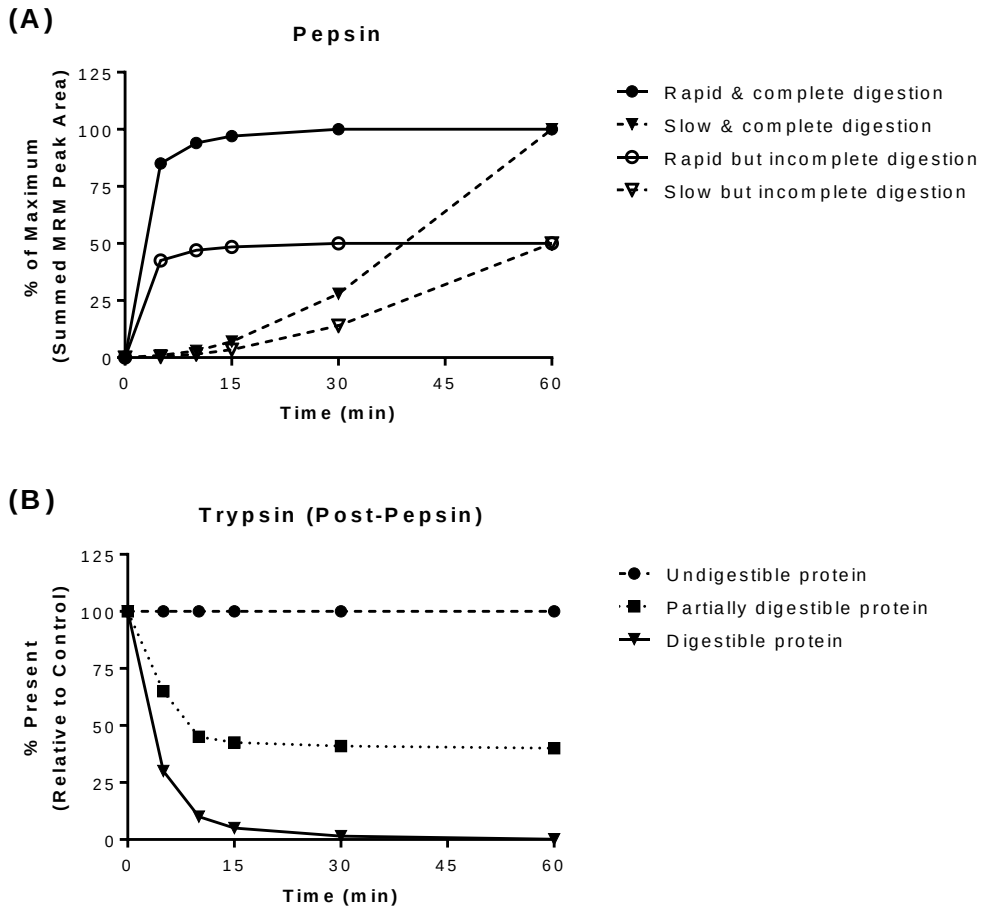

Figure 10. Theoretical digestion curves for pepsin (A) and trypsin post-pepsin (B) as characterized using LC-MS

The thermal stability of the trans-membrane enzyme proteins, such as desaturases and elongases, is difficult to characterize *in vitro*. Membrane-associated proteins, and especially trans-membrane proteins, are thermally unstable and difficult to refold once partially or fully denatured (Bowie, 2001). In the processing of canola seeds to produce oil, seed material reaches temperatures ranging from 80 ºC to 115 ºC. It is improbable that any trans-membrane proteins retain their native, folded state at this temperature range, and unlikely that any such proteins will refold correctly upon cooling. Therefore, transgenic desaturases and elongases are considered thermally unstable.

A. *L. kluyveri* Δ12-desaturase

The translated *L. kluyveri* Δ12-desaturase (Lackl-Δ12D) contained 416 AA residues (Figure 11). Its molecular weight is predicted as 48.2 kDa, with an estimated isoelectric point (pI) of 7.84. The Lackl-Δ12D protein catalyzes the desaturation of OA at Δ12 position to LA ($18{:}1^{\Delta 9} \rightarrow 18{:}2^{\Delta 9,12}$). The Lackl-Δ12D protein contains one potential glycosylation site, indicated in **bold** in Figure 11, the AA sequence derived from the transgenic DNA sequence.

MSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAIPHECYERRLA
TSLYYVFRDIFCMLTTGYLTHKILYPLLISYTSNSIIKFTFWALYTYVQGLFGTGIWVLA
HECGHQAFSDYGIVNDFVGWTLHSYLMVPYFSWKYSHGKHHKATGHMTRDMVFVPATKEE
FKKSRNFFGNLAEYSEDSPLRTLYELLVQQLGGWIAYLFV**NVT**GQPYPDVPSWKWNHFWL
TSPLFEQRDALYIFLSDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWVNHWLVFITFL
QHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFHDIIETHVLHHYCSRIPFYNARP
ASEAIKKVMGKHYRSSDENMWKSLWKSFRSCQYVDGDNGVLMFRNINNCGVGAAEK

Figure 11. AA sequence of Lackl-Δ12D

Similarity to other proteins in consumed foods, food production or in animal feeds

Fatty acid Δ12-desaturases have been cloned from a wide range of organisms, including thraustochytrid (Matsuda et al., 2012), diatom (Domergue et al., 2003), fungus (Sakuradani et al., 1999), plant (Okuley et al., 1994), nematode (Peyou-Ndi et al., 2000) and insect (Zhou et al., 2008).

Lackl-Δ12D shares homology (33-69%) to other Δ12-desaturase proteins from yeasts, fungi, and various crop plants, such as canola, rice, soybean, flax, sunflower and sesame (Table 20), which (except rice) are typical oil crops for food application. Specifically, the introduced Lackl-Δ12D protein in DHA canola shared 36% sequence identity with the endogenous canola Δ12-desaturase.

Microbial food cultures are live bacteria, yeasts or molds used in food production (Bourdichon et al., 2012). At least 69 species of yeasts and molds are listed in the current "Inventory of microbial food cultures," including many isolates that express Δ12-desaturase proteins. Yeasts are essential microorganisms in the production of various foods and drinks such as bread, beer, wine, and cider. The yeast strain *L. kluyveri* itself, from which the present gene was cloned, is used widely in Emmental, Roquefort, Damietta and Greek cheeses, and fermented milk. The closely related strain, *L. lanzarotensis*, naturally present in grape must, contributes to spontaneous alcoholic fermentation during the early phases of wine fermentation, before *Saccharomyces cerevisiae* becomes dominant and completes the process. A number of food proteins and enzymes have also been expressed in *P. pastoris* (Batt, 2014).

Table 20: AA sequence identity between Lackl-Δ12D in DHA canola and other desaturases

No.	Protein	Accession	Common Name	Sequence identity												
				1	2	3	4	5	6	7	8	9	10	11	12	13
1	NS-B50027-4 Lackl-Δ12D			100	64.4	58.1	67.6	69.0	66.4	37.5	36.1	34.6	36.1	33.9	35.5	36.7
2	Picpa-Δ12D	AAX20125	Yeast		100	62.3	59.2	51.5	52.5	38.0	33.9	33.8	33.6	34.1	33.1	35.1
3	Pican-Δ12D	BAN63793	Yeast			100	57.2	53.7	54.7	38.0	37.4	35.9	35.6	35.1	35.9	36.0
4	Kluma-Δ12D	BAO38850	Fungus				100	64.7	65.1	37.8	37.1	36.4	35.3	35.0	34.1	36.0
5	Zygba-Δ12D	CDH15170	Fungus					100	78.0	36.8	35.3	35.8	35.4	34.3	33.7	34.3
6	Zygro-Δ12D	XP_002495271	Fungus						100	38.3	35.2	36.3	35.2	36.6	34.4	36.1
7	Moral-Δ12D	BAAB1754	Fungus							100	39.5	40.1	40.5	41.0	40.7	42.5
8	Brana-Δ12D	AFJ19031	Canola								100	69.3	78.9	77.6	72.7	75.8
9	Orysa-Δ12D	ACN87220	Rice									100	71.6	68.5	66.8	71.9
10	Glyma-Δ12D	ABF84063	Soybean										100	79.4	74.0	79.4
11	Linus-Δ12D	ACF49508	Flax											100	73.7	78.3
12	Helan-Δ12D	AF251844	Sunflower												100	75.0
13	Sesin-Δ12D	AAX11454	Sesame													100

Brana, *B. napus*; Glyma, *G. max;* Helan, *Helianthus annus;* Kluma, *Kluyveromyces marxianus*; Lackl, *L. kluyveri;*

Linus, *L. usitatissmum;* Moral, *Mortierella alpina*; Orysa, *Oryza sativa;* Pican, *Pichia angusta*; Picpa, *P. pastoris*; Sesin, *Sesamum indicum;* Zygba, *Zygosaccharomyces bailii*; Zygro, *Zygosaccharomyces rouxii.*

Functional activity of Lackl-Δ12D

The enzyme functionality of Lackl-Δ12D has been confirmed in different heterologous expression systems. For the functional assays, Lackl-Δ12D was expressed in *P. pastoris*, as fusion proteins designated as SP:His_{10}::Lackl-Δ12D or His_{10}::Lackl-Δ12D. Table 21 shows the enzyme activity of Lackl-Δ12D expressed as fusion proteins in *P. pastoris* with or without secretion peptide. Over-expression of Lackl-Δ12D fusion protein in *P. pastoris* substantially increased the desaturation of OA to LA compared with the vector alone. In addition, the His_{10}::Lackl-Δ12D had higher activity than SP::His_{10}::Lackl-Δ12D.

Table 21. Activity of Lackl-Δ12D fusion protein in *P. pastoris* cells

Sample	Substrate (%)		Product (%)		Conversion (%)	
Vector	18:1	30.9 ± 6.4	18:2	37.3 ± 4.0	58.7 ± 8.0	n=10
SP::His_{10}::Lackl-Δ12D		24.8 ± 5.2		41.9 ± 3.2	66.6 ± 6.5	n=10
Vector		34.1 ± 1.0		26.4 ± 0.9	47.8 ± 1.4	n=3
His_{10}::Lackl-Δ12D		3.3 ± 0.5		56.9 ± 0.7	94.9 ± 0.8	n=3

Substrate and product are shown in percentage of total FA in cells. Conversion rate based on all products (including 18:2 further desaturated by *P. pastoris* host cell ω3-desaturase) compared with remaining substrate 18:1. SP, signal peptide. n = repeats with individual colonies.

The genome sequence including the T-DNA insertions in DHA canola was analyzed. The translated AA sequence of Lackl-Δ12D in the transgene insert was confirmed to be identical to the original sequence.

Δ12D sequence translated from sequenced T-DNA insert in DHA canola was identical to the original Δ12D sequence from *L. kluyveri* in binary vector (Lackl-Δ12D_vec in Figure 12).

```
                     1                                                  50
Lackl-Δ12D_vec   (1) MSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAI
NS-B50027-4      (1) MSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAI
                     51                                                 100
Lackl-Δ12D_vec  (51) PHECYERRLATSLYYVFRDIFCMLTTGYLTHKILYPLLISYTSNSIIKFT
NS-B50027-4     (51) PHECYERRLATSLYYVFRDIFCMLTTGYLTHKILYPLLISYTSNSIIKFT
                     101                                                150
Lackl-Δ12D_vec (101) FWALYTYVQGLFGTGIWVLAHECGHQAFSDYGIVNDFVGWTLHSYLMVPY
NS-B50027-4    (101) FWALYTYVQGLFGTGIWVLAHECGHQAFSDYGIVNDFVGWTLHSYLMVPY
                     151                                                200
Lackl-Δ12D_vec (151) FSWKYSHGKHHKATGHMTRDMVFVPATKEEFKKSRNFFGNLAEYSEDSPL
NS-B50027-4    (151) FSWKYSHGKHHKATGHMTRDMVFVPATKEEFKKSRNFFGNLAEYSEDSPL
                     201                                                250
Lackl-Δ12D_vec (201) RTLYELLVQQLGGWIAYLFVNVTGQPYPDVPSWKWNHFWLTSPLFEQRDA
NS-B50027-4    (201) RTLYELLVQQLGGWIAYLFVNVTGQPYPDVPSWKWNHFWLTSPLFEQRDA
                     251                                                300
Lackl-Δ12D_vec (251) LYIFLSDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWVNHWLVFITFL
NS-B50027-4    (251) LYIFLSDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWVNHWLVFITFL
           301                                                          350
Lackl-Δ12D_vec (301) QHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFHDIIETHVLHHYC
NS-B50027-4    (301) QHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFHDIIETHVLHHYC
                     351                                                400
Lackl-Δ12D_vec (351) SRIPFYNARPASEAIKKVMGKHYRSSDENMWKSLWKSFRSCQYVDGDNGV
NS-B50027-4    (351) SRIPFYNARPASEAIKKVMGKHYRSSDENMWKSLWKSFRSCQYVDGDNGV
                     401            416
Lackl-Δ12D_vec (401) LMFRNINNCGVGAAEK
NS-B50027-4    (401) LMFRNINNCGVGAAEK
```

Figure 12. Alignment of protein sequences of Lackl-Δ12D

Digestibility of Lackl-Δ12D

Lackl-Δ12D is a yeast acyl-CoA type FA desaturase (as is Picpa–ω3D), which introduces a double bond at position 12 of the FA (Zhou et al., 2008). Another representative of this group, Picpa–ω3D, was analyzed for protein stability (see section B below).

B. *P. pastoris* ω3-desaturase

Picpa–ω3D contains 415 AA residues (Figure 13). Its molecular weight is predicted as 47.8 kDa, with estimated pI as 7.67. The Picpa–ω3D protein catalyzes the desaturation of LA at the Δ15 position (18:$2^{\Delta 9,12}$ to 18:$3^{\Delta 9,12,15}$). The Picpa-ω3D protein includes two potential glycosylation sites indicated in **bold** within this AA sequence derived from the nucleotide sequence of the transgene:

MSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIKDILDAI
PKHCYERSLVKSMSYVVRDIVAISAIAYVGLTYIPLLPNEFLRFAAWSAYVFSISC
FGFGIWILGHECGHSAFSNYGWV**NDT**VGWVLHSLVMVPYFSWKFSHAKHHKATGHM
TRDMVFVPYTAEEFKEKHQVTSLHDIAEETPIYSVFALLFQQLGGLSLYLAT**NAT**G
QPYPGVSKFFKSHYWPSSPVFDKKDYWYIVLSDLGILATLTSVYTAYKVFGFWPTF
ITWFCPWILVNHWLVFVTFLQHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIF
HDIIETHVLHHYVSRIPFYHAREATECIKKVMGEHYRHTDENMWVSLWKTWRSCQF
VENHDGVYMFRNCNNVGVKPKDT

Figure 13. AA sequence of Picpa-ω3D

Similarity to other proteins in consumed foods, food production or in animal feeds

The FA ω3/Δ15-desaturases have been cloned from a wide range of organisms and share high homology to other Δ15-desaturase proteins, including cyanobacteria (Sakamoto et al., 1994), protozoon (Sayanova et al., 2006), thraustochytrid (Meesapyodsuk and Qiu, 2016), nematode (Spychalla et al., 1997), plant (Arondel et al., 1992) and fungus (Pereira et al., 2004).

The Picpa-ω3D shared homology (26-63%) to other ω3/Δ15-desaturase proteins from yeasts, fungi and various crop plants, such as canola, soybean, flax and sesame (Table 22), of which sesame, soybean, flax and canola are typical seed oil crops for food application. The transgenic Picpa-ω3D protein in DHA canola shares 28% sequence identity with the endogenous canola Δ15-desaturase.

Yeasts are essential microorganisms in the production of various foods and drinks such as bread, beer, wine and cider. *P. pastoris* is a species of methylotrophic yeast. *Pichia* itself is widely used for protein production using recombinant DNA techniques (Ahmad et al., 2014). A number of food proteins and enzymes have been expressed in *P. pastoris* (Batt, 2014).

Candida utilis (anamorph of *Cyberlindernera jadinii*, also misnamed *Lindnera jadinii*) is a yeast strain very important for the food and feed industry. Its industrial utilization started in World War I, when common protein sources became scarce. Following its initial use as a dietary supplement, different endogenous compounds were isolated from *C. utilis* including invertase, glutathione, ribonucleic acids, glucomannan, phospholipase B, or biotin (Buerth et al., 2016). Because of its long and safe history in the food industry *C. utilis*, as *S. cerevisiae*, has been classified as GRAS.

The yeast *Lipomyces starkeyi* and *L. kononenkoae* have been used in food related applications because of their ability to produce α-amylase and/or endo-dectranase, and are not known to

produce antibiotics or toxic metabolites (Kang et al., 2004). *Ogataea angusta* is another methylotrophic yeast used as a protein factory for pharmaceuticals. *L. kluyveri* is widely used in Emmental, Roquefort, Damietta and Greek cheeses, and in fermented milk.

Table 22. AA sequence identity between Picpa-ω3D in DHA canola and other desaturases

No.	Protein	Accession	Common Name	Sequence identity												
				1	2	3	4	5	6	7	8	9	10	11	12	13
1	NS-B50027-4 Picpa-ω3D			100	63.1	63.4	59.8	58.9	48.8	35.5	50.2	55.2	27.6	25.6	25.6	25.9
2	Wicci-ω3D	XP_011275445	Fungus		100	65.2	72.1	64.0	49.2	33.9	51.1	58.0	24.8	25.6	23.5	25.6
3	Lackl-ω3D	BAD11952	Fungus			100	57.4	61.5	51.5	33.3	52.2	54.8	24.5	23.4	23.9	23.6
4	Cybjan-ω3D	BAJ78984	Fungus				100	62.1	44.9	30.9	46.8	53.4	24.9	23.8	22.6	24.0
5	Lipko-Δ12D/Δ15D	ACF36357	Fungus					100	46.7	31.8	49.8	56.6	24.4	25.9	25.6	23.7
6	Yarli-Δ12D	CAG82952	Yeast						100	39.3	47.3	52.5	28.1	28.1	27.4	26.7
7	Moral-ω3D	BAD91495	Fungus							100	32.6	32.6	29.3	29.5	30.5	26.4
8	Zygba-Δ12D	CDH15170	Fungus								100	64.7	26.7	26.6	26.2	25.2
9	Kluma-Δ12D	BAO38850	Fungus									100	26.5	26.7	25.9	26.9
10	Brana-ω3D	P48624	Canola										100	68.7	66.3	65.5
11	Glyma-ω3D	NP_001236943	Soybean											100	67.9	62.2
12	Linus-Δ15D	ABA02172	Flax												100	65.0
13	Sesin-ω3D	XP_011080789	Sesame													100

Brana, *B. napus*; Cybji, *C. jadinii*; Glyma, *G. max;* Kluma, *K. marxianus*; Lackl, *L. kluyveri*; Linus, *L. usitatissmum;* Lipko, *L. kononenkoae*; Moral, *M. alpina*; Picpa, *P. pastoris*; Sesin, *S. indicum;* Wicci, *Wickerhamomyces ciferrii*; Yarli, *Yarrowia lipolytica*; Zygba, *Z. bailii.*

Functional activity of Picpa-ω3D

The enzyme functionality of Picpa-ω3D has been confirmed in different heterologous expression systems, including yeast (Zhang et al., 2008), *Arabidopsis* seed (Petrie et al., 2012) and *Camelina* seed (Petrie et al., 2014). In this study, Picpa-ω3D was expressed in *P. pastoris*, as fusion proteins designated either SP::His_{10}::Picpa-ω3D or His_{10}::Picpa-ω3D. Table 23 shows the enzyme activity of Picpa-ω3D expressed as fusion proteins in *P. pastoris* with or without secretion peptide. Over-expression of Picpa-ω3D fusion protein in *P. pastoris* substantially increased the desaturation of LA to ALA compared with vector alone. In addition, the His_{10}::Picpa-ω3D had higher activity than SP::His_{10}::Picpa-ω3D.

Table 23. Activity of Picpa-ω3D fusion protein in *P. pastoris* cells

Sample	Substrate (%)		Product (%)		Conversion (%)	
Vector	18:2	26.0 ± 1.1	18:3	4.2 ± 0.2	14.0 ± 0.8	n=3
SP::His_{10}::Picpa-ω3D		12.7 ± 1.7		11.2 ± 2.4	46.5± 8.1	n=10
Vector		26.4 ± 0.9		2.0 ± 0.2	15.3 ± 0.8	n=3
His_{10}::Picpa-ω3D		3.8 ± 0.5		21.9 ± 1.8	85.4 ± 0.9	n=3

Substrate and product are shown in percentage of total FA in cells. Conversion rate is based on the product 18:3 compared to the total of product 18:3 and remaining substrate 18:2. SP, signal peptide. n = repeats with individual colonies.

The genome sequence including the T-DNA insertions in DHA canola was analyzed. The translated AA sequence of Picpa-ω3D in the insert was confirmed to be identical to the original sequence. Figure 14 shows that the Picpa-ω3D sequence translated from sequenced T-DNA insert

in DHA canola NS-B50027-4 event was identical to the original ω3D sequence from *P. pastoris* in binary vector (Picpa-ω3D_vec).

```
                     1                                                  50
Picpa-ω3D_vec    (1) MSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIK
NS-B50027-4      (1) MSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIK
                     51                                                 100
Picpa-ω3D_vec   (51) DILDAIPKHCYERSLVKSMSYVVRDIVAISAIAYVGLTYIPLLPNEFLRF
NS-B50027-4     (51) DILDAIPKHCYERSLVKSMSYVVRDIVAISAIAYVGLTYIPLLPNEFLRF
                     101                                                150
Picpa-ω3D_vec  (101) AAWSAYVFSISCFGFGIWILGHECGHSAFSNYGWVNDTVGWVLHSLVMVP
NS-B50027-4    (101) AAWSAYVFSISCFGFGIWILGHECGHSAFSNYGWVNDTVGWVLHSLVMVP
                     151                                                200
Picpa-ω3D_vec  (151) YFSWKFSHAKHHKATGHMTRDMVFVPYTAEEFKEKHQVTSLHDIAEETPI
NS-B50027-4    (151) YFSWKFSHAKHHKATGHMTRDMVFVPYTAEEFKEKHQVTSLHDIAEETPI
                     201                                                250
Picpa-ω3D_vec  (201) YSVFALLFQQLGGLSLYLATNATGQPYPGVSKFFRSHYWPSSPVFDKKDY
NS-B50027-4    (201) YSVFALLFQQLGGLSLYLATNATGQPYPGVSKFFRSHYWPSSPVFDKKDY
                     251                                                300
Picpa-ω3D_vec  (251) WYIVLSDLGILATLTSVYTAYKVFGFWPTFITWFCPWILVNHWLVFVTFL
NS-B50027-4    (251) WYIVLSDLGILATLTSVYTAYKVFGFWPTFITWFCPWILVNHWLVFVTFL
                     301                                                350
Picpa-ω3D_vec  (301) QHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIFHDIIETHVLHHYVS
NS-B50027-4    (301) QHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIFHDIIETHVLHHYVS
                     351                                                400
Picpa-ω3D_vec  (351) RIPFYHAREATECIKKVMGEHYRHTDENMWVSLWKTWRSCQFVENHDGVY
NS-B50027-4    (351) RIPFYHAREATECIKKVMGEHYRHTDENMWVSLWKTWRSCQFVENHDGVY
                     401         415
Picpa-ω3D_vec  (401) MFRNCNNVGVKPKDT
NS-B50027-4    (401) MFRNCNNVGVKPKDT
```

Figure 14. Alignment of protein sequences of Picpa-ω3D

Digestibility of Picpa-ω3D

The Picpa-ω3D protein was used as the representative of both yeast acyl-CoA type FA desaturases (Picpa-ω3D and Lackl-Δ12D; see Figure 1, Section A.) engineered into DHA canola, for stability analysis.

The results of this study demonstrated that greater than 80% of the full-length Picpa-ω3D protein was digested within 5 min and greater than 97% of it was digested within 60 min of incubation in pepsin. The combined pepsin-trypsin assay showed a rapid decline in the tryptic peptides that were used as a proxy for the presence of intact protein. Overall, it was observed that the peptides from the termini (both N- and extreme C-termini) of the protein were liberated rapidly with <6% remaining after 5 min (Table 24).

Table 24. Percentage of each tryptic peptide remaining during pepsin time course

Tryptic Peptide Sequence	Incubation time (minutes)				
	5	10	15	30	60
VTVSGSEILEGSTK	2.8	1.5	1.8	1.3	0.7
SGNVASFK	1.3	1.1	1.0	1.0	0.5
TAIDTFGNVFK	2.5	1.7	2.6	1.4	0.9
VPDYTIK	3.8	2.7	3.6	2.3	1.3
DILDAIPK	6.0	6.1	5.5	3.7	1.4
EATEC{CAM}IK	17.5	17.1	18.1	9.4	2.7
HTDENMWVSLWK	16.5	9.2	9.9	8.2	1.1
SC{CAM}QFVENHDGVYMFR	2.0	1.3	1.1	0.9	0.3

C. *Micromonas pusilla* Δ6-Desaturase

The translated *M. pusilla* Δ6-desaturase (Micpu-Δ6D, EEH58637) contained 463 AA residues (Figure 15). The molecular weight of Micpu-Δ6D is predicted as 52.9 kDa, with estimated pI of 9.00. The Micpu-Δ6D protein catalyzes the desaturation of ALA at the Δ6 position (18:3$^{\Delta 9,12,15}$ to 18:4$^{\Delta 6,9,12,15}$). For the Micpu-Δ6D protein, there is no potential glycosylation site within this AA sequence derived from the nucleotide sequence of the inserted DNA.

MCPPKTDGRSSPRSPLTRSKSSAEALDAKDASTAPVDLKTLEPHELAATFETRWVR
VEDVEYDVTNFKHPGGSVIFYMLANTGADATEAFKEFHMRSLKAWKMLRALPSRPA
EIKRSESEDAPMLEDFARWRAELERDGFFKPSITHVAYRLLELLATFALGTALMYA
GYPIIASVVYGAFFGARCGWVQHEGGHNSLTGSVYVDKRLQAMTCGFGLSTSGEMW
NQMHNKHHATPQKVRHDMDLDTTPAVAFFNTAVEDNRPRGFSRAWARLQAWTFVPV
TSGLLVQAFWIYVLHPRQVLRKKNYEEASWMLVSHVVRTAVIKLATGYSWPVAYWW
FTFGNWIAYMYLFAHFSTSHTHLPVVPSDKHLSWVNYAVDHTVDIDPSRGYVNWLM
GYLNCQVIHHLFPDMPQFRQPEVSRRFVPFAKKWGLNYKVLSYYGAWKATFSNLDK
VGQHYYVNGKAEKAH

Figure 15. AA sequence of Micpu-Δ6D

Similarity to other proteins in consumed foods, food production or animal feeds

The FA Δ6-desaturases have been cloned from bacteria (Reddy et al., 1993), alga (Domergue et al., 2005), diatom (Domergue et al., 2002), fungus (Huang et al., 1999), nematode (Napier et al., 1998), moss (Girke et al., 1998), plant (Sayanova et al., 1997), mouse and human (Cho et al., 1999). The Δ6-desaturases have been widely studied in vertebrates, including many fish species (Vagner and Santigosa, 2011; Tanomman et al., 2013). The Δ6-desaturase enzymes can desaturate both ω3 ALA and omega-6 (ω6) LA at the Δ6 position, producing ω3 SDA and ω6 GLA (see Figure 1, Petrie et al., 2010b). DHA canola contains marine microalga Micpu-Δ6D, which exhibits ω3-preference (Petrie et al., 2010b).

The Micpu-Δ6D protein shares about 17-21% sequence identity to other Δ6-desaturase proteins from fungi, salmon, evening primrose and canola (Table 25). The Micpu-Δ6D protein shares ~20% AA sequence identity with plant Δ6-desaturase proteins from *E. plantagineum* (echium, AAZ08559), *Borago officinalis* (borage, AAC49700) and *Oenothera biennis* (evening primrose, ACB47482). These species have been used to produce oils that are relatively high in GLA and/or SDA for human consumption. The oils produced by these species have been studied extensively for their anti-inflammatory effects on leukotriene and prostaglandin biosynthesis (Fan and Chapkin, 1998), and are sold as cold-pressed oils for use as dietary supplements. Evening primrose plants have been used as ornamentals, food sources, and as medicinal herbs for more than 50 years.

Table 25. AA sequence identity between Micpu-Δ6D in DHA canola and other desaturases

No.	Protein	NCBI Accession	Common Name	Sequence identity					
				1	2	3	4	5	6
	NS-B50027-4								
1	Micpu-Δ6D			100	20.5	21.1	19.1	17.5	19.1
2	Moral-Δ6D	BAC82359	Fungus		100	37.3	21.9	26.6	26.6
3	Thaps-Δ6D	AAX14505	Diatom			100	23.5	25.5	26.5
4	Salsa-Δ6D	AAR21624	Salmon				100	23.7	23.2
5	Onebi-Δ6D	ACB47482	Evening primrose					100	61.2
6	Brana-Δ8D	NP_001302507	Canola						100

Micpu, *M. pusilla*; Brana, *B. napus* ; Moral, *M. alpina* ; Onebi, *O. biennis*; Salsa, *Salmo salar*; Thaps, *Thalassiosira pseudonana.*

Functional activity of Micpu-Δ6D

The enzyme functionality of Micpu-Δ6D has been confirmed in different heterologous expression systems, including yeast cell and *Nicotiana benthamiana* leaf (Petrie et al., 2010b), Arabidopsis seed (Petrie et al., 2012) and Camelina seed (Petrie et al., 2014). In this study, Micpu-Δ6D was expressed in *P. pastoris,* as fusion proteins designated as SP::His_{10}::Micpu-Δ6D or His_{10}::Micpu-Δ6D. In SP::His_{10}::Micpu-Δ6D, the Micpu-Δ6D sequence was fused to *S. cerevisiae* SP, followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal. Over-expression of Micpu-Δ6D fusion proteins in *P. pastoris* with or without secretion peptide demonstrated the desaturation of ALA to SDA compared to the vector alone (Table 26). In addition, the His_{10}::Micpu-Δ6D had higher activity than SP::His_{10}::Micpu-Δ6D in *P. pastoris*.

Table 26. Activity of Micpu-Δ6D fusion protein in *P. pastoris* cells

Sample	**Substrate (%)**		**Product (%)**		**Conversion (%)**	
Vector	18:3	6.4 ± 1.8	18:4	0.0 ± 0.0	0.0 ± 0.0	n=10
SP::His_{10}::Micpu-Δ6D		6.1 ± 1.0		0.2 ± 0.2	3.3 ± 2.8	n=10
Vector		24.0 ± 1.7		0.0 ± 0.0	0.0 ± 0.0	n=3
His_{10}::Micpu-Δ6D		19.0 ± 0.3		5.8 ± 1.7	23.2 ± 5.4	n=3

Substrate and product are shown in percentage of total FA in cells. Conversion rate is based on the product 18:4 compared to the total of product 18:4 and remaining substrate 18:3. n = repeats with individual colonies. In His_{10}::Micpu-Δ6D activity assay, yeast cell culture was fed with 0.5 mM 18:3 substrate, while in SP::His_{10}::Micpu-Δ6D activity assay, no extra 18:3 substrate added.

The genome sequence including the T-DNA insertions in DHA canola was analyzed. The translated AA sequence of Micpu-Δ6D in the insert was confirmed to be identical to the original sequence. Figure 16 shows that the Micpu-Δ6D sequence translated from sequenced T-DNA insert in DHA canola was identical to the original Δ6D sequence from *M. pusilla* in binary vector (Micpu-Δ6D_vec).

```
                    1                                                   50
Micpu-Δ6D_vec   (1) MCPPKTDGRSSPRSPLTRSKSSAEALDAKDASTAPVDLKTLEPHELAATF
NS-B50027-4     (1) MCPPKTDGRSSPRSPLTRSKSSAEALDAKDASTAPVDLKTLEPHELAATF
                    51                                                  100
Micpu-Δ6D_vec  (51) ETRWVRVEDVEYDVTNFKHPGGSVIFYMLANTGADATEAFKEFHMRSLKA
NS-B50027-4    (51) ETRWVRVEDVEYDVTNFKHPGGSVIFYMLANTGADATEAFKEFHMRSLKA
                    101                                                 150
Micpu-Δ6D_vec (101) WKMLRALPSRPAEIKRSESEDAPMLEDFARWRAELERDGFFKPSITHVAY
NS-B50027-4   (101) WKMLRALPSRPAEIKRSESEDAPMLEDFARWRAELERDGFFKPSITHVAY
                    151                                                 200
Micpu-Δ6D_vec (151) RLLELLATFALGTALMYAGYPIIASVVYGAFFGARCGWVQHEGGHNSLTG
NS-B50027-4   (151) RLLELLATFALGTALMYAGYPIIASVVYGAFFGARCGWVQHEGGHNSLTG
                    201                                                 250
Micpu-Δ6D_vec (201) SVYVDKRLQAMTCGFGLSTSGEMWNQMHNKHHATPQKVRHDMDLDTTPAV
NS-B50027-4   (201) SVYVDKRLQAMTCGFGLSTSGEMWNQMHNKHHATPQKVRHDMDLDTTPAV
                    251                                                 300
Micpu-Δ6D_vec (251) AFFNTAVEDNRPRGFSRAWARLQAWTFVPVTSGLLVQAFWIYVLHPRQVL
NS-B50027-4   (251) AFFNTAVEDNRPRGFSRAWARLQAWTFVPVTSGLLVQAFWIYVLHPRQVL
                    301                                                 350
Micpu-Δ6D_vec (301) RKKNYEEASWMLVSHVVRTAVIKLATGYSWPVAYWWFTFGNWIAYMYLFA
NS-B50027-4   (301) RKKNYEEASWMLVSHVVRTAVIKLATGYSWPVAYWWFTFGNWIAYMYLFA
                    351                                                 400
Micpu-Δ6D_vec (351) HFSTSHTHLPVVPSDKHLSWVNYAVDHTVDIDPSRGYVNWLMGYLNCQVI
NS-B50027-4   (351) HFSTSHTHLPVVPSDKHLSWVNYAVDHTVDIDPSRGYVNWLMGYLNCQVI
                    401                                                 450
Micpu-Δ6D_vec (401) HHLFPDMPQFRQPEVSRRFVPFAKKWGLNYKVLSYYGAWKATFSNLDKVG
NS-B50027-4   (401) HHLFPDMPQFRQPEVSRRFVPFAKKWGLNYKVLSYYGAWKATFSNLDKVG
                    451        463
Micpu-Δ6D_vec (451) QHYYVNGKAEKAH
NS-B50027-4   (451) QHYYVNGKAEKAH
```

Figure 16. Alignment of protein sequences of Micpu-Δ6D

Digestibility of Micpu-Δ6D

Micpu-Δ6D is an algae front-end FA desaturase (as are Pavsa-Δ5D and Pavsa-Δ4D), which introduces a double bond between an existing double bond and the carboxyl end of FAs (Zhou et al., 2007). Another representative of this group, Pavsa-Δ4D, was analyzed for protein stability (see section G. below).

D. *P. cordata* Δ6-Elongase

The translated *P. cordata* Δ6-elongase (Pyrco-Δ6E, ACR53359) contained 288 AA residues (Figure 17). The molecular weight of Pyrco-Δ6E is predicted as 33.1 kDa, with estimated pI of 9.09. The Pyrco-Δ6E protein catalyzes the elongation (adds two carbons) of SDA ($18:4^{\Delta 6,9,12,15}$) to ETA ($20:4^{\Delta 8,11,14,17}$). For the Pyrco-Δ6E protein, there is no potential glycosylation site within this AA sequence, which is shown on Figure 17 as derived from the nucleotide sequence of the inserted transgene.

MEFAQPLVAMAQEQYAAIDAVVAPAIFSATDSIGWGLKPISSATKDLPLVESPTPL
ILSLLAYFAIVGSGLVYRKVFPRTVKGQDPFLLKALMLAHNVFLIGLSLYMCLKLV
YEAYVNKYSFWGNAYNPAQTEMAKVIWIFYVSKIYEFMDTFIMLLKGNVNQVSFLH
VYHHGSISGIWWMITYAAPGGDAYFSAALNSWVHVCMYTYYFMAAVLPKDEKTKRK
YLWWGRYLTQMQMFQFFMNLLQAVYLLYSSSPYPKFIAQLLVVYMVTLLMLFGNFY
YMKHHASK

Figure 17. AA sequence of Pyrco-Δ6E

Similarity to other proteins in consumed foods food production or in animal feeds

Pyrco-Δ6E shares AA sequence identities (22-35%) to many other elongases presented in food that is consumed, used in food production or in animal feeds (Table 27). Several human PUFA elongases have been isolated (Leonard et al., 2004), including the SDA elongation (Δ6-elongation). They share the 25~27% of sequence identities with Pyrco-Δ6E. Pyrco-Δ6E also shares 26% sequence identity to salmon elongase (AAO13175). Salmon is a well-known salt-water fish consumed as food.

M. alpina is currently used for the commercial production of arachidonic acid for fortification of baby food. Several LC-PUFAs are also commercially produced by using *Mortierella* fungi species (Sakuradani and Shimizu, 2009). Pyrco-Δ6E shares 35% of sequence identity to *M. alpina* Δ6E (AAF70417). Finally, Pyrco-Δ5E shares 22% sequence identity to soybean FA elongase (XP_003531583). Soybean is one of the major oil crops produced for food oil.

Table 27. AA sequence identity between Pyrco-Δ6E in DHA canola and other desaturases

No.	Protein	NCBI Accession	Common Name	Sequence identity						
				1	2	3	4	5	6	7
1	NS-B50027-4 Pyrco-Δ6E			100	35.2	25.4	27.1	26.8	25.9	21.9
2	Moral-Δ6E	AAF70417	Fungus		100	22.5	24.6	23.7	24.6	21.5
3	Thaps-Δ6E	AAV67799	Alga			100	63.8	21.3	24.2	22.5
4	Phatr-Δ6E	CAM55851	Diatom				100	23.2	25.6	22.5
5	Homsa-Elo1	XP_002040	Human					100	31.7	22.1
6	Salsa-Elo	AAO13175	Salmon						100	19.2
7	Glyma-Elo	XP_003531583	Soybean							100

Glyma, *G. max*; Homsa, *Homo sapiens* (human); Moral, *M. alpina*; Phatr, *Phaeodactylum tricornutum*; Pyrco, *P. cordata*; Salsa, *S. salar;* Thaps, *T. pseudonana*.

Functional activity of Pyrco-Δ6E

The enzyme functionality of Pyrco-Δ6E has been confirmed in different heterologous expression systems, including yeast cell (Petrie et al., 2010a), Arabidopsis seed (Petrie et al., 2012) and Camelina seed (Petrie et al., 2014). In this study, Pyrco-Δ6E was expressed in *P. pastoris*, as fusion proteins designated as SP::His_{10}::Pyrco-Δ6E or His_{10}::Pyrco-Δ6E. In SP::His_{10}::Pyrco-Δ6E, the Pyrco-Δ6E sequence was fused to *S. cerevisiae* SP, followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal. Over-expression of Pyrco-Δ6E fusion proteins in *P. pastoris* with secretion peptide confirmed the elongation activity (adds two carbons) of SDA to ETA compared to the vector alone where there was not any ETA (Table 28). In addition, the His_{10}::Pyrco-Δ6E had higher activity than SP::His_{10}::Pyrco-Δ6E in *P. pastoris*.

Table 28. Activity of Pyrco-Δ6E fusion protein in *P. pastoris* cells

Sample	Substrate (%)		Product (%)		Conversion (%)	
Vector	18:4	8.1 ± 0.0	20:4	0.0 ± 0.0	0.0 ± 0.0	n=3
SP::His_{10}::Pyrco-Δ6E		4.5 ± 1.1		3.8 ± 1.1	46.5 ± 9.8	n=7
Vector		17.9 ± 0.9		0.0 ± 0.0	0.0 ± 0.0	n=3
His_{10}::Pyrco-Δ6E		4.6 ± 2.1		9.9 ± 2.2	68.2 ± 14.7	n=3

Substrate and product are shown in percentage of total FA in cells. Conversion rate is based on the product 20:4 compared to the total of product 20:4 and remaining substrate 18:4. n = repeats with individual colonies. In His_{10}::Pyrco-Δ6E activity assay, yeast cell culture was fed with 0.5 mM 18:4 substrate.

The genome sequence including the T-DNA insertions in DHA canola was analyzed. The translated AA sequence of Pyrco-Δ6E in the insert was confirmed to be identical to the original sequence. Figure 18 shows the Pyrco-Δ6E sequence translated from sequenced T-DNA insert in DHA canola was identical to the original Pyrco-Δ6E sequence from *P. cordata* in binary vector (Pyrco-Δ6E_vec).

```
                    1                                                   50
Pyrco-Δ6E_vec     (1) MEFAQPLVAMAQEQYAAIDAVVAPAIFSATDSIGWGLKPISSATKDLPLV
NS-B50027-4       (1) MEFAQPLVAMAQEQYAAIDAVVAPAIFSATDSIGWGLKPISSATKDLPLV
                   51                                                  100
Pyrco-Δ6E_vec    (51) ESPTPLILSLLAYFAIVGSGLVYRKVFPRTVKGQDPFLLKALMLAHNVFL
NS-B50027-4      (51) ESPTPLILSLLAYFAIVGSGLVYRKVFPRTVKGQDPFLLKALMLAHNVFL
                  101                                                  150
Pyrco-Δ6E_vec   (101) IGLSLYMCLKLVYEAYVNKYSFWGNAYNPAQTEMAKVIWIFYVSKIYEFM
NS-B50027-4     (101) IGLSLYMCLKLVYEAYVNKYSFWGNAYNPAQTEMAKVIWIFYVSKIYEFM
                  151                                                  200
Pyrco-Δ6E_vec   (151) DTFIMLLKGNVNQVSFLHVYHHGSISGIWWMITYAAPGGDAYFSAALNSW
NS-B50027-4     (151) DTFIMLLKGNVNQVSFLHVYHHGSISGIWWMITYAAPGGDAYFSAALNSW
                  201                                                  250
Pyrco-Δ6E_vec   (201) VHVCMYTYYFMAAVLPKDEKTKRKYLWWGRYLTQMQMFQFFMNLLQAVYL
NS-B50027-4     (201) VHVCMYTYYFMAAVLPKDEKTKRKYLWWGRYLTQMQMFQFFMNLLQAVYL
                  251                                 288
Pyrco-Δ6E_vec   (251) LYSSSPYPKFIAQLLVVYMVTLLMLFGNFYYMKHHASK
NS-B50027-4     (251) LYSSSPYPKFIAQLLVVYMVTLLMLFGNFYYMKHHASK
```

Figure 18. Alignment of protein sequences of Pyrco-Δ6E

Digestibility of Pyrco-Δ6E

Pyrco-Δ6E protein is an alga FA elongase that adds two carbons to the carboxyl end of FAs including Pyrco-Δ6E and Pyrco-Δ5E. One representative of this group, Pyrco-Δ5E, was analyzed for protein stability (see Section F).

E. *P. salina* Δ5-Desaturase

The translated *P. salina* Δ5-desaturase (Pavsa-Δ5D, ABL96295) contained 425 AA residues (Figure 19). The molecular weight of Pavsa-Δ5D is predicted as 48.2 kDa, with estimated pI of 8.18. The Pavsa-Δ5D protein catalyzes the desaturation of ETA at the Δ5 position ETA ($20:4^{\Delta 8,11,14,17}$) to EPA ($20:5^{\Delta 5,8,11,14,17}$). For the Pavsa-Δ5D protein, there are two potential glycosylation sites within this AA sequence derived from the nucleotide sequence of the inserted DNA (highlighted in **bold** in Figure 19).

MPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGGKIIAYQ
VGTDATDAYKQFHVRSAKADKMLKSLPSRPVHKGYSPRRADLIADFQEFTKQLEAE
GMFEPSLPHVAYRLAEVIAMHVAGAALIWHGYTFAGIAMLGVVQGRCGWLMHEGGH
YSLTGNIAFDRAIQVACYGLGCGMSGAWWRNQHNKHHATPQKLQHDVDLDTLPLVA
FHERIAAKVKSPAMKAWLSMQAKLFAPVTTLLVALGWQLYLHPRHMLRTKHYDELA
MLGIRYGLVGYLAANYGAGYVLACYLLYVQLGAMYIFCNFAVSHTHLPVVEPNEHA
TWVEYAA**NHT**T**NCS**PSWWCDWWMSYLNYQIEHHLYPSMPQFRHPKIAPRVKQLFEK
HGLHYDVRGYFEAMADTFANLDNVAHAPEKKMQ

Figure 19. AA sequence of Pavsa-Δ5D

Similarity to other proteins in consumed foods, food production or in animal feeds

The Pavsa-Δ5D protein shares similarity (18-53%) to desaturase proteins consumed in food, animal feed, or used in food production (*see* Table 29). Pavsa-Δ5D was cloned from *P. salina*. which is one of several microalgae used in mariculture (Brown, 1991). For example, *Pavlova lutheri* is used for oyster larvae and clam larvae feeds (Brown et al., 1997). Pavsa-Δ5D shares 53% sequence identity with *P. lutheri* Δ5-desaturase (ALE15225, partial sequence) in an overlapping region.

Pavsa-Δ5D also shares AA sequence identities to many other FA desaturases from wide range of species. For example, it shares 53% sequence identity to marine microalga *Isochrysis galbana* Δ5-desaturase (AIA24277). *I. galbana* is used to make functional sweet biscuits with enriched LC-PUFA (Gouveia et al., 2008).

Pavsa-Δ5D shares 21% sequence identity to Δ6-desaturases from *O. biennis* (ACB47482), *E. plantagineum* (AAZ08559) and *B. officinalis* (borage, O04353), represented by *O. biennis* in Table 29. These species have been used to produce oils that are relatively high in GLA and/or SDA for human consumption. The oils produced by these species have been studied extensively for their

anti-inflammatory effects on leukotriene and prostaglandin biosynthesis (Fan and Chapkin, 1998), and are sold as cold-pressed oils for use as dietary supplements.

Table 29. AA sequence identity between Pavsa-Δ5D in DHA canola and other desaturases

No.	Protein	NCBI Accession	Common Name	Sequence identity								
				1	2	3	4	5	6	7	8	9
1	NS-B50027-4 Pavsa-Δ5D			100	52.7	52.6	46.8	24.9	22.9	22.9	18.0	20.8
2	Pavlu-Δ5D*	ALE15225	Alga		100	86.6	43.3	26.8	24.7	26.6	18.7	20.7
3	Isoga-Δ5D	AIA24277	Alga			100	42.5	25.2	24.1	24.6	17.4	19.4
4	Thrau-Δ5D	BAK08911	Protist				100	23.8	24.2	23.0	16.1	21.4
5	Octvu-Δ5D	AEK20864	Octopus					100	49.7	51.0	19.9	24.7
6	Homsa-Δ5D	AAF70457	Human						100	57.2	21.8	23.7
7	Salsa-Δ5D	AAL82631	Salmon							100	20.0	23.0
8	Moral-Δ5D	AAC72755	Fungus								100	20.7
9	Onebi-Δ6D	ACB47482	Evening primrose									100

Homsa, *H. sapiens*; Isoga, *I. galbana*; Moral, *M. alpine*; Octvu, *Octopus vulgaris*; Onebi, *O. biennis*; Pavlu, *P. lutheri*; Pavsa, *P. salina*; Salsa, *S. salar*; Thrau, *Thraustochytrium aureum*. *Pavlu-Δ5D is partial sequence. The sequence identity was based on the overlap region only.

Functional activity of Pavsa-Δ5D

The enzyme functionality of Pavsa-Δ5D has been confirmed in different heterologous expression systems, including yeast cell (Zhou et al., 2007), *N. benthamiana* leaf (Wood et al., 2009), *Arabidopsis* seed (Petrie et al., 2012) and *Camelina* seed (Petrie et al., 2014). In this study, Pavsa-Δ5D was expressed in *P. pastoris,* as a fusion protein designated as SP::His_{10}::Pavsa-Δ5D. In SP::His_{10}::Pavsa-Δ5D, the Pavsa-Δ5D sequence was fused to *S. cerevisiae* SP, followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal. The enzyme activity of SP::His_{10}::Pavsa-Δ5D fusion protein was confirmed in *P. pastoris* yeast cells, as shown in Table 30. The result showed the desaturation activity on ETA to produce EPA. Although the conversion efficiency was low, vector alone produced no EPA.

Table 30. Activity of Pavsa-Δ5D fusion protein in *P. pastoris* cells

Sample	Substrate (%)		Product (%)		Conversion (%)	
Vector	20:4	6.2 ± 1.1	20:5	0.0 ± 0.0	0.0 ± 0.0	n=3
SP::His_{10}::Pavsa-Δ5D		5.3 ± 0.7		0.05 ± 0.01	0.9 ± 0.3	n=8

Substrate and product are shown in percentage of total FA in cells. Conversion rate is based on substrate ETA (20:4 $^{\Delta 8,11,14,17}$) (ω3) compared with the total of EPA (20:5$^{\Delta 5,8,11,14,17}$) (ω3) product and remaining substrate. n = repeats with individual colonies. The yeast cell culture was fed with 0.1 mM 20:4$^{\Delta 8,11,14,17}$ (ω3) substrate.

The genome sequence including the T-DNA insertions in DHA canola was analyzed. The translated AA sequence of Pavsa-Δ5D in the insert was confirmed to be identical to the original sequence. Figure 20 shows Pavsa-Δ5D sequence translated from sequenced T-DNA insert in DHA

canola was identical to the original Pavsa-Δ5D sequence from *P. salina* in binary vector (Pavsa-Δ5D_vec).

```
                     1                                                  50
Pavsa-D5D_vec    (1) MPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGG
NS-B50027-4      (1) MPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGG
                    51                                                 100
Pavsa-D5D_vec   (51) KIIAYQVGTDATDAYKQFHVRSAKADKMLKSLPSRPVHKGYSPRRADLIA
NS-B50027-4     (51) KIIAYQVGTDATDAYKQFHVRSAKADKMLKSLPSRPVHKGYSPRRADLIA
                   101                                                 150
Pavsa-D5D_vec  (101) DFQEFTKQLEAEGMFEPSLPHVAYRLAEVIAMHVAGAALIWHGYTFAGIA
NS-B50027-4    (101) DFQEFTKQLEAEGMFEPSLPHVAYRLAEVIAMHVAGAALIWHGYTFAGIA
                   151                                                 200
Pavsa-D5D_vec  (151) MLGVVQGRCGWLMHEGGHYSLTGNIAFDRAIQVACYGLGCGMSGAWWRNQ
NS-B50027-4    (151) MLGVVQGRCGWLMHEGGHYSLTGNIAFDRAIQVACYGLGCGMSGAWWRNQ
                   201                                                 250
Pavsa-D5D_vec  (201) HNKHHATPQKLQHDVDLDTLPLVAFHERIAAKVKSPAMKAWLSMQAKLFA
NS-B50027-4    (201) HNKHHATPQKLQHDVDLDTLPLVAFHERIAAKVKSPAMKAWLSMQAKLFA
                   251                                                 300
Pavsa-D5D_vec  (251) PVTTLLVALGWQLYLHPRHMLRTKHYDELAMLGIRYGLVGYLAANYGAGY
NS-B50027-4    (251) PVTTLLVALGWQLYLHPRHMLRTKHYDELAMLGIRYGLVGYLAANYGAGY
                   301                                                 350
Pavsa-D5D_vec  (301) VLACYLLYVQLGAMYIFCNFAVSHTHLPVVEPNEHATWVEYAANHTTNCS
NS-B50027-4    (301) VLACYLLYVQLGAMYIFCNFAVSHTHLPVVEPNEHATWVEYAANHTTNCS
                   351                                                 400
Pavsa-D5D_vec  (351) PSWWCDWWMSYLNYQIEHHLYPSMPQFRHPKIAPRVKQLFEKHGLHYDVR
NS-B50027-4    (351) PSWWCDWWMSYLNYQIEHHLYPSMPQFRHPKIAPRVKQLFEKHGLHYDVR
                   401                   425
Pavsa-D5D_vec  (401) GYFEAMADTFANLDNVAHAPEKKMQ
NS-B50027-4    (401) GYFEAMADTFANLDNVAHAPEKKMQ
```

Figure 20. Alignment of protein sequences of Pavsa-Δ5D

Digestibility of Pavsa-Δ5D

Pavsa-Δ5D is an algae front-end FA desaturase that introduces a double bond between an existing double bond and the carboxyl end of FAs (Zhou et al., 2007), including Micpu-Δ6D, Pavsa-Δ5D and Pavsa-Δ4D. One representative of this group, Pavsa-Δ4D, was analyzed for protein stability (See Section G).

F. *P. cordata* Δ5-Elongase

The translated *P. cordata* Δ5-elongase (Pyrco-Δ5E, ACR53360) contained 267 AA residues (Figure 21). The Pyrco-Δ5E protein adds 2 carbons, elongates EPA ($20{:}5^{\Delta5,8,11,14,17}$) to DPA ($22{:}5^{\Delta7,10,13,16,19}$). The molecular weight of Pyrco-Δ5E is predicted as 31.3 kDa, with estimated pI of 9.33, and no potential glycosylation sites.

MASIAIPAALAGTLGYVTYNVANPDIPASEKVPAYFMQVEYWGPTIGTIGYLLFIY
FGKRIMQNRSQPFGLKNAMLVYNFYQTFFNSYCIYLFVTSHRAQGLKVWGNIPDMT
ANSWGISQVIWLHYNNKYVELLDTFFMVMRKKFDQLSFLHIYHHTLLIWSWFVVMK
LEPVGDCYFGSSVNTFVHVIMYSYYGLAALGVNCFWKKYITQIQMLQFCICASHSI
YTAYVQNTAFWLPYLQLWVMVNMFVLFANFYRKRYKSKGAKKQ

Figure 21. AA sequence of Pyrco-Δ5E

Similarity to other proteins in consumed foods, food production or in animal feeds

The FA Δ5-elongases have been cloned from a wide range of organisms, including moss (Eiamsa-ard et al., 2013), alga (Robert et al., 2005), marine protist *Thraustochytrid*, kinetoplastid parasite (Livore et al., 2007) and liverwort (Kajikawa et al., 2006). In addition, FA elongases involved in the PUFA with similar function of Δ6-elongases are also isolated from many animals like frog, fish, sea squirt (Meyer et al., 2004) and human (Leonard et al., 2004). Human PUFA elongase, Elo5, converts a wide range of exogenously added long-chain PUFA substrates into their respective elongated FA products, including SDA into ETA (Δ6-elongation) and EPA into DPA (Δ5-elongation) (Leonard et al., 2000, 2004). The Pyrco-Δ5E shared homology (23-37%) to other Δ5-elongase, Δ6-elongase or PUFA elongase proteins.

Pyrco-Δ5E shares AA sequence identity with other elongases consumed in food, animal feed, or used in food production (Table 31). Several human PUFA elongases have also been isolated (Leonard et al., 2004), including the EPA elongation (Δ5-elongation). They share 24%~28% sequence identity with Pyrco-Δ5E. Pyrco-Δ5E also shares 27%-28% sequence identity to salmon (AAO13175) bifunctional Δ5/Δ6-elongases. Salmon is a well-known salt-water fish consumed as food. Pyrco-Δ5E also shares 23% sequence identity to soybean FA elongase (XP_003531583). Soybean is one of the major oil crops produced for food oil.

Table 31. Amino acid sequence identity between Pyrco-Δ5E in DHA canola and other elongases

No.	Protein	NCBI Accession	Common Name	Sequence identity							
				1	2	3	4	5	6	7	8
1	NS-B50027-4 Pyrco-Δ5E			100	37.5	25.1	27.6	27.5	24.9	25.1	22.9
2	Pavlu-Δ5E	AAV33630	Alga		100	24.4	24.2	25.5	22.0	31.0	22.4
3	Moral-Δ6E	AAF70417	Fungus			100	24.0	23.7	24.6	18.1	21.5
4	Homsa-Elo1	XP_002040	Human				100	31.7	23.2	32.2	22.1
5	Salsa-Elo	AAO13175	Salmon					100	25.6	23.8	19.2
6	Phatr-Δ6E	CAM55851	Diatom						100	19.0	22.5
7	Thaps-Δ5E	AAV67800	Alga							100	18.2
8	Glyma-Elo	XP_003531583	Soybean								100

Glyma, *G. max*; Homsa, *H. sapiens*; Moral, *M. alpina*; Pavlu, *P. lutheri*; Phatr, *P. tricornutum*; Pyrco, *P. cordata*; Salsa, *S. salar;* Thaps, *T. pseudonana*.

Functional activity of Pyrco-Δ5E

The enzyme functionality of Pyrco-Δ5E has been confirmed in different heterologous expression systems, including yeast cell (Petrie et al., 2010a), *Arabidopsis* seed (Petrie et al., 2012) and *Camelina* seed (Petrie et al., 2014). In this study, Pyrco-Δ5E was expressed in *P. pastoris*, as fusion proteins designated as SP::His_{10}::Pyrco-Δ5E or His_{10}::Pyrco-Δ5E. In SP::His_{10}::Pyrco-Δ5E, the Pyrco-Δ5E sequence was fused to *S. cerevisiae* SP, followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal. Over-expression of Pyrco-Δ5E fusion proteins in *P. pastoris* with secretion peptide demonstrated the elongation of ω3 EPA to ω3 DPA, compared to the vector alone where there was no ω3 DPA (Table 32).

Table 32. Activity of Pyrco-Δ5E fusion protein in *P. pastoris* cells

Sample	Substrate (%)		Product (%)		Conversion (%)	
Vector	20:5	2.8 ± 0.0	22:5	0.0 ± 0.0	0.0 ± 0.0	n=3
SP::His_{10}::Pyrco-Δ5E		1.8 ± 1.3		2.7 ± 0.6	62.8 ± 18.5	n=9

Substrate and product are shown in percentage of total FA in cells. Conversion rate is based on the product DPA compared to the total of product DPA and remaining substrate EPA. n = repeats with individual colonies. Yeast cell culture was fed with 0.5 mM EPA (20:$5^{\Delta5,8,11,14,17}$) substrate.

The genome sequence including the T-DNA insertions in DHA canola was analyzed. The translated AA sequence of Pyrco-Δ5E in the insert was confirmed to be identical to the original sequence (Figure 22). Pyrco-Δ5E sequence translated from sequenced T-DNA insert in DHA canola was identical to the original Pyrco-Δ5E sequence from *P. cordata* in binary vector (Pyrco-Δ5E_vec).

```
                    1                                                  50
Pyrco-d5E_vec   (1) MASIAIPAALAGTLGYVTYNVANPDIPASEKVPAYFMQVEYWGPTIGTIG
NS-B50027-4     (1) MASIAIPAALAGTLGYVTYNVANPDIPASEKVPAYFMQVEYWGPTIGTIG
                    51                                                 100
Pyrco-d5E_vec  (51) YLLFIYFGKRIMQNRSQPFGLKNAMLVYNFYQTFFNSYCIYLFVTSHRAQ
NS-B50027-4    (51) YLLFIYFGKRIMQNRSQPFGLKNAMLVYNFYQTFFNSYCIYLFVTSHRAQ
                    101                                                150
Pyrco-d5E_vec (101) GLKVWGNIPDMTANSWGISQVIWLHYNNKYVELLDTFFMVMRKKFDQLSF
NS-B50027-4   (101) GLKVWGNIPDMTANSWGISQVIWLHYNNKYVELLDTFFMVMRKKFDQLSF
                    151                                                200
Pyrco-d5E_vec (151) LHIYHHTLLIWSWFVVMKLEPVGDCYFGSSVNTFVHVIMYSYYGLAALGV
NS-B50027-4   (151) LHIYHHTLLIWSWFVVMKLEPVGDCYFGSSVNTFVHVIMYSYYGLAALGV
                    201                                                250
Pyrco-d5E_vec (201) NCFWKKYITQIQMLQFCICASHSIYTAYVQNTAFWLPYLQLWVMVNMFVL
NS-B50027-4   (201) NCFWKKYITQIQMLQFCICASHSIYTAYVQNTAFWLPYLQLWVMVNMFVL
                    251            267
Pyrco-d5E_vec (251) FANFYRKRYKSKGAKKQ
NS-B50027-4   (251) FANFYRKRYKSKGAKKQ
```

Figure 22. Alignment of protein sequences of Pyrco-Δ5E

Digestibility of Pyrco-Δ5E

The results of the digestibility / stability study demonstrated that greater than 75% of the Pyrco-Δ5E protein digested within 5 minutes and full-length protein was rapidly digested within 60 minutes of incubation in pepsin producing a suite of pepsin products <3,000 daltons that spanned the entire peptide sequence when analyzed by LC-MS/MS.

The rapid degradation of the His_{10}::Pyrco-Δ5E protein demonstrated by the rapid liberation of peptic peptides was further confirmed by the decline of the single tryptic peptide after trypsin digestion (in the combined pepsin-trypsin digestion). Four of the peptides characterized and quantified after pepsin digestion were cleavage variants (Fig. 22A-D). The black arrows in Figure 23 indicate that the peptide in the left panel is cleaved further by pepsin to yield the peptide in the right panel. The N-terminal peptic peptides monitored were produced rapidly (<5 min) and reached an equilibrium over the experimental duration. The peptic peptides monitored may not represent the fully cleaved final product as pepsin is relatively non-specific. All of the displayed pepsin proteolysis products in Figure 23 contained missed cleavages (indicated by red font in peptide sequence) and are therefore susceptible to further degradation. The only peptide that might be considered a final product of pepsin digestion is NVANPDIPASEKVPAY wherein the lysine (K) located in the P3 position is likely to hinder further cleavage before Y (Figure 23B). This peptide reaches an equilibrium (plateau) by 15 min. The appearance of these peptides in the digest is taken as evidence of the degradation and therefore digestibility of the His_{10}::Pyrco-Δ5E protein.

In the case of the Pyrco-Δ5E protein, only a single tryptic product could be detected owing to both the small-scale (6.7 µg load) digest and the distribution of trypsin sites within the protein sequence that resulted in few peptides amenable to LC-MS. The single peptide monitored, SQPF$^{\downarrow}$GL$^{\downarrow}$K, contained two pepsin cleavage sites (as indicated by the arrows) and it was expected that pepsin would cleave this peptide resulting in a decrease in peptide abundance over the time course of the pepsin digestion. After 5 minutes, however, the peptide peak area was noted to increase 3-fold and remained relatively constant over the next 5 minutes before proceeding to decline slowly over the next 50 minutes (Figure 24). A similar phenomenon was observed previously for a peptide derived from Δ4D wherein a 2-fold increase in the peak area of a tryptic peptide (WEGEPISK) was noted after 5 minutes incubation of the protein with pepsin. Both scenarios are postulated to arise from the peptides monitored residing within the core of the molecule, which in its native conformation is partially protected from trypsin digestion.

After a short incubation with pepsin (5 minutes), the tertiary structure of the protein (Pyrco-Δ5E) is destroyed allowing full access to the tryptic sites and hence liberation of the tryptic peptide (SQPFGLK) at its maximal level (Figure 24). The absence of detectable tryptic peptides derived from the Pyrco-Δ5E protein precluded the determination of the final percentage degradation as determined for ω3D and Δ4D, (see Section B, Table 24 and Section G, Table 35), however the appearance of peptic products (Figure 23) demonstrated that the Pyrco-Δ5E protein is digested by

pepsin over the time course of the experiment with >75% cleavage of the N-terminal region achieved in <5 minutes (Figure 23A-23B).

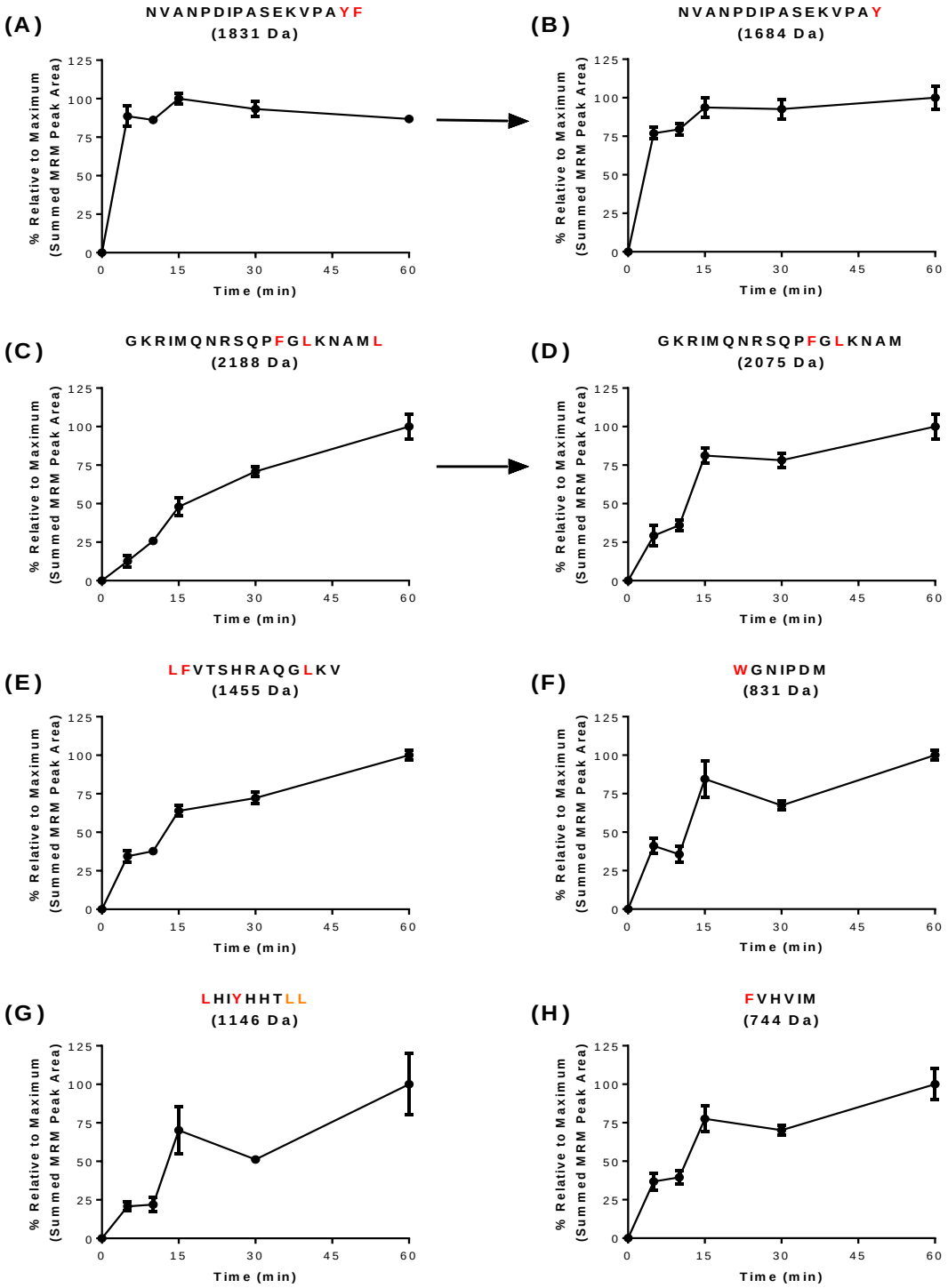

Figure 23. Quantification of the peptide products of His_{10}::Pyrco-Δ5E after pepsin digestion

Figure 23 illustrates LC-MRM-MS analysis of pepsin proteolytic fragments. The response in the LC-MS system (measured as peak area) was converted to a percentage relative to the maximum peak area observed

during pepsin digestion. The experimental control was time 0 with no pepsin addition. The peptides are graphed in order from protein N- to C-terminus. The peptide sequence (and calculated molecular weight) are denoted above each graph. Arrows indicate a subsequent cleavage to yield a secondary cleavage variant. The potential sites for secondary pepsin cleavage are indicated in red (expected cleavage) or orange (potentially hindered) font within the sequence. The error bars denote SD.

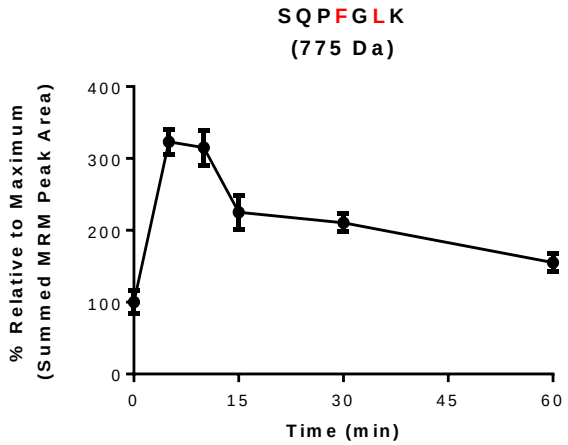

Figure 24. Quantification of the single tryptic peptide product of His_{10}::Pyrco-Δ5E after combined pepsin-trypsin digestion.

LC-MRM-MS analysis of the single trypsin proteolytic fragment that was detected. The response in the LC-MS system (measured as peak area) was converted to a percentage reduction relative to the experimental control (time 0, no pepsin addition). The peptide sequence (and calculated molecular weight) is denoted above the graph. The potential sites for pepsin cleavage of these peptide sequences are indicated in red font.

The results of this study show that the integral membrane protein Pyrco-Δ5E was readily digestible in pepsin and/or trypsin. The Pyrco-Δ5E protein was used as the representative of the two microalgae FA elongases engineered into DHA canola, Pyrco-Δ5E and Pyrco-Δ6E (see Section D.), for stability analysis in this report.

G. *P. salina* Δ4-Desaturase

The translated *P. salina* Δ4-desaturase (Pavsa-Δ4D) contains 447 AA residues (Figure 25). The AA sequence of Pavsa-Δ4D shares high homology to other Δ4-desaturases. The Pavsa-Δ4D protein catalyzes the desaturation of EPA at the Δ4 position ($22{:}5^{\Delta 7,10,13,16,19}$) to DHA ($22{:}6^{\Delta 4,7,10,13,16,19}$). Pavsa-Δ4D has a predicted molecular weight of 49.3 kDa, and an estimated pI of 8.66. The Pavsa-Δ4D protein contains no potential glycosylation site. Figure 25 shows the AA sequence derived from the nucleotide sequence of the inserted DNA.

MPPSAAKQMGASTGVHAGVTDSSAFTRKDVADRPDLTIVGDSVYDAKAFRSEHPGG
AHFVSLFGGRDATEAFMEYHRRAWPKSRMSRFHVGSLASTEEPVAADEGYLQLCAR
IAKMVPSVSSGFAPASYWVKAGLILGSAIALEAYMLYAGKRLLPSIVLGWLFALIG
LNIQHDANHGALSKSASVNLALGLCQDWIGGSMILWLQEHVVMHHLHTNDVDKDPD
QKAHGALRLKPTDAWSPMHWLQHLYLLPGETMYAFKLLFLDISELVMWRWEGEPIS
KLAGYLFMPSLLLKLTFWARFVALPLYLAPSVHTAVCIAATVMTGSFYLAFFFFIS
HNFEGVASVGPDGSITSMTRGASFLKRQAETSSNVGGPLLATLNGGLNYQIEHHLF
PRVHHGFYPRLAPLVKAELEARGIEYKHYPTIWSNLASTLRHMYALGRRPRSKAE

Figure 25. Amino acid sequence of Pavsa-Δ4D

Similarity to other proteins in foods, food production or in animal feeds

The Pavsa-Δ4D protein shares similarity (16-66%) to desaturase proteins present in food that is consumed, used in food production or in animal feeds (Table 33).

Table 33. Comparison of AA sequence identity of Pavsa-Δ4D in DHA canola to other desaturases

No.	Protein	Accession	Common Name	Sequence identity											
				1	2	3	4	5	6	7	8	9	10	11	12
1	NS-B50027-4 Pavsa-Δ4D			100	65.5	23.3	26.6	19.8	24.9	20.0	18.9	19.8	17.7	15.6	17.4
2	Pavlu-Δ4D	AQ98793	Alga		100	23.9	25.8	19.2	23.2	20.1	20.9	18.5	19.1	18.5	16.2
3	Euggr-Δ4D	AAQ19605	Alga			100	22.8	34.4	24.2	27.9	16.0	16.7	16.7	15.7	18.3
4	Phatr-Δ5D	AAL92562	Alga				100	20.6	21.7	21.9	21.0	17.9	18.9	20.1	19.6
5	Thaps-Δ4D	AAX14506	Alga					100	23.8	16.4	16.8	17.2	15.8	15.5	15.3
6	Moral-Δ5D	O74212	Fungus						100	20.9	18.1	22.5	20.7	20.9	21.5
7	Moral-Δ6D	AAF08685	Fungus							100	21.0	15.5	27.9	27.1	27.9
8	Isoga-Δ4D	AAV33631	Alga								100	12.0	20.2	20.5	19.3
9	Artpl-Δ6D	CAA60573	Cyanobacteria									100	16.7	13.9	14.5
10	Echpl-Δ6D	AAZ08559	Paterson's curse										100	85.3	62.5
11	Borof-Δ6D	O04353	Borage											100	64.3
12	Oenbi-Δ6D	ACB47482	Evening primrose												100

Artpl, *Arthrospira platensis*; Borof, *B. officinalis*; Echpl, *E. plantagineum*; Euggr, *Euglena gracilis*; Isoga, *I. galbana*; Moral, *M. alpina*; Moral, *M. alpina*; Oenbi, *O. biennis*; Pavlu, *Pavola luthri*; Phatr, *P. tricornutum*; Thaps, *T. pseudonana*.

The Pavsa-Δ4D protein belongs to the subfamily of front-end desaturases that introduce a double bond between an existing double bond and the carboxyl end of FAs. The front-end desaturases include Δ4-, Δ5-, Δ6- and Δ8-desaturases, which exist in a wide range of organisms including algae, diatoms, fungi, mosses and bacteria. Microalgae such as *Spirulina*, *Chlorella*, *Dunaliella*, *Haematococcus* and *Schizochytrium* are classified as food sources considered GRAS (Chacón-Lee and González-Marino, 2010).

Spirulina and *Chlorella* are major microalgal genera cultivated in China for health food (Liang et al., 2004). Algal biomass is supplemented to noodles, breads, biscuits, candies, ice cream, bean curd and other common foods to enhance their nutritive values. The so-called blue-green algae, *Spirulina*, are cyanobacteria. Cyanobacteria have been part of the human diet for centuries (Gantar and Svirčev, 2008). Additionally, the flowers of *Echium* spp. have been consumed as medicinal plants in countries such as Iran (Heidari et al., 2006), and evening primrose oil is commonly sold

in Australian health food shops. Evening primrose plants have been used as ornamentals, food sources, and as medicinal herbs for more than 50 years.

Functional activity of Pavsa-Δ4D

The enzyme functionality of Pavsa-Δ4D has been confirmed in many different heterologous expression systems. Table 34 shows the enzymatic activity of Pavsa-Δ4D expressed in yeast S288C cells when exogenously supplied with ω3 or ω6 substrates (Zhou et al., 2007). Pavsa-Δ4D can desaturate both DTA ($22:4^{\Delta7,10,13,16}$) and DPA ($22:5^{\Delta7,10,13,16,19}$) substrates at the Δ4 position, producing DPA ($22:5^{\Delta4,7,10,13,16}$) or DHA ($22:6^{\Delta4,7,10,13,16,19}$). The conversion rates with the yeast cell expressed the Pavsa-Δ4D protein were 3.0% or 2.4%, respectively.

Table 34. Fatty acid composition of yeast S288C cells expressing Pavsa-Δ4D showing Δ4-desaturase activity

Fatty acid	Substrate (% of total FA)		Product (% of total FA)		Conversion (%)
ω6	$22:4^{\Delta7,10,13,16}$	0.97	$22:5^{\Delta4,7,10,13,16}$	0.03	3.0
ω3	$22:5^{\Delta7,10,13,16,19}$	1.66	$22:6^{\Delta4,7,10,13,16,19}$	0.04	2.4

Additionally, the genome sequence including the T-DNA insertions in DHA canola was analyzed. The translated AA sequence of Pavsa-Δ4D in the insert was confirmed to be identical to the original sequence (Figure 26). The Pavsa-Δ4D sequence translated from sequenced T-DNA insert in DHA canola was identical to the original Pavsa-Δ4D sequence from *P. salina* in the binary vector (Pavsa-Δ4D_vec).

```
                    1                                                   50
Pavsa-Δ4D_vec   (1) MPPSAAKQMGASTGVHAGVTDSSAFTRKDVADRPDLTIVGDSVYDAKAFR
NS-B50027-4     (1) MPPSAAKQMGASTGVHAGVTDSSAFTRKDVADRPDLTIVGDSVYDAKAFR
                   51                                                  100
Pavsa-Δ4D_vec  (51) SEHPGGAHFVSLFGGRDATEAFMEYHRRAWPKSRMSRFHVGSLASTEEPV
NS-B50027-4    (51) SEHPGGAHFVSLFGGRDATEAFMEYHRRAWPKSRMSRFHVGSLASTEEPV
                  101                                                  150
Pavsa-Δ4D_vec (101) AADEGYLQLCARIAKMVPSVSSGFAPASYWVKAGLILGSAIALEAYMLYA
NS-B50027-4   (101) AADEGYLQLCARIAKMVPSVSSGFAPASYWVKAGLILGSAIALEAYMLYA
                  151                                                  200
Pavsa-Δ4D_vec (151) GKRLLPSIVLGWLFALIGLNIQHDANHGALSKSASVNLALGLCQDWIGGS
NS-B50027-4   (151) GKRLLPSIVLGWLFALIGLNIQHDANHGALSKSASVNLALGLCQDWIGGS
                  201                                                  250
Pavsa-Δ4D_vec (201) MILWLQEHVVMHHLHTNDVDKDPDQKAHGALRLKPTDAWSPMHWLQHLYL
NS-B50027-4   (201) MILWLQEHVVMHHLHTNDVDKDPDQKAHGALRLKPTDAWSPMHWLQHLYL
                  251                                                  300
Pavsa-Δ4D_vec (251) LPGETMYAFKLLFLDISELVMWRWEGEPISKLAGYLFMPSLLLKLTFWAR
NS-B50027-4   (251) LPGETMYAFKLLFLDISELVMWRWEGEPISKLAGYLFMPSLLLKLTFWAR
                  301                                                  350
Pavsa-Δ4D_vec (301) FVALPLYLAPSVHTAVCIAATVMTGSFYLAFFFFISHNFEGVASVGPDGS
NS-B50027-4   (301) FVALPLYLAPSVHTAVCIAATVMTGSFYLAFFFFISHNFEGVASVGPDGS
                  351                                                  400
Pavsa-Δ4D_vec (351) ITSMTRGASFLKRQAETSSNVGGPLLATLNGGLNYQIEHHLFPRVHHGFY
NS-B50027-4   (351) ITSMTRGASFLKRQAETSSNVGGPLLATLNGGLNYQIEHHLFPRVHHGFY
                  401                                                448
Pavsa-Δ4D_vec (401) PRLAPLVKAELEARGIEYKHYPTIWSNLASTLRHMYALGRRPRSKAE
NS-B50027-4   (401) PRLAPLVKAELEARGIEYKHYPTIWSNLASTLRHMYALGRRPRSKAE
```

Figure 26. Alignment of protein sequences of Pasva-Δ4D

Digestibility of Pavsa-Δ4D

The results of the study demonstrated that greater than 80% was digested within 10 minutes and greater than 93% of the full-length Pavsa-Δ4D protein was digested within 60 minutes of incubation in pepsin when analyzed by LC-MS/MS (Table 35). The results of this study show that the integral membrane protein Pavsa-Δ4D was readily digestible in pepsin and/or trypsin. The Pavsa-Δ4D protein was used as the representative of the three front-end desaturases engineered in DHA canola, Micpu-Δ6D (see Section C), Pavsa-Δ5D (see Section E) and Pavsa-Δ4D, for stability analysis in this report. Rapid digestion of the full-length protein is one of many factors that indicate protein safety.

Table 35. Percentage of each tryptic peptide remaining during pepsin time course

Peptide Sequence	Time (min)				
	5	10	15	30	60
QMGASTGVHAGVTDSSAFTR	46.9	14.8	10.4	10.2	5.8
DVADRPDLTIVGDSVYDAK	28.6	6.2	5.0	3.6	3.0
SEHPGGAHFVSLFGGR	12.3	1.0	1.2	0.6	0.4
FHVGSLASTEEPVAADEGYLQLC{CAM}AR	23.2	2.8	1.5	1.2	0.4
DATEAFMEYHR	34.2	4.8	3.2	1.8	0.9
VHHGFYPR	51.2	18.6	12.4	11.3	5.1
LAPLVK	47.4	16.3	11.4	12.1	6.7
HMYALGR	45.1	8.2	7.7	5.4	3.0

H. Phosphinothricin acetyltransferase

Acetyltransferases are abundant and ubiquitous in nature and are present in microbes, plants and animals. They share the common function of transferring an acetyl group from acetyl-CoA to a substrate. Acetyltransferases differ in substrates and the metabolic pathways in which they function (Webb, 1992). For example, S-acetyltransferases perform vital functions in FA biosynthesis. PAT is an N-acetyltransferase. Although they are not commonly considered to be food, acetyltransferases are consumed as components of food. There are no known naturally occurring glufosinate resistant plants that express PAT.

The biochemical characterization, allergenic and toxic potential of the PAT protein (encoded by either the *bar* or *pat* genes) was addressed in at least 20 different previous food and feed safety assessment summaries provided to the regulatory authorities around the world (e.g. events T14/T25 maize, LLRICE06, LLRICE62, LLCotton25, Topas 19/2 canola, SYN-00098-3, DeKalb's B16 maize, etc). The *bar* and *pat* genes produce very similar proteins (Wehrmann et al., 1996) and should be considered together. A detailed safety assessment, including the physio-chemical properties of PAT, allergen and toxin evaluation along with digestibility and toxicity data of the PAT protein has been published (Hérouet et al., 2005). No further review will be given here.

4. Protein Content in DHA Canola

Each protein has been fully characterized and quantitated in DHA canola. The expressed proteins in DHA canola were quantified in multiple tissue types collected over a growing season (Table 36). Typically, traditional Western blot analysis would be used for protein quantitation in plant tissues. However, in the absence of functioning antibodies against these membrane integral proteins, an alternative approach using high sensitivity LC MRM mass spectrometry (MS) was developed to quantify the concentration of the expressed proteins in DHA canola at a variety of growth stages, as noted from here, forward expressed using the Bayer, BASF, Ciba-Geigy and Hoechst Growth Scale (BBCH scale) for canola.

Table 36. Canola tissues sampled at different stages

Growth stage*	Timing	Tissue sampled	Replicate
BBCH15	5 True leaves	3 Whole plants	3
BBCH35	3 Visibly extended internodes	1 Whole plant	3
BBCH65	50% Full flowering	All flowers from 1 plant	3
		All roots from 1 plant	3
		All leftover from 1 plant	3
BBCH79	Developing seed	All pods from 1 plant	3
BBCH90	Senescence	All grain from 1 plant	3

*Developmental stage at which each tissue was collected. Canola growth stages are based on the BBCH scale.

Protein quantification by LC-MRM-MS, using a triple quadrupole mass spectrometer (MS), is an approach that has been successfully applied to clinical laboratory studies (Rauh, 2012; Gillette and Carr, 2013). Analysis of proteins by MRM is based on detection of peptides derived from proteolytic digestion of the target protein, typically by trypsin. The measurement of the peptides in a complex sample matrix is achieved by adding a known concentration of an isotope-labeled peptide isomer as an internal standard to the plant sample before analysis. The labeled peptide isomer (typically referred to as "heavy") contains an AA labeled with the stable isotopes ^{15}N and/or ^{13}C, resulting in a mass increase compared to that of the native peptide isomer (typically referred to as "light"). The heavy and light peptides, when subjected to chromatography, show identical elution profiles allowing the detection of the light peptides in the plant matrix. The endogenous peptide response was measured and the concentration was interpolated from a calibration curve, allowing the quantification of the peptide as femtomoles per 100 µg total protein. This value was converted to a nanogram equivalent per mg total protein based on the molecular mass of each protein.

The total protein extracts from DHA canola seed or from recombinant proteins expressed in either yeast, bacterial or baculovirus expression systems were first analyzed by non-targeted LC-MS for detection of the tryptic peptides generated for each target protein (DHA biosynthesis pathway desaturase or elongase). The total protein from *N. benthamiana* leaf with transiently expressed PAT protein was used for detection of the tryptic peptides of PAT. Data generated was searched against the custom protein database and two peptides were selected from each target protein as proxies for quantification of the proteins. The selection of peptides was based on having:

(1) a good MS response (high intensity);
(2) the absence of AAs within the peptide sequence that would likely be modified (for example, oxidation of methionine) or miscleaved (presence of dibasic residues at either terminus);
(3) specific/unique sequence to the target protein; and
(4) size amenable to LC-MS (~6-20 AAs in length).

For each selected peptide, both the endogenous light peptides and heavy peptides were synthesized. The purity of synthesized peptides was analyzed by LC-MS. Dilutions equivalent to ~5 pmol/µL were prepared in aqueous solution (1% formic acid) and analyzed by LC positive ion electrospray

ionization tandem MS. Any peptides showing significant contamination including the presence of truncated, modified and/or synthesis by-products were excluded from further analysis. The amounts of synthesized peptides were determined by high sensitivity AA analysis (AAA) at Australian Proteomics Analysis Facility (Sydney, Australia). All samples were analyzed in duplicate. The calculated amount of AA (µg/mL) is based on the AA residue mass in the protein (molecular weight minus H_2O). Using the determined concentrations, stock solutions were prepared at 100 pmol/µL.

5. Expression levels

AV Jade and DHA canola were planted at two field trial sites. Tissue samples from AV Jade and DHA canola were collected from each site at seven sampling times, representative of specific growth stages of canola allowing for collection of various tissue types, including leaves, roots, pods and reproductive tissues. Protein was extracted from these tissues and subjected to the LC-MRM-MS quantification method using peptides derived from the AA sequences of the expressed enzymes in DHA canola.

LC-MRM-MS quantification confirmed that none of the targeted peptides were detected in protein extracts from AV Jade tissues collected or were they detected in protein extracts from the non-seed tissues of DHA canola (Table 37). All seven peptides representing the DHA biosynthesis pathway enzymes were detected in developing and/or mature seeds of DHA canola (Tables 37, 38, 39). The Pyrco-Δ5E and Pyrco-Δ6E proteins revealed the lowest protein abundance in the DHA canola. The Pyrco-Δ5E was below the limit of detection (LOD) in developing seeds, while the Pyrco-Δ6E protein was below the LOD in mature seeds. In mature seeds, the Pavsa-Δ4D protein had the highest abundance of the seven enzymes. The molecular mass of each protein was used to determine the level of each transgenic protein. The lowest protein was 20 ng/mg of Pyrco-Δ5E per in total protein, and highest was Pavsa-Δ4D at 740 ng/mg total protein.

The results from this study demonstrated that the enzymatic proteins that drive the production of DHA using seed-specific promoters were only detected in developing seed and mature seed at very low levels (20-740 ng/mg total protein; Table 39), while none of the DHA biosynthesis pathway enzymes were detected in the non-seed tissues of DHA canola.

Table 37. Detection of peptides of transgene proteins in canola plant parts

Protein	Peptide Sequence	Whole plant BBCH15*	Whole plant BBCH35	Root BBCH65	Flower BBCH65	Other BBCH65	Develop. Seed BBCH79	Mature Seed BBCH90
Lackl-Δ12D	GSSSNTEQEVPK	ND**	ND	ND	ND	ND	✓	✓
Picpa-ω3D	IPFYHAR	ND	ND	ND	ND	ND	✓	✓
Micpu-Δ6D	DASTAPVDLK	ND	ND	ND	ND	ND	✓	✓
Pyrco-Δ6E	GQDPFLLK	ND	ND	ND	ND	ND	✓	ND
Pavsa-Δ5D	AYDVTNFVK	ND	ND	ND	ND	ND	✓	✓
Pyrco-Δ5E	SQPFGLK	ND	ND	ND	ND	ND	ND	✓
Pavsa-Δ4D	LAPLVK	ND	ND	ND	ND	ND	✓	✓

*Canola growth stages based on the BBCH scale; **ND = non-detectable

Table 38. Transgenic proteins in developing and mature DHA canola seed (moles)

Protein	Peptide Sequence	Developing seed (BBCH79)		Mature seed (BBCH90)	
		Site 1506	Site 1508	Site 1506	Site 1508
Lackl-Δ12D	GSSSNTEQEVPK	507.1 ± 14.1	461.6 ± 149.5	441.0 ± 89.6	551.0 ± 87.3
Picpa-ω3D	IPFYHAR	351.1 ± 51.9	352.0 ± 148.7	469.3 ± 189.1	551.2 ± 55.1
Micpu-Δ6D	DASTAPVDLK	166.0 ± 28.7	257.1 ± 57.3	85.6 ± 7.5	80.9 ± 14.9
Pyrco-Δ6E	GQDPFLLK	79.0 ± 5.3	89.9 ± 19.6	ND	ND
Pavsa-Δ5D	AYDVTNFVK	131.6 ± 34.1	136.4 ± 65.7	129.2 ± 31.6	155.5 ± 41.6
Pyrco-Δ5E	SQPFGLK	ND	ND	64.1 ± 38.7	89.7 ± 15.7
Pavsa-Δ4D	LAPLVK	974.6 ± 296.6	888.7 ± 629.1	1500 ± 408.7	1470 ± 313.7

The amount of peptide detected is reported in units of femtomole/100 µg total protein, as mean ± SD, n=3. ND, not detected *Developmental stage at which each tissue was collected. Canola growth stages based on the BBCH scale.

Table 39. Abundance of transgenic proteins in developing and mature DHA canola seed (weight/weight)

Protein	MW (Da)	Developing Seed (BBCH79*)		Mature Seed (BBCH90)	
		Site 1506	Site 1508	Site 1506	Site 1508
Lackl-Δ12D	48,158	244.2 ± 6.8	222.3 ± 72.0	212.4 ± 43.2	265.4 ± 42.0
Picpa-ω3D	47,760	167.7 ± 24.8	168.1 ± 71.0	224.1 ± 90.3	263.3 ± 26.3
Micpu-Δ6D	52,935	87.9 ± 15.2	136.1 ± 30.3	45.3 ± 4.0	42.8 ± 7.9
Pyrco-Δ6E	33,078	26.1 ± 1.8	29.7 ± 6.5	ND	ND
Pavsa-Δ5D	48,215	63.4 ± 16.4	65.8 ± 31.7	62.3 ± 15.2	75.0 ± 20.0
Pyrco-Δ5E	31,268	ND	ND	20.0 ± 12.1	28.0 ± 4.9
Pavsa-Δ4D	49,307	480.5 ± 146.2	438.2 ± 310.2	739.5 ± 201.5	724.7 ± 154.7

*Developmental stage at which each tissue was collected. Canola growth stages are based on the BBCH scale; Units are ng transgene protein per mg total protein extracted.

6. Assessment of potential allergenicity and toxicity

Bioinformatics searches were conducted to compare the AA sequence of each expressed protein in DHA canola against the curated AllergenOnline.org database version 16 (January 2016), which is maintained at the University of Nebraska, as well as searches against the NCBI Protein database using BLASTP with keyword limits for allergy and toxicity (FARRP, 2016). The search criteria were set to identify matches likely to represent minimal risks with identity matches of >35% identity over 80 AA and exact identity matches across 8 AA for allergenicity, as well as comparisons with sequences in NCBI Protein database for sequence matches of 50% or more with keyword limits (e.g. allergen, toxin) for risks of allergy and toxicity.

None of the results from the bioinformatics searches of the microorganisms or inserted AA sequences, compared to known and putative allergens or toxins, identified any significant sequence identity match to a protein likely to cause an adverse effect in consumers.

7. Safety assessment summary

In conclusion, the safety of the introduced proteins is supported by the history of safe use of proteins similar to those in DHA canola that have been routinely consumed for many years, their quick digestion in pepsin and/or trypsin and their lack of similarity to known allergens of toxins using *in silico* analysis. Each protein has been fully characterized and quantitated in DHA canola. The enzymatic proteins that drive the production of DHA using seed-specific promoters were only detected in developing seed and mature seed at low levels (20-740 ng/mg total protein), while none of the DHA biosynthesis pathway enzymes were detected in the non-seed tissues of the transgenic canola, irrespective of the sampling time or the tissues tested.

Characterization of the DHA canola event demonstrated that there are no safety concerns. The ORFs created by insertion of the DNA will not express a toxin or allergen, the expressed DHA pathway enzymes are very low in concentration and are only expressed in the seed, and the agronomic properties of the DHA canola are no different than AV Jade. In conclusion, there is a reasonable certainty of no harm resulting from DHA canola, including the introduced genes and proteins, in human foods, animal feed or environmentally.

VII. Compositional Assessment

Compositional comparisons between transgenic crops and conventional varieties are a key part of a nutritional and safety assessment and provide assurance of the food safety of transgenic crops. Compositional assessments are performed in accordance with the principles outlined in the OECD consensus document (OECD, 2011). This document emphasizes quantitative measurements of essential nutrients, and known anti-nutrients and toxicants. These analyses will effectively highlight any compositional changes that may indicate potential safety and anti-nutritional concerns.

For DHA canola grain samples, 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.

Levels of the analytes for DHA canola were compared to corresponding levels in AV Jade comparator (CMP) and were also compared to statistical tolerance intervals generated from non-modified conventional commercial canola reference varieties (REF). These comparisons formed the basis for determining compositional comparability of DHA canola to conventional canola. Canola seed was chosen as the test material for the compositional analysis of DHA canola because oil fractions are derived from seed. Compositional evaluation of seed would be representative of these derived materials.

Seed samples were collected from field trials conducted in 2015 at eight locations in major canola growing regions of Australia for compositional analysis. The experimental sites were located across varying environments for soil type and rainfall and agronomic management practices.

Rainfall and temperature were recorded at each location for the period from planting to harvest. Irrigation was occasionally used when available and required to supplement rainfall.

Each trial was designed as a randomized complete block experiment consisting of five replicates (block) with AV Jade and eight reference cultivars. Grain samples of 350-400g were collected and pooled from seedpods taken from the middle two rows of each plot and analyzed (Eurofins Nutritional Analysis Center).

A summary of each parameter, its method of analysis, appropriate units and the LOQs are included in Table 40. Data means, standard deviations, ranges and p-values were determined for the compositional data. Data were analyzed using SAS v9.4 (SAS, 2017) on the final, rounded results. The results of each analyte are summarized for each canola variety, namely CMP; DHA canola (GMO), and REF, including mean, minimum (min), maximum (max), and SD. A comparison was made of GMO and CMP on seeds across all sites with a linear mixed model with genotype as a fixed factor and site as a random factor. Across all sites and each analyte, the difference between GMO and CMP was estimated after conducting an ANOVA analysis using the site and line main effects and the site by line interaction. Single degree of freedom contrasts were used to test for differences between the experimental line and the parental line. P-values (≤0.05% indicates a significant difference) are not reported where the means were below the LOD, when >30% of the values were <LOQ, or where missing data made the effect non-testable. When data points were at or below the LOQ, the LOQ value was used to calculate the averages, standard deviations and data ranges. The range of determined values for each of the analytes for the reference lines is also reported.

Calculations of dry weights and fatty analysis were done as described below. Conversion from a fresh weight (FW) basis to dry weight (DW) basis:

$$\%DW = \%FW \times (100/(100-\text{moisture}))$$

Conversion from FW basis to a percent relative (Rel) basis for individual FA:

$$\%FA\ Rel = (\%FA / \%\text{total FA}) \times 100$$

When FA results were below LOQ, the LOQ value was used for % FA.

Due to limited sample availability, no vitamin or mineral analyses were performed on some samples, therefore these samples were excluded in the statistical analysis of those analytes. The Nº column reflects which sample numbers were reduced, but did not have any impact on the overall analyses of minerals and vitamins. Detailed compositional analysis of DHA canola meal was also conducted in accordance with the revised OECD consensus document (OECD, 2011). This analysis was conducted to investigate the nutritional elements of meal processed from DHA canola, and AV Jade. Compositional analysis of meal samples included protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber, ash, carbohydrates, FAs, AAs, vitamin E, minerals, phytosterols and key anti-nutrients.

Table 40. Compositional analysis of DHA canola, methods, units and LOQ values

Parameters	Eurofins Method	Units	LOQ
Protein, Crude	MET-PR-002	%	0.1%
Fat, Crude	MET-LI-001	%	0.1%
Ash	MET-PR-004	%	0.4%
Carbohydrates, Calculated	OPS-024	%	N/A
Crude Fiber	MET-PR-003	%	0.2%
AAs by Acid Hydrolysis	MET-LC-006	%	Aspartic Acid: 0.02% Threonine: 0.02% Serine: 0.01% Glutamic Acid: 0.01% Glycine: 0.01% Alanine: 0.01% Valine: 0.02% Isoleucine: 0.02% Leucine: 0.02% Tyrosine: 0.04% Phenylalanine: 0.03% Total Lysine: 0.01% Histidine: 0.01% Arginine: 0.05% Proline: 0.05%
Cystine and Methionine by Performic Acid Oxidation	MET-LC-005	%	Cystine: 0.01% Methionine: 0.01%
Tryptophan by Alkaline Hydrolysis	MET-LC-024	%	0.01%
Vitamin E (α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol)	MET-VT-009 MET-VT-030	mg/100g	0.1 mg/100g*
Vitamin K1	MET-VT-028	mg/100g	0.000625 mg/100g
Phenolic Acids	MET-LC-004	Sinapine (%) µg/g (ppm)	Sinapine: 0.05% Ferulic acid: 10 µg/g Coumaric acid: 10 µg/g
Glucosinolates	MET-LC -026	µmol/g	0.05 µmol/g*
Tannins – Soluble Condensed	MET-AN-012	%	0.05%
Phytic acid	MET-EL-011	%	0.14%
Fatty Acid Profile	MET-LI-002/MET-LI-025	%	C16:0: 0.02%. All others at 0.01%

1. Compositional analysis of DHA canola seed

DHA canola seed was evaluated for food/feed composition and other important nutrients and toxicants that naturally occur in canola, as described above.

a.1 PROXIMATES

No statistically significant differences were identified for acid detergent fiber, crude fiber, neutral detergent fiber and protein (Table 41). Although statistical differences in the calculated means were identified for ash, carbohydrates and crude fat, the calculated means are very close numerically and the SD and ranges overlap.

Table 41. Proximate analysis of DHA canola seed

Analyte	Test Material	Nº	Mean (% DW)	SD	Range	P-value
Acid Detergent Fiber	CMP	39	11.2	1.4	8.7-14.6	0.4709
	GE	40	11.4	1.4	9.6-16.6	
	REF	280	11.5	1.5	8.6-16.5	NA
Ash	CMP	39	3.7	0.5	2.9-4.5	<0.0001
	GE	40	3.8	0.4	3.1-4.6	
	REF	280	3.5	0.4	2.7-4.5	NA
Carbohydrates	CMP	39	33.0	2.3	27.0-37.0	<0.0001
	GE	40	35.4	2.0	31.4-38.4	
	REF	280	34.8	2.5	27.3-42.3	NA
Crude Fat	CMP	39	33.2	2.9	27.8-39.5	<0.0001
	GE	40	30.5	2.7	25.8-35.9	
	REF	280	32.8	2.9	25.5-42.1	NA
Crude Fiber	CMP	39	14.9	1.9	10.1-17.5	0.5936
	GE	40	14.7	2.0	11.3-17.9	
	REF	280	15.8	2.2	10.9-22.6	NA
Neutral Detergent Fiber	CMP	39	15.6	1.6	12.6-18.8	0.9676
	GE	40	15.6	1.1	13.6-18.1	
	REF	280	15.7	1.7	12.1-21.8	NA
Protein	CMP	39	30.1	1.2	26.9-32.2	0.3797
	GE	40	30.4	1.2	27.5-32.5	
	REF	280	28.8	1.6	23.5-32.1	NA

Nº = number of samples analyzed; SD = Standard Deviation; DHA canola = GMO, AV Jade = CMP, commercial references = REF

a.2 AAs

No statistically significant differences were identified for the following AAs: arginine, cystine, glutamic acid, histidine, isoleucine, leucine, phenyalanine, serine, tryptophan, valine. Although statistical differences in the calculated means were identified for alanine, aspartic acid, glycine, lysine, methionine, proline, threonine and tyrosine, the calculated means are close numerically and the SD and ranges overlap and fell within the reference data ranges. For alanine, aspartic acid, glycine, lysine, threonine and tyrosine the levels were slightly higher in DHA canola than AV Jade and commercial varieties, but this does not raise any nutritional concerns (see Table 42).

a.3 GLUCOSINOLATES

No statistically significant differences were identified for the following glucosinolates: epiprogoitrin, glucoalyssin, glucobrassicanapin, gluconapin, gluconapoleiferin, gluconasturtin, neoglucobrassicin, progoitrin, 4-hydroxyglucobrassicin (Table 42). Although statistical differences in the calculated means were identified for glucobrassicin, the calculated means are close numerically and the SD and ranges overlap and fell within the reference data ranges. Furthermore, when the total glucosinolates were combined, the totals are 12.071, 11.883 and 10.718 µmol/g for the CMP, GMO and REF, respectively, well below the limits for the definition of canola (30 µmol/g) (USDA-GIPSA, 1992).

a.4 PHYTOSTEROLS

No statistically significant differences were identified for cholesterol (Table 42). Although statistical differences in the calculated means were identified for brassicasterol, campesterol, clerosterol, delta-5-avenasterol, sitosterol, stigmasterol, 24-methylene cholesterol, delta-5 24-stigmastyadienol and total phytosterols, the calculated means are close numerically and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. It should also be noted that for five of the thirteen phytosterols the values were very low and close to the LOQ; cholesterol, clerosterol, delta-7-avenasterol, 24-methylene cholesterol, delta-5 24-stigmastadienol. Taken together, it is unlikely that these differences indicate any biological significance.

a.5 ORGANIC COMPOUNDS

Finally, no statistically significant differences were identified for the organic compound ferulic acid (Table 42). Although statistical differences in the calculated means were identified for sinapine, the calculated means are close numerically, and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference and CMP data ranges for those analytes. Taken together, it is unlikely that these differences indicate any biological significance.

Table 42. Amino acid and sterol analysis in DHA canola seed

Analyte	Test Material*	Nº	Mean	Std Dev	Range	p-value
Alanine	CMP	39	1.239	0.049	1.130-1.360	0.0053
	GE	40	1.268	0.046	1.160-1.350	
	REF	278	1.190	0.064	0.999-1.340	NA
Arginine	CMP	39	1.923	0.092	1.700-2.090	0.8397
	GE	40	1.919	0.087	1.720-2.060	
	REF	278	1.823	0.115	1.480-2.090	NA
Aspartic Acid	CMP	39	2.164	0.106	1.920-2.350	<0.0001
	GE	40	2.282	0.097	2.070-2.440	
	REF	278	2.080	0.134	1.680-2.420	NA
Brassicasterol	CMP	39	0.112	0.005	0.097-0.120	<0.0001
	GE	40	0.052	0.004	0.045-0.066	
	REF	280	0.118	0.022	0.045-0.170	NA
Campesterol	CMP	39	0.287	0.010	0.268-0.310	<0.0001
	GE	40	0.385	0.018	0.352-0.425	
	REF	280	0.317	0.043	0.226-0.397	NA
Cholesterol	CMP	39	0.002	0.002	0.000-0.006	0.7472
	GE	40	0.002	0.003	0.000-0.020	
	REF	280	0.003	0.004	0.000-0.050	NA
Clerosterol	CMP	39	0.006	0.000	0.005-0.007	0.0009
	GE	40	0.006	0.000	0.006-0.007	
	REF	280	0.005	0.000	0.004-0.006	NA
Cystine	CMP	39	0.754	0.037	0.680-0.840	0.1520
	GE	40	0.743	0.038	0.630-0.820	
	REF	278	0.734	0.044	0.580-0.820	NA
Delta_5_avenasterol	CMP	39	0.036	0.006	0.026-0.046	<0.0001
	GE	40	0.044	0.008	0.030-0.064	
	REF	280	0.018	0.005	0.008-0.037	NA
Epiprogoitrin	CMP	39	0.094	0.052	0.000-0.200	0.9182
	GE	40	0.096	0.053	0.000-0.200	
	REF	278	0.071	0.066	0.000-0.300	NA
Ferulic Acid	CMP	39	137.238	23.680	101.10-184.60	0.1768
	GE	40	130.084	20.960	98.72-171.70	
	REF	278	139.527	24.031	88.91-217.50	NA
Glucoalyssin	CMP	39	0.349	0.147	0.062-0.730	0.7523

Analyte	Test Material*	Nº	Mean	Std Dev	Range	p-value
	GE	40	0.363	0.150	0.130-0.670	
	REF	278	0.347	0.286	0.000-1.800	NA
Glucobrassicanapin	CMP	39	0.311	0.155	0.073-0.680	0.0905
	GE	40	0.250	0.121	0.061-0.530	
	REF	278	0.251	0.204	0.000-1.300	NA
Glucobrassicin	CMP	39	0.205	0.055	0.000-0.280	<0.0001
	GE	40	0.282	0.073	0.081-0.420	
	REF	278	0.241	0.088	0.090-0.550	NA
Gluconapin	CMP	39	2.166	0.723	0.664-3.650	0.2947
	GE	40	1.972	0.681	0.627-3.510	
	REF	278	1.770	1.087	0.417-6.390	NA
Gluconasturtin	CMP	39	0.094	0.052	0.000-0.180	0.1092
	GE	40	0.135	0.084	0.000-0.380	
	REF	278	0.151	0.127	0.000-0.520	NA
Glutamic Acid	CMP	39	5.681	0.258	5.090-6.210	0.1426
	GE	40	5.599	0.269	4.930-6.030	
	REF	278	5.408	0.353	4.360-6.170	NA
Glycine	CMP	39	1.519	0.062	1.380-1.660	<0.0001
	GE	40	1.584	0.061	1.440-1.690	
	REF	278	1.471	0.081	1.240-1.660	NA
Histidine	CMP	39	0.843	0.032	0.774-0.910	0.8145
	GE	40	0.845	0.036	0.755-0.900	
	REF	278	0.810	0.044	0.677-0.922	NA
Isoleucine	CMP	39	1.218	0.052	1.080-1.320	0.9692
	GE	40	1.218	0.048	1.100-1.290	
	REF	278	1.159	0.069	0.931-1.310	NA
Leucine	CMP	39	2.129	0.092	1.890-2.300	0.6732
	GE	40	2.120	0.086	1.920-2.280	
	REF	278	2.019	0.124	1.660-2.300	NA
Lysine	CMP	39	1.890	0.107	1.670-2.130	0.0171
	GE	40	1.948	0.129	1.730-2.240	
	REF	278	1.833	0.123	1.490-2.140	NA
Methionine	CMP	39	0.611	0.023	0.570-0.660	0.0197
	GE	40	0.623	0.027	0.560-0.660	
	REF	278	0.592	0.031	0.490-0.670	NA

Analyte	Test Material*	Nº	Mean	Std Dev	Range	p-value
Phenyalanine	CMP	39	1.217	0.054	1.080-1.320	0.1830
	GE	40	1.202	0.046	1.100-1.290	
	REF	278	1.154	0.069	0.949-1.310	NA
Progoitrin	CMP	39	4.914	1.895	0.933-8.680	0.9660
	GE	40	4.936	1.874	1.590-9.120	
	REF	278	4.422	2.606	0.838-17.000	NA
Proline	CMP	39	1.925	0.086	1.700-2.110	0.0010
	GE	40	1.865	0.091	1.670-2.090	
	REF	278	1.832	0.110	1.460-2.050	NA
Serine	CMP	39	1.279	0.050	1.150-1.370	0.2342
	GE	40	1.292	0.051	1.180-1.390	
	REF	278	1.216	0.068	1.020-1.380	NA
Sinapine	CMP	39	1.264	0.078	1.089-1.415	0.0002
	GE	40	1.191	0.070	1.031-1.330	
	REF	278	1.167	0.095	0.876-1.463	NA
Sitosterol	CMP	39	0.551	0.028	0.501-0.616	<0.0001
	GE	40	0.579	0.036	0.512-0.650	
	REF	280	0.477	0.032	0.346-0.580	NA
Stigmasterol	CMP	39	0.003	0.000	0.002-0.004	<0.0001
	GE	40	0.000	0.001	0.000-0.006	
	REF	280	0.002	0.001	0.000-0.005	NA
Threonine	CMP	39	1.280	0.044	1.170-1.380	<0.0001
	GE	40	1.318	0.045	1.220-1.400	
	REF	278	1.231	0.060	1.040-1.360	NA
Tryosine	CMP	39	0.789	0.035	0.702-0.854	<0.0001
	GE	40	0.817	0.029	0.756-0.878	
	REF	278	0.756	0.041	0.644-0.839	NA
Tryptophan	CMP	39	0.456	0.020	0.410-0.500	0.4308
	GE	40	0.453	0.021	0.400-0.500	
	REF	278	0.432	0.026	0.340-0.490	NA
Valine	CMP	39	1.562	0.063	1.400-1.690	0.7330
	GE	40	1.566	0.068	1.420-1.680	
	REF	278	1.492	0.088	1.160-1.650	NA
24-Methylene cholesterol	CMP	39	0.013	0.005	0.008-0.020	0.0013
	GE	40	0.011	0.004	0.007-0.020	

Analyte	Test Material*	Nº	Mean	Std Dev	Range	p-value
	REF	280	0.008	0.003	0.003-0.020	NA
4-Hydroxyglucobrassicin	CMP	39	3.938	0.769	1.360-5.220	0.6366
	GE	40	3.849	0.964	1.120-5.730	
	REF	278	3.465	0.785	0.000-5.540	NA
Delta-5 24-Stigmastadienol	CMP	39	0.007	0.001	0.006-0.008	<0.0001
	GE	40	0.009	0.001	0.008-0.010	
	REF	280	0.005	0.001	0.003-0.009	NA
Total Phytosterols	CMP	39	1.025	0.040	0.966-1.118	<0.0001
	GE	40	1.106	0.061	1.013-1.249	
	REF	280	0.965	0.059	0.702-1.097	NA

*DHA canola (GMO), AV Jade (CMP) and commercial references (REF). Units for amino acids (% DW), glucosinolates (µmol/g), phytosterols (µg/g), organic compounds (% DW)

a.6. MINERALS

No statistically significant differences were identified for the following minerals: copper, magnesium, manganese, phosphorus, phytic acid, sodium and sulfur (Table 43). Although statistical differences in the calculated means were identified for calcium, iron, potassium and zinc, the calculated means are close numerically, and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. It should also be noted that for four of eleven of these analytes the values were very low and close to the LOQ: copper, manganese, sodium, zinc. Therefore, it is unlikely that these differences indicate any biological significance.

Molybdenum and chloride mineral results were below the LOQ, with the exception of three chloride data points that had dry weight basis values of 0.065%, which are equivalent to the LOQ on a dry weight basis. These analytes were excluded from the statistical analyses.

Table 43. Mineral composition in DHA canola seed

Analyte	Test Material*	Nº	Mean (% DW)	Std Dev	Range	p-value
Calcium	CMP	39	0.3563	0.0613	0.2230-0.4640	<0.0001
	GE	40	0.3116	0.0484	0.2060-0.3950	
	REF	278	0.3454	0.0564	0.2040-0.4880	NA
Copper	CMP	39	0.0002	0.0001	0.0001-0.0004	0.1285
	GE	40	0.0003	0.0003	0.0001-0.0020	
	REF	278	0.0003	0.0001	0.0002-0.0020	NA
Iron	CMP	39	0.0055	0.0008	0.0040-0.0070	<0.0001
	GE	40	0.0068	0.0010	0.0050-0.0090	
	REF	278	0.0057	0.0010	0.0040-0.0080	NA
Magnesium	CMP	39	0.3077	0.0211	0.2610-0.3510	0.9910
	GE	40	0.3081	0.0210	0.2620-0.3510	
	REF	278	0.3069	0.0222	0.2460-0.3700	NA
Manganese	CMP	39	0.0032	0.0006	0.0020-0.0040	0.7774
	GE	40	0.0032	0.0007	0.0020-0.0050	
	REF	278	0.0029	0.0006	0.0020-0.0040	NA
Phosphorus	CMP	39	0.6549	0.1224	0.4180-0.8820	0.0639
	GE	40	0.6686	0.1234	0.4370-0.8860	
	REF	278	0.5793	0.1165	0.3650-0.8690	NA
Phytic Acid	CMP	39	1.9179	0.4334	1.1000-2.7000	0.7961
	GMO	40	1.8950	0.4403	1.1000-2.7000	
	REF	278	1.6119	0.4056	0.8400-2.5000	NA
Potassium	CMP	39	0.6655	0.0929	0.4850-0.8660	<0.0001
	GE	40	0.7816	0.0816	0.6210-0.9680	
	REF	278	0.6996	0.0761	0.5320-0.9150	NA
Sodium	CMP	39	0.0025	0.0014	0.0000-0.0050	0.9508
	GE	40	0.0025	0.0016	0.0000-0.0070	
	REF	278	0.0037	0.0021	0.0000-0.0100	NA
Sulfur	CMP	39	0.5121	0.0309	0.4400-0.5700	0.7002
	GE	40	0.5100	0.0332	0.4300-0.5800	
	REF	278	0.4879	0.0422	0.3800-0.6500	NA
Zinc	CMP	39	0.0043	0.0007	0.0030-0.0060	0.0026
	GE	40	0.0046	0.0008	0.0030-0.0060	
	REF	278	0.0041	0.0007	0.0030-0.0060	NA

*DHA canola (GMO), AV Jade (CMP) and commercial references (REF)

a.7. VITAMINS

No statistically significant differences were identified for the following vitamins: δ-tocopherol, folic acid and γ-tocopherol (Table 44). Although statistical differences in the calculated means were identified for α-tocopherol, β-tocopherol, biotin, choline, niacin, pantothenic acid, pyridoxine, riboflavin, thiamin, total tocopherols and vitamin K, the calculated means are close numerically, and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference or CMP data ranges for those analytes. It should also be noted that for half of these analytes the values were very low and close to the LOQ: β-tocopherol, biotin, folic acid, pantothenic acid, pyridoxine, riboflavin and vitamin K. Therefore, it is unlikely that these differences indicate any biological significance.

Table 44. Vitamin composition in DHA canola seed

Analyte	Test Material*	Nº	Mean (% DW)	Std Dev	Range	p-value
α-Tocopherol Vitamin E	CMP	39	11.94	6.61	9.17-51.70	<0.0001
	GE	40	15.69	5.78	12.40-49.70	
	REF	278	15.44	2.48	10.90-31.30	NA
β-Tocopherol Vitamin E	CMP	39	0.08	0.10	0.0-0.45	0.0021
	GE	40	0.12	0.05	0.0-0.24	
	REF	278	0.16	0.06	0.0-0.65	NA
Biotin	CMP	34	0.05	0.00	0.05-0.07	<0.0001
	GE	40	0.07	0.00	0.06-0.08	
	REF	267	0.06	0.01	0.05-0.09	NA
Choline	CMP	34	262.73	21.59	220.51-312.25	0.0249
	GE	40	276.05	23.33	229.14-328.40	
	REF	267	283.72	30.78	195.37-381.31	NA
δ-Tocopherol Vitamin E	CMP	39	0.46	0.53	0.18-3.35	0.2990
	GE	40	0.28	0.09	0.11-0.57	
	REF	278	0.37	0.81	0.00-13.50	NA
Folic Acid	CMP	34	0.12	0.04	0.09-0.23	0.9802
	GE	40	0.12	0.03	0.04-0.21	
	REF	267	0.14	0.07	0.04-0.63	NA
γ-Tocopherol Vitamin E	CMP	39	21.21	1.83	17.70-25.00	0.0648
	GE	40	22.78	1.92	17.80-26.20	
	REF	278	20.53	4.24	10.20-72.20	NA
Niacin Vitamin B3	CMP	34	9.66	0.96	7.89-11.50	<0.0001
	GE	40	15.14	1.91	10.60-18.90	
	REF	267	12.70	1.52	8.41-16.80	NA

Analyte	Test Material*	Nº	Mean (% DW)	Std Dev	Range	p-value
Panothenic Acid Vitamin B5	CMP	34	0.46	0.10	0.22-0.81	<0.0001
	GE	40	0.56	0.11	0.34-0.75	
	REF	267	0.45	0.10	0.20-0.82	NA
Pyridoxine Vitamin B6	CMP	34	0.54	0.06	0.45-0.68	<0.0001
	GE	40	0.85	0.10	0.63-1.10	
	REF	267	0.71	0.11	0.44-0.98	NA
Riboflavin Vitamin B2	CMP	34	0.32	0.06	0.26-0.58	0.0241
	GE	40	0.35	0.03	0.29-0.43	
	REF	267	0.31	0.04	0.20-0.58	NA
Thiamin Vitamin B1	CMP	34	1.29	0.20	0.79-1.71	0.0023
	GE	40	1.48	0.23	1.05-1.95	
	REF	267	1.37	0.28	0.19-2.27	NA
Total Tocopherols Vitamin E	CMP	39	33.68	7.20	28.60-75.10	0.0001
	GE	40	38.88	5.90	31.20-71.00	
	REF	278	36.51	5.79	24.50-96.90	NA
Vitamin K1	CMP	34	0.05	0.01	0.04-0.06	0.0205
	GE	40	0.05	0.01	0.04-0.07	
	REF	267	0.04	0.01	0.03-0.07	NA

*DHA canola (GMO), AV Jade (CMP) and commercial references (REF)

a.8 FAs

Because DHA canola expresses seven FA pathway enzymes, it is not surprising that many of the FAs are different from conventional canola, including C18:2 n-6 (LA, reduced by approximately a factor of 2), C18:1 n-9 (OA, reduced slightly), C18:3 n-3 (ALA, increased by a factor of 2) and most notably, C22:6 n-3 (DHA) is greatly increased as expected from the introduced trait in the GMO. Specifically, the DHA mean for the GMO was 8.38% substantially higher than 0.24/0.11% for the parental and reference varieties, respectively. There is also an increase in *trans* FAs in DHA canola, which are FAs with one or more double bonds in the *trans* configuration, largely due to C18:3 n-3. However, the amount of *trans* FA in DHA canola is quite low (<1%).

While conventional canola does not express DHA, because the samples were taken from a mixed plot layout, some cross-pollination is possible leading to some DHA in conventional lines. A DHA value observed for a REF variety indicated a max value of 7.76, which was highly unusual in a conventional canola line. Although conventional canola does not express DHA, the samples were taken from a mixed plot layout where cross-pollination was possible. When the data was reviewed in detail, however, it was noted that a single replication of a REF variety at a single site gave this high value. Therefore, this value was removed from all FA analysis.

No statistically significant differences were identified for the following FAs: C14:0, C16:1 n-7, C17:0, C18:0, C22:1 n-9, C22:1 total and C24:0 (Table 45). Although statistical differences in the calculated means were identified for C16:0, C16:1 n-7, C16:1 total, C17:0, C18:1 n-7, C20:0, C20:1 n-9 and C22:0, the calculated means are close numerically, and the standard deviation and ranges overlap. The means and data ranges fell largely within the reference data ranges for those analytes. Therefore, it is unlikely that these differences indicate any biological significance.

Statistical analysis could not be conducted on C18:1 *trans*, C18:2 n-9, C18:2 *trans*, C18:3 n-6, C18:4 n-3, C18 total, C20:3 n-3, C20:3 total, C20:4 n-3, C20:4 total, C20:5 n-3, C22:1 n-9, C22:1 total, C22:4 n-3, C22:5 n-3, C22:5 n-6 and C22:5 total, because more than 30% of the values were <LOQ. And for FA profile analysis, data for C16:1 *trans*, C16:3 n-3, C20:2 n-9, C20:3 n-6, C20:3 n-9, C20:4 n-6, C22:2 n-6, and C22:4 n-6 were omitted as ALL results were below the LOQ.

Table 45. Fatty acid composition in seed

Analyte	Test Material	Nº	Mean (%)	Std Dev	Range	p-value
C14:0 Myristic acid	CMP	39	0.075	0.005	0.067-0.089	0.2289
	GMO	40	0.077	0.004	0.070-0.086	
	REF	279	0.069	0.008	0.055-0.096	NA
C16:0 Palmitic acid	CMP	39	4.310	0.110	4.093-4.582	<0.0001
	GMO	40	4.503	0.086	4.368-4.692	
	REF	279	4.125	0.241	3.625-4.818	NA
C16:1 n-7 Palmitoleic acid	CMP	39	0.191	0.010	0.180-0.220	0.6390
	GMO	40	0.193	0.007	0.180-0.210	
	REF	279	0.185	0.020	0.150-0.250	NA
C16:1 n-9 Palmitoleic acid	CMP	39	0.053	0.003	0.046-0.061	<0.0001
	GMO	40	0.082	0.007	0.073-0.110	
	REF	279	0.055	0.007	0.041-0.079	NA
C16:1 total	CMP	39	0.267	0.012	0.250-0.302	<0.0001
	GMO	40	0.294	0.012	0.272-0.325	
	REF	279	0.262	0.025	0.214-0.335	NA
C17:0 Margaric acid	CMP	39	0.047	0.005	0.040-0.060	0.0248
	GMO	40	0.049	0.004	0.040-0.060	
	REF	279	0.049	0.004	0.040-0.060	NA
C17:1	CMP	39	0.059	0.004	0.050-0.068	<0.0001
	GMO	40	0.044	0.008	0.000-0.054	
	REF	279	0.064	0.004	0.052-0.079	NA
C18:0	CMP	39	2.212	0.082	2.050-2.340	0.0868

Analyte	Test Material	Nº	Mean (%)	Std Dev	Range	p-value
Stearic acid	GMO	40	2.151	0.078	2.020-2.460	
	REF	279	1.772	0.182	1.410-2.260	NA
C18:1 n-7 Vaccenic acid	CMP	39	2.678	0.079	2.503-2.872	<0.0001
	GMO	40	2.870	0.096	2.701-3.079	
	REF	279	2.760	0.228	0.000-3.100	NA
C18:1 n-9 Oleic acid (OA)	CMP	39	57.069	1.477	54.591-59.906	<0.0001
	GMO	40	42.031	2.429	37.231-47.382	
	REF	279	61.809	5.248	49.157-72.679	NA
C18:1 total	CMP	39	59.823	1.441	57.404-62.601	<0.0001
	GMO	40	45.005	2.379	40.443-50.278	
	REF	279	64.646	5.105	51.934-74.358	NA
C18:2 n-6 Linoleic acid (LA)	CMP	39	19.341	0.827	16.600-20.580	<0.0001
	GMO	40	8.502	0.237	8.037-9.075	
	REF	279	16.951	4.085	11.590-23.260	NA
C18:2 total	CMP	39	19.441	0.825	16.737-20.680	<0.0001
	GMO	40	8.762	0.232	8.367-9.328	
	REF	279	17.060	4.085	11.706-23.361	NA
C18:3 n-3 Alpha Linolenic acid (ALA)	CMP	39	11.185	0.743	9.960-12.620	<0.0001
	GMO	40	21.040	1.081	18.810-22.870	
	REF	279	9.146	1.883	3.905-12.080	NA
C18:3 total	CMP	39	11.278	0.767	10.020-12.730	<0.0001
	GMO	40	22.207	1.141	19.810-24.190	
	REF	279	9.208	1.890	3.930-12.190	NA
C20:0 Arachidic acid	CMP	39	0.475	0.012	0.455-0.500	<0.0001
	GMO	40	0.589	0.012	0.568-0.624	
	REF	279	0.548	0.069	0.422-0.730	NA
C20:1 n-9 Eicosenoic acid	CMP	39	0.951	0.025	0.903-1.040	<0.0001
	GMO	40	1.185	0.032	1.130-1.250	
	REF	279	1.179	0.152	0.877-1.590	NA
C20:2 n-6 Eicosadienoic acid	CMP	39	0.060	0.005	0.049-0.073	<0.0001
	GMO	40	0.091	0.004	0.084-0.100	
	REF	279	0.068	0.012	0.050-0.190	NA
C22:0 Behenic acid	CMP	39	0.189	0.007	0.176-0.204	<0.0001
	GMO	40	0.253	0.007	0.242-0.273	
	REF	279	0.290	0.050	0.180-0.387	NA

Analyte	Test Material	Nº	Mean (%)	Std Dev	Range	p-value
C22:6 n-3 Docosahexaenoic acid (DHA)	CMP	39	0.239	0.284	0.034-1.550	<0.0001
	GMO	40	8.376	0.810	6.500-10.300	
	REF*	279	0.112	0.126	0.000-1.390	NA
C24:0 Lignoceric acid	CMP	39	0.100	0.006	0.089-0.110	0.1906
	GMO	40	0.094	0.005	0.084-0.100	
	REF	279	0.144	0.024	0.110-0.210	NA
C24:1 n-9 Nervonic acid	CMP	39	0.096	0.007	0.084-0.110	<0.0001
	GMO	40	0.059	0.004	0.050-0.069	
	REF	279	0.120	0.013	0.000-0.170	NA
C24:1 total	CMP	39	0.096	0.007	0.084-0.110	<0.0001
	GMO	40	0.059	0.004	0.050-0.069	
	REF	279	0.120	0.013	0.000-0.170	NA
FA Total	CMP	39	29.906	1.950	26.267-34.073	<0.0001
	GMO	40	27.265	1.883	22.229-31.021	
	REF	279	29.541	2.228	23.937-35.778	NA
Total Trans Fatty Acids	CMP	39	0.108	0.032	0.077-0.253	<0.0001
	GMO	40	0.839	0.050	0.727-0.933	
	REF	279	0.084	0.018	0.046-0.232	NA

*DHA canola (GMO), AV Jade (CMP) and commercial references (REF)

a.7 OTHER ANALYTES

In addition to the OECD recommended compositional components to be analyzed for food use of canola seed, the mineral content, phytosterols, and several other vitamins were evaluated and no biologically significant differences were found when DHA canola constituents were compared to AV Jade or other commercial canola varieties.

Conclusion

The results demonstrate that, aside from the expected changes in the FA profile, none of the compositional analytes showed any biologically significant differences between AV Jade, the commercial varieties and DHA canola. Although statistical differences in the calculated means were identified for several analytes, the calculated means were typically very close numerically and the SD and ranges overlapped. The means and data ranges fell largely within the reference data ranges for those analytes. Therefore, it is unlikely that any of these differences indicate any biological significance.

This compositional analysis also demonstrates that important healthy FAs in DHA canola seed oil is similar to the same important FA content in fish oil (Table 46), thus supporting the safe use of DHA canola oil in foods, feed and nutraceutical.

Table 46. Canola oil and fish oil FA composition (%)

Fatty acid	Rapeseed oil*	Canola oil*	Anchovy oil**
C6:0	ND	ND	ND
C8:0	ND	ND	ND
C10:0	ND	ND	ND
C12:0	ND	ND	ND
C14:0	ND-0.2	ND-0.2	2.7-11.5
C16:0	1.5-6.0	2.5-7.0	13.0-22.0
C16:1	ND-3.0	ND-0.6	4.0-12.6
C17:0	ND-0.1	ND-0.3	ND-2.0
C17:1	ND-0.1	ND-0.3	ND
C18:0	0.5-3.1	0.8-3.0	1.0-7.0
C18:1	8.0-60.0	51.0-70.0	5.3-20.7
C18:2	11.0-23.0	15.0-30.0	ND-3.5
C18:3	5.0-13.0	5.0-14.0	ND-12.0
C20:0	ND-3.0	0.2-1.2	ND-1.8
C20:1	3.0-15.0	0.1-4.3	NS-8.0
C20:2	ND-1.0	ND-0.1	ND
C20:4	ND	ND	ND-4.5
C20:5	ND	ND	5.0-26.0
C21:5	ND	ND	ND-4.0
C22:0	ND-2.0	ND-0.6	NR
C22:1	> 2.0-60.0	ND-2.0	ND-7.9
C22:2	ND-2.0	ND-0.1	NR
C22:5	ND	ND	ND-4.0
C22:6	ND	ND	4.0-26.5
C24: 0	ND-2.0	ND-0.3	NR
C24:1	ND-3.0	ND-0.4	NR

ND – non-detectable, defined as <0.05%; NR = not reported

*http://www.fao.org/docrep/004/y2774e/y2774e04.htm

**http://www.fao.org/fao-who-codexalimentarius/.pdf

2. Compositional Analyses of DHA canola meal

Compositional analyses were also conducted on various nutritional characteristics for processed meal fractions from DHA canola and the parental AV Jade variety. DHA canola was harvested in 2015 at one location in a major canola growing region of Australia to collect grain for processing into meal, commonly used for animal feeds. AV Jade was harvested in 2015 from two locations near to the DHA canola site. Processed meal was prepared from canola grain that was divided into two batches, which were crushed separately at CSIRO Agriculture and Food facility. There were a total of eight samples created and analyzed:

- 2 crushes of crude meal from DHA canola
- 1 crush of crude meal from AV Jade, location 1
- 1 crush of crude meal from AV Jade, location 2
- 2 crushes of hexane-extracted meal from DHA canola
- 1 crush of hexane-extracted meal from AV Jade, location 1
- 1 crush of hexane-extracted meal from AV Jade, location 2

Compositional analysis of meal samples included protein, fat, ADF, NDF, crude fiber, ash, carbohydrates, FAs, AAs, vitamin E, minerals, phytosterols and key anti-nutrients. Compositional analyses were conducted at Eurofins Nutritional Analysis Center in Des Moines, Iowa, U.S.A.

A summary of each parameter, its method of analysis, appropriate units and LOQ are included in Table 47 below. When data points were at or below the LOQ, zero was used to calculate the averages, SDs and data ranges. The meal samples were compared for each constituent and expressed in percent difference between AV Jade and DHA canola.

Table 47. Compositional analysis of DHA canola meal

Parameter	Eurofins Method	LOQ
Protein, Crude	MET-PR-002	0.1%
Fat, Crude	MET-LI-001	0.1%
Ash	MET-PR-004	0.4%
Carbohydrates, Calculated	OPS-024	N/A
Crude Fiber	MET-PR-003	0.2%
Acid Detergent Fiber	MET-PR-007	0.3%
Neutral Detergent Fiber	MET-PR-008	0.3%
AAs by Acid Hydrolysis	MET-LC-006	Serine, Glutamic Acid, Glycine, Alanine, Histidine, Total Lysine: 0.01% Aspartic Acid, Threonine, Valine, Isoleucine, Leucine: 0.02% Tyrosine: 0.04% Phenylalanine: 0.03% Arginine, Proline: 0.05%
Cystine and Methionine by Performic Acid Oxidation	MET-LC-005	Cystine: 0.01% Methionine: 0.01%
Tryptophan by Alkaline Hydrolysis	MET-LC-024	0.01%
Vitamin E (α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol)	MET-VT-009 MET-VT-030	0.1 mg/100g*
Phenolic Acids	MET-LC-004	Sinapine: 0.05% Ferulic acid: 10 µg/g Coumaric acid: 10 µg/g
Glucosinolates	MET-LC-026	0.05 µmol/g*
Tannins – Soluble Condensed	MET-AN-012	0.05%
Phytic acid	MET-EL-011	0.14%
Calcium	MET-EL-002/MET-EL-003	0.004%
Phosphorus	MET-EL-002/MET-EL-003	0.004%
Phytosterols	MET-LI-034	0.002%*
Fatty Acid Profile	MET-LI-002/MET-LI-025	C16:0: 0.02% All others at 0.01%
Listed LOQ applies to all analyte parameters		

b.1 PROXIMATES

The levels of proximate and minerals were measured in all meal samples of DHA canola and AV Jade and then compared. When compared, the mean values of crude and hexane-extracted meals from DHA canola and AV Jade, are within 10% of each other for protein, ash, phosphorus and phytic acid. The crude meals were within 10% of each other for crude fiber, acid detergent fiber and neutral detergent fiber as were the carbohydrate values for hexane-extracted meal. Differences of 13.4% were identified for the carbohydrate values for crude meal and 11.7% to 19% differences

were observed for crude fiber, acid detergent fiber and neutral detergent fiber in hexane-extracted meals. Calcium was lower for DHA canola, 16.9% and 25% for crude and hexane-extracted meals, respectively, when compared to levels in AV Jade meals. As expected, crude fat was higher in AV Jade, 37% and 64% higher for crude and hexane-extracted meals, respectively, when compared to DHA canola (Table 48).

Table 48. Proximates and minerals composition of meal

Analyte	Test Material	Sample	%	Average of both crushes (%)
Crude Fat	CMP	Crude meal	16.95	21.44
			25.92	
		Hexane extracted meal	0.55	0.69
			0.83	
	GMO	Crude meal	15.42	15.66
			15.91	
		Hexane extracted meal	0.20	0.42
			0.63	
Protein	CMP	Crude meal	44.38	42.64
			40.89	
		Hexane extracted meal	55.76	54.59
			53.42	
	GMO	Crude meal	44.93	44.21
			43.49	
		Hexane extracted meal	52.67	53.04
			53.42	
Ash	CMP	Crude meal	4.86	5.04
			5.23	
		Hexane extracted meal	5.79	6.28
			6.76	
	GMO	Crude meal	5.13	5.03
			4.93	
		Hexane extracted meal	6.00	5.90
			5.80	
Carbohydrate	CMP	Crude meal	33.91	30.93
			27.95	
		Hexane extracted meal	37.90	39.22
			40.55	
	GMO	Crude meal	34.52	35.09
			35.66	
		Hexane extracted meal	41.14	40.64
			40.15	
Crude Fiber	CMP	Crude meal	9.02	8.59

Analyte	Test Material	Sample	%	Average of both crushes (%)
			8.16	
		Hexane extracted meal	10.1	10.4
			10.6	
	GMO	Crude meal	8.74	8.74
			8.75	
		Hexane extracted meal	9.82	9.23
			8.65	
Acid Detergent Fiber	CMP	Crude meal	15.9	15.8
			15.6	
		Hexane extracted meal	19.7	21.1
			22.5	
	GMO	Crude meal	16.6	17.0
			17.4	
		Hexane extracted meal	21.6	18.2
			14.9	
Neutral Detergent Fiber	CMP	Crude meal	24.6	24.5
			24.4	
		Hexane extracted meal	32.2	31.6
			30.9	
	GMO	Crude meal	21.6	23.3
			25.0	
		Hexane extracted meal	26.7	28.3
			29.9	
Calcium	CMP	Crude meal	0.504	0.519
			0.534	
		Hexane extracted meal	0.605	0.648
			0.692	
	GMO	Crude meal	0.455	0.444
			0.434	
		Hexane extracted meal	0.530	0.520
			0.519	
Phosphorus	CMP	Crude meal	0.854	0.926
			0.998	
		Hexane extracted meal	1.02	1.14

Analyte	Test Material	Sample	%	Average of both crushes (%)
			1.27	
	GMO	Crude meal	0.955	0.923
			0.891	
		Hexane extracted meal	1.12	1.10
			1.07	
Phytic Acid	CMP	Crude meal	2.2	2.5
			2.8	
		Hexane extracted meal	2.7	3.2
			3.6	
	GMO	Crude meal	2.6	2.5
			2.4	
		Hexane extracted meal	3.2	3.0
			2.8	

*****DHA canola (GMO) and AV Jade (CMP); crude and hexane-extracted meals

b.2 AAs

As reflected in Table 49, comparison of the AA profile of AV Jade with DHA canola for crude and hexane-extracted meal showed few differences (all below 8%).

Table 49. Amino acid composition of meal

Analyte	Test Material	Sample	%	Average of both crushes (%)
Alanine	CMP	Crude meal	1.94	1.86
			1.77	
		Hexane extracted meal	2.35	2.33
			2.31	
	GMO	Crude meal	1.91	1.92
			1.93	
		Hexane extracted meal	2.34	2.35
			2.36	
Arginine	CMP	Crude meal	2.84	2.73
			2.62	
		Hexane extracted meal	3.47	3.42
			3.38	
	GMO	Crude meal	2.77	2.78
			2.80	
		Hexane extracted meal	3.39	3.40
			3.40	
Aspartic Acid	CMP	Crude meal	3.22	3.12
			3.01	
		Hexane extracted meal	3.93	3.90
			3.87	
	GMO	Crude meal	3.27	3.32
			3.37	
		Hexane extracted meal	4.02	3.94
			3.87	
Cystine	CMP	Crude meal	1.1	1.05
			1.0	
		Hexane extracted meal	1.4	1.35
			1.3	
	GMO	Crude meal	1.1	1.10
			1.1	
		Hexane extracted meal	1.3	1.30
			1.3	

Analyte	Test Material	Sample	%	Average of both crushes (%)
Glutamic Acid	CMP	Crude meal	8.57	8.17
			7.77	
		Hexane extracted meal	10.4	10.25
			10.1	
	GMO	Crude meal	8.25	8.30
			8.36	
		Hexane extracted meal	10.1	10.15
			10.2	
Glycine	CMP	Crude meal	2.26	2.16
			2.06	
		Hexane extracted meal	2.74	2.70
			2.67	
	GMO	Crude meal	2.29	2.30
			2.31	
		Hexane extracted meal	2.80	2.81
			2.82	
Histidine	CMP	Crude meal	1.26	1.20
			1.14	
		Hexane extracted meal	1.53	1.50
			1.47	
	GMO	Crude meal	1.22	1.23
			1.24	
		Hexane extracted meal	1.49	1.50
			1.51	
Isoleucine	CMP	Crude meal	1.87	1.80
			1.72	
		Hexane extracted meal	2.27	2.24
			2.22	
	GMO	Crude meal	1.81	1.80
			1.80	
		Hexane extracted meal	2.21	2.22
			2.23	

Analyte	Test Material	Sample	%	Average of both crushes (%)
Leucine	CMP	Crude meal	3.15	3.01
			2.87	
		Hexane extracted meal	3.82	3.78
			3.74	
	GMO	Crude meal	3.04	3.04
			3.05	
		Hexane extracted meal	3.70	3.72
			3.75	
Lysine	CMP	Crude meal	3.08	2.79
			2.50	
		Hexane extracted meal	3.37	3.40
			3.44	
	GMO	Crude meal	3.23	3.01
			2.79	
		Hexane extracted meal	3.42	3.57
			3.72	
Methionine	CMP	Crude meal	0.89	0.84
			0.80	
		Hexane extracted meal	1.1	1.05
			1.0	
	GMO	Crude meal	0.90	0.90
			0.89	
		Hexane extracted meal	1.0	1.0
			1.0	
Phenyalanine	CMP	Crude meal	1.81	1.74
			1.66	
		Hexane extracted meal	2.21	2.18
			2.15	
	GMO	Crude meal	1.74	1.74
			1.75	
		Hexane extracted meal	2.10	2.12
			2.13	

Analyte	Test Material	Sample	%	Average of both crushes (%)
Proline	CMP	Crude meal	2.86	2.70
			2.55	
		Hexane extracted meal	3.48	3.42
			3.35	
	GMO	Crude meal	2.70	2.74
			2.77	
		Hexane extracted meal	3.29	3.30
			3.32	
Serine	CMP	Crude meal	1.85	1.76
			1.67	
		Hexane extracted meal	2.24	2.21
			2.18	
	GMO	Crude meal	1.81	1.83
			1.85	
		Hexane extracted meal	2.18	2.20
			2.21	
Threonine	CMP	Crude meal	1.85	1.78
			1.70	
		Hexane extracted meal	2.25	2.22
			2.20	
	GMO	Crude meal	1.84	1.86
			1.89	
		Hexane extracted meal	2.24	2.24
			2.25	
Tryosine	CMP	Crude meal	1.18	1.13
			1.08	
		Hexane extracted meal	1.41	1.38
			1.36	
	GMO	Crude meal	1.17	1.18
			1.18	
		Hexane extracted meal	1.41	1.41
			1.41	

Analyte	Test Material	Sample	%	Average of both crushes (%)
Tryptophan	CMP	Crude meal	0.71	0.67
			0.63	
		Hexane extracted meal	0.87	0.86
			0.84	
	GMO	Crude meal	0.69	0.68
			0.67	
		Hexane extracted meal	0.82	0.83
			0.84	
Valine	CMP	Crude meal	2.36	2.26
			2.16	
		Hexane extracted meal	2.85	2.83
			2.81	
	GMO	Crude meal	2.30	2.29
			2.28	
		Hexane extracted meal	2.81	2.81
			2.81	

*****DHA canola (GMO) and AV Jade (CMP); crude and hexane-extracted meals

b.3 GLUCOSINOLATES

The following glucosinolates had values that are below 1 µmol/g for all meal samples: epiprogoitrin, glucoalyssin, glucobrassicanapin, glucobrassicin, gluconapoleiferin, gluconasturtiin and neoglucobrassicin. Values are not provided for glucoraphanin and glucoiberin, because they were below the LOQ.

Although the values for the remaining glucosinolates (glucoalyssin, progoitrin and 4-hydroxyglucobrassicin) did show differences between AV Jade and DHA canola, the range of values overlapped and in every case, the highest value was linked to AV Jade. Importantly, the sum of means of these three glucosinolates for all samples was below 20 µmol/g, well below the limits included in the definition of canola (30 µmol/g). Finally, when all the glucosinolates values are combined, the levels in all samples remained below this same limit (Table 50).

Table 50. Glucosinolate composition of meal

Analyte	Test Material	Sample	µmol/g	Average of both crushes
Epi-progoitrin	CMP	Crude meal	0.1	0.15
			0.2	
		Hexane extracted meal	0.2	0.2
			0.2	
	GMO	Crude meal	0.2	0.2
			0.2	
		Hexane extracted meal	0.2	0.2
			0.2	
Glucoalyssin	CMP	Crude meal	0.43	0.54
			0.66	
		Hexane extracted meal	0.55	0.70
			0.85	
	GMO	Crude meal	0.63	0.60
			0.57	
		Hexane extracted meal	0.71	0.66
			0.62	
Glucobrassicanapin	CMP	Crude meal	0.59	0.70
			0.80	
		Hexane extracted meal	0.68	0.84
			1.00	
	GMO	Crude meal	0.57	0.58
			0.59	
		Hexane extracted meal	0.63	0.63
			0.63	
Glucobrassicin	CMP	Crude meal	0.38	0.37
			0.36	
		Hexane extracted meal	0.45	0.44
			0.44	
	GMO	Crude meal	0.42	0.42
			0.41	
		Hexane extracted meal	0.49	0.48
			0.47	
Gluconapin	CMP	Crude meal	3.75	4.05

Analyte	Test Material	Sample	µmol/g	Average of both crushes
			4.35	
		Hexane extracted meal	4.42	4.94
			5.46	
	GMO	Crude meal	3.70	3.58
			3.46	
		Hexane extracted meal	4.05	3.85
			3.75	
Gluconapoleiferin	CMP	Crude meal	0.06	0.08
			0.1	
		Hexane extracted meal	0.1	0.15
			0.2	
	GMO	Crude meal	0.1	0.1
			0.1	
		Hexane extracted meal	0.2	0.15
			0.1	
Gluconasturtiin	CMP	Crude meal	0.25	0.24
			0.24	
		Hexane extracted meal	0.29	0.31
			0.33	
	GMO	Crude meal	0.28	0.26
			0.24	
		Hexane extracted meal	0.33	0.30
			0.27	
Neoglucobrassicin	CMP	Crude meal	<LOQ	<LOQ
			<LOQ	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.07	0.08
			0.08	
		Hexane extracted meal	0.08	0.08
			0.09	
Progoitrin	CMP	Crude meal	6.10	7.89
			9.68	
		Hexane extracted meal	7.72	10.01

Analyte	Test Material	Sample	µmol/g	Average of both crushes
			12.3	
	GMO	Crude meal	8.98	8.78
			8.57	
		Hexane extracted meal	10.2	9.91
			9.62	
4-Hydroxyglucobrassicin	CMP	Crude meal	7.77	7.12
			6.47	
		Hexane extracted meal	9.42	8.90
			8.39	
	GMO	Crude meal	6.56	6.66
			6.76	
		Hexane extracted meal	7.44	7.48
			7.52	

*****DHA canola (GMO) and AV Jade (CMP); crude and hexane-extracted meals

b.4 ORGANIC COMPOUNDS

The means for *p*-coumaric acid were very low, below the LOQ for meals samples from DHA canola and 2-3 times the LOQ for meal samples from AV Jade. All values for soluble tannins were below the LOQ and are not represented herein. Mean values for ferulic acid were 8.2% and 11.0% higher for crude and hexane-extracted meals, respectively, in AV Jade meal compared to DHA canola meal. Mean values for sinapine were only 1.8% difference for crude meals of AV Jade and DHA canola, but were 13.6% higher in the AV Jade hexane-extracted meals (Table 51).

Table 51. Organic compound composition of meal

Analyte	Test Material	Sample	mg/100g	Average of crushes
Ferulic acid	CMP	Crude meal	179.2	173.0
			166.8	
		Hexane extracted meal	221.3	215.9
			210.5	
	GMO	Crude meal	160.8	159.9
			159.0	
		Hexane extracted meal	178.2	194.4
			210.5	
p-Coumaric acid	CMP	Crude meal	20.78	22.38
			23.97	
		Hexane extracted meal	25.79	28.79
			31.79	
	GMO	Crude meal	<LOQ	<LOQ
			<LOQ	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
Sinapine	CMP	Crude meal	1.842	1.767
			1.692	
		Hexane extracted meal	2.446	2.338
			2.231	
	GMO	Crude meal	1.735	1.736
			1.736	
		Hexane extracted meal	1.999	2.058
			2.117	

*****DHA canola (GMO) and AV Jade (CMP); crude and hexane-extracted meals

b.5 VITAMINS

Mean values for crude meals from both AV Jade and DHA canola were 11.8%, 12.7% and 2.6% for alpha, gamma and total tocopherols, respectively. The ranges overlapped and therefore the differences are not biologically significant. Mean values for the hexane-extracted meals from AV Jade and DHA canola were quite different with DHA canola being approximately 50% of the mean AV Jade values. Hexane-extraction greatly diminishes the tocopherols for both AV Jade and DHA canola, and in most cases, greater than a 90% reduction was observed, resulting in more variability

in the data since levels are very low. Both beta and Δ tocopherol values were below the LOQ (Table 52).

Table 52. Vitamin E composition of meal

Analyte	Test Material*	Sample	mg/100g	Average of crushes
Alpha Tocopherol Vitamin E	CMP	Crude meal	8.15	9.12
			10.1	
		Hexane extracted meal	1.38	1.48
			1.59	
	GMO	Crude meal	10.2	10.2
			10.1	
		Hexane extracted meal	0.817	0.780
			0.743	
Gamma Tocopherol Vitamin E	CMP	Crude meal	12.5	14.2
			15.9	
		Hexane extracted meal	0.888	0.922
			0.956	
	GMO	Crude meal	12.6	12.6
			12.7	
		Hexane extracted meal	0.459	0.454
			0.450	
Total Tocopherols Vitamin E	CMP	Crude meal	20.7	23.4
			26.0	
		Hexane extracted meal	2.27	2.40
			2.54	
	GMO	Crude meal	22.8	22.8
			22.9	
		Hexane extracted meal	1.28	1.24
			1.19	

*****DHA canola (GMO) and AV Jade (CMP); crude and hexane-extracted meals

b.6 FATTY ACIDS

As expected, hexane extraction resulted in a drastic reduction in FA in all meal samples, regardless of the canola variety. In all cases, the amount of FA is less than 5% of that measured in crude oil. Thus, further comparisons of the FA profile of hexane-extracted oil for AV Jade and DHA canola would not be meaningful.

The quantities of C14:0, C16:0 and C16:1, C22:0, C20:2 n-6, C24:0 were very low for both AV Jade and DHA canola crude meals. Mean values of C18:0 and C18:1n-7 were 27.6% and 10.6%, respectively, for AV Jade and DHA canola. Because DHA canola expresses seven FA pathway enzymes, it is not surprising that the FAs profile is different when AV Jade and DHA canola meals are compared. The crude meal from DHA canola was expected to be different from AV Jade crude meal for the following FAs: LA (reduced by approximately a factor of 3), OA (decreased by a factor of 2), ALA (increased by a factor of 1.5) while SDA, ETA, DPA and DHA were greatly increased due to the introduction of DHA biosynthetic pathway in DHA canola (Table 53).

Table 53. Fatty acid composition of meals

FA	Test Material	Sample	%	Average of both crushes
C14:0 Myristic acid	CMP	Crude meal	0.015	0.018
			0.020	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.014	0.014
			0.013	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C16:0 Palmitic acid	CMP	Crude meal	0.742	0.864
			0.985	
		Hexane extracted meal	0.041	0.042
			0.043	
	GMO	Crude meal	0.700	0.694
			0.688	
		Hexane extracted meal	0.023	0.024
			0.024	
C16:1 n-7 Palmitoleic acid	CMP	Crude meal	0.052	0.056
			0.061	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.041	0.051
			0.061	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C16:1 total	CMP	Crude meal	0.070	0.076
			0.081	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.058	0.059
			0.060	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	

FA	Test Material	Sample	%	Average of both crushes
C18:0 Stearic acid	CMP	Crude meal	0.340	0.393
			0.446	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.308	0.308
			0.307	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C18:1 n-7 Vaccenic acid	CMP	Crude meal	0.646	0.706
			0.766	
		Hexane extracted meal	0.086	0.080
			0.074	
	GMO	Crude meal	0.632	0.638
			0.643	
		Hexane extracted meal	0.032	0.030
			0.028	
C18:1 n-9 Oleic acid (OA)	CMP	Crude meal	8.754	10.114
			11.473	
		Hexane extracted meal	0.172	0.212
			0.253	
	GMO	Crude meal	5.165	5.174
			5.184	
		Hexane extracted meal	0.036	0.055
			0.074	
C18:1 total	CMP	Crude meal	9.404	10.82
			12.244	
		Hexane extracted meal	0.260	0.164
			0.068	
	GMO	Crude meal	5.807	5.820
			5.832	
		Hexane extracted meal	0.068	0.085
			0.102	
C18:2 n-6 Linoleic acid (LA)	CMP	Crude meal	3.126	3.638
			4.150	
		Hexane extracted meal	0.108	0.118

FA	Test Material	Sample	%	Average of both crushes
			0.128	
	GMO	Crude meal	1.184	1.177
			1.171	
		Hexane extracted meal	<LOQ	0.014
			0.028	
C18:2 total	CMP	Crude meal	3.141	3.656
			4.171	
		Hexane extracted meal	0.108	0.118
			0.128	
	GMO	Crude meal	1.223	1.212
			1.201	
		Hexane extracted meal	<LOQ	0.014
			0.028	
C18:3 n-3 Alpha Linolenic acid (ALA)	CMP	Crude meal	1.670	1.992
			2.313	
		Hexane extracted meal	0.034	0.042
			0.051	
	GMO	Crude meal	3.063	3.026
			2.988	
		Hexane extracted meal	0.020	0.021
			0.022	
C18:3 total	CMP	Crude meal	1.681	2.008
			2.336	
		Hexane extracted meal	0.034	0.042
			0.051	
	GMO	Crude meal	3.236	3.198
			3.159	
		Hexane extracted meal	0.020	0.021
			0.022	
C18:4 n-3 Stearidonic acid (SDA)	CMP	Crude meal	0.013	0.022
			0.031	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.439	0.436
			0.433	

FA	Test Material	Sample	%	Average of both crushes
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C20:0 Arachidic acid	CMP	Crude meal	0.076	0.088
			0.100	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.085	0.084
			0.084	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C20:1 n-9 Gondoic acid	CMP	Crude meal	0.145	0.172
			0.198	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.153	0.153
			0.153	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C20:2 n-6 Eicosadienoic acid	CMP	Crude meal	<LOQ	0.07
			0.014	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.012	0.012
			0.012	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C20:4 n-3 Eicosatetraenoic acid (ETA)	CMP	Crude meal	<LOQ	0.007
			0.014	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.185	0.181
			0.178	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	

FA	Test Material	Sample	%	Average of both crushes
C20:4 total	CMP	Crude meal	<LOQ	0.008
			0.015	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.188	0.184
			0.180	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C22:0	CMP	Crude meal	0.032	0.037
			0.042	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.037	0.040
			0.042	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C22:5 n-3 Docosapentiaenoic acid (DPA)	CMP	Crude meal	<LOQ	0.006
			0.012	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.172	0.169
			0.166	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C22:5 total	CMP	Crude meal	<LOQ	0.008
			0.015	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.211	0.207
			0.203	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C22:6 n-3	CMP	Crude meal	0.028	0.058
			0.087	

FA	Test Material	Sample	%	Average of both crushes
Docosahexaenoic acid (DHA)		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	1.33	1.31
			1.29	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
C24:0	CMP	Crude meal	0.018	0.022
			0.025	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.015	0.014
			0.014	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
Total Trans-Fatty Acids	CMP	Crude meal	<LOQ	0.010
			0.019	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
	GMO	Crude meal	0.127	0.122
			0.117	
		Hexane extracted meal	<LOQ	<LOQ
			<LOQ	
FA Total	CMP	Crude meal	15.783	18.336
			20.889	
		Hexane extracted meal	0.474	0.530
			0.585	
	GMO	Crude meal	14.039	13.956
			13.874	
		Hexane extracted meal	0.152	0.176
			0.200	

*****DHA canola (GMO) and AV Jade (CMP); crude and hexane-extracted meals

Conclusion

The results of the analyses of compositional components in crude and hexane-extracted meals from AV Jade and DHA canola are within 10% of each other. Although some differences were above this 10% level, all were within the ranges usually observed in canola meal. Differences were expected and observed in the FA profile due to the intended effects of engineering the DHA biosynthetic pathway into DHA canola. Protein, fat and fiber are the key indicators of livestock feed quality along with AAs and digestibility. Based on the comparison of these important feed formulation parameters in meal samples from AV Jade and DHA canola, meal derived from DHA canola can be used for animal feed in the same manner as meal from any commercial canola variety.

3. Summary of Oil and Meal Composition

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 FAs; delta- and total tocopherols; magnesium; the glucosinolates 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, we have concluded that DHA canola was compositionally comparable to conventional canola except for the intentional production of the ω3 FAs.

VIII. Agronomic, Phenotypic and Environmental Interactions

Agronomic and ecological evaluations were conducted to compare DHA canola to conventional canola, to help show that DHA canola is no more likely to pose a plant pest risk than conventional canola. Agronomic evaluations were based on laboratory and replicated, multi-site field trials conducted by agronomists and scientists whom are experts in the production and evaluation of canola. To evaluate its agronomic characteristics, data were collected on representative characteristics that influence reproduction, crop survival, and potential weediness. In each of these assessments, DHA canola was compared to AV Jade and commercial references.

There are many similarities in agronomic practices used in canola production between North America and Australia, including weed, insect and disease control practices. Canola varieties have been adapted over years to a wide range of climatic conditions, and data from this study suggests that Australian varieties can also be grown in North America. The data generated in the field trials conducted in Australia and Canada are equally applicable in support of environmental as well as food and feed safety of DHA canola.

In Australia, canola is grown across the southern dryland cropping zone and mostly within winter-dominant rainfall environments. Australian production is mostly from spring type canola cultivars that have low vernalization requirements. In general, Australian cultivars typically retain some minor delay in the onset of flowering and have relatively high plant vigor or biomass production over winter months. The canola crop in Australia is typically sown after the first major rainfall event from April to May. Yield is primarily determined by available water during the growing season and water use efficiency of the cultivar. Blackleg of canola is a disease complex of at least two fungal species: *Leptosphaeria maculans* and *Leptosphaeria biglobosa*. Major pathotype gene resistance to blackleg can differentiate cultivars in terms of seedling survival and stem cankering, however, in general Australian cultivars are considered to have high resistance. Seed development occurs in late spring or early summer, following a growing season of 5 to 7 months. Apart from water availability, yield can be negatively impacted from large temperature extremes (+35 ºC to < 0 ºC) that may cause seed and pod abortion.

In North America, spring canola is grown in the western provinces of Alberta, Saskatchewan and Manitoba. British Columbia, Ontario and Quebec also grow a substantial amount. New varieties are pushing the boundaries of where canola is being successfully grown. It is now grown in the Pacific Northwest states and across other U.S.A. states that border Canada, with most of the production in North Dakota (Brown et al., 2008). In Canada, seeding in late April is fairly common in the brown and dark brown soil zones, and has been tried in black and grey wooded zones. April-seeded canola faces higher risks from early insect infestations, seedling disease pressures, water ponding in the field, soil crusting, frost damage, and slow emergence due to cold soil.

All field trials of DHA canola were observed for naturally occurring disease or insect biotic stressors. The agronomic analysis of DHA canola was tested across ten different growing locations in Australia and Canada. DHA canola was compared to AV Jade and at least 6 different commercial reference standard varieties. Across all measured parameters (e.g. early vigor, plant height,

reproductive timing, etc.), the DHA canola values fell within the range of the commercial reference standard varieties and were comparable to AV Jade.

1. Agronomic and Phenotypic Evaluations in Replicated Field Trials

The environmental safety of DHA canola was shown to be comparable to AV Jade and other conventional canola reference through the evaluation of agronomic, phenotypic, phenologic, and ecological interaction characteristics. Field trials were conducted under field permits granted by the OGTR and CFIA at eight different locations in Australia during the 2015 growing season and at two trial locations in Canada during the 2016 growing season (Table 54). In all field trials, the DHA canola was compared to AV Jade and to several other conventional canola varieties commonly cultivated in Australia and Canada. The field trial locations were representative of the different regions within those countries where canola is commercially cultivated and each trial location consisted of five replications of the test, AV Jade and conventional canola varieties that were organized in a randomized complete block design.

Table 54. Field trial permits approved by OGTR and CFIA

Agency	Permit reference	Trial year	Approved	Sites	References
OGTR	DIR 123	2015/16	11/13/2013	8	http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/DIR123
CFIA	16-RPC1-434-CAN	2016	5/11/2016	2	Locations approved under this permit included: 2016-RPC1-434-CAN01-AB111-01 2016-RPC1-434-CAN01-SK123-01

Agronomic performance assessments were conducted in multi-site field studies to measure characteristics such as emergence, seedling vigor, plant height, lodging, and yield. All field trials were also observed for opportunistic disease or insect stressors as well as normal phenotypic characteristics. Based on field observations at these sites, pathogen susceptibility or resistance characteristics of the DHA canola were unchanged when compared to those of AV Jade. The DHA canola remains tolerant to blackleg. There were no meaningful differences observed between DHA canola and AV Jade for plant pest characteristics and no indication of a selective advantage that could result in increased weediness potential of DHA canola.

Six DHA-expressing transgenic lines were compared to eight Australian cultivars across ten experimental locations (sites) for a range of important agronomic and seed traits. The ten_sites represented diverse environmental yield potentials as indicated by the wide range of site mean yields. Agronomic performance of DHA canola was comparable to that of the commercial cultivars evaluated across all environments tested

DHA canola was compared with AV Jade and other conventional canola varieties with respect to key vegetative and reproductive development characteristics associated with the competitiveness and survival of plant species. In field trials, characteristics including seed germination rate, seedling vigor, days to reach key developmental stages, plant height, and grain yield, as well as

observations on susceptibility to and interactions with diseases and insects were evaluated. In addition, seed germination studies were conducted to compare DHA canola and AV Jade and other conventional canola varieties.

Agronomic Data Collection

Agronomic characteristics (Table 55) were measured and recorded for all test entries at each location on a per plot basis.

Table 55. Agronomic characteristics measured

General Characteristic	Characteristic Measured	Evaluation description (measurement endpoints)	Scale
Germination / Emergence	Early Population	Number of plants emerged per square meter	1-9, where 1 = short plants and 9 = tall plants
	Seedling Vigor	Visual estimate of emerged plants per plot during plant cabbage stage	1-9, <10% leaf area coverage and 9 = >90%
Vegetative Parameters	Plant Height	Height at dry seed maturity measured from base to growing tip in center of plot	Height in cm
	Lodging	Visual estimate	1-9, where 1 = plants >90% of ground (standing straight) and 9 = plants flat (<10% of ground
	Shattering	Seeds shattered counted per $1/8^{th}$ mt^2 over 2 weeks	1-9, where 1 = 0% shattered and 9 = >40% shattered
	Final Population	Number of plants per plot in two 1 mt^2 quadrants	Number of plants
Reproductive Parameters	Days to Flowering	Days from sowing to 50% flowering	Days
	Flowering Duration	Difference between flowering time and end of flowering time[1]	Days
	Flowering End	90% of plants with no flowers	Days from sowing
	Days to Maturity	Planting to date when 60% of pods are ripe	Days
	Yield	Weight (corrected for moisture) harvested	Tons/ha
Ecological Interactions	Blackleg Disease Incidence	Visual estimate of blackleg foliar severity	1-9, where 1 = poor resistance, and 9 = low infection
	Insect Damage	Visual estimate	% damage

[1]Flowering end (days from sowing) based on 90% of plants having no flowers.

Data was analyzed using ASReml, a statistical package that fits linear mixed models using residual maximum likelihood (REML) (Gilmour et al., 2009) procedures in GenStat (Version 17). A linear mixed model statistical method was used to account for field spatial variation as extensively described and used for field plant breeding and genetics research (Smith et al., 2001, Welham et al., 2013). A meta-REML analysis across site analysis was further undertaken for all traits measured. The treatment line mean, variance (VAR), standard error (SE), least significant difference (LSD), confidence interval (CI), coefficient of variation (CV%) and F distribution probability of significant difference (F pr) are presented for both site specific and across site analysis in Table 56 and summarized in Table 57. The commercial varieties tested are described in Tables 2 and 3. In addition, they were compared with various DHA events closely related to DHA canola, designated in Table 66 as Line, followed by a letter from A to E.

Table 56. Across site META-REML analysis of agronomic traits

Trait	Emerg.[1]	Harvest count[2]	Emerg. [1]	Plant vigor	Flower start	Flower end	Flower duration	Height	Shatter[5]	Yield	Moisture [6]
Line name	Plants/m^2	Plants/ m^2	Score[3]	Score [3]	Day	Day	Days	cm[4]	No.	t/ha	%
ATR Bonito	18.2	16.0	7.3	6.8	103.8	131.2	27.5	90.0	13.0	1.35	10.6
ATR GEM	17.9	16.6	7.1	6.7	105.3	133.6	28.2	91.0	10.9	1.21	13.0
ATR Stingray	17.6	17.3	7.1	5.9	100.9	129.7	28.8	82.7	14.4	1.34	8.2
ATR WAHOO	11.2	11.8	5.9	6.1	108.2	136.0	27.3	92.3	10.7	1.12	18.7
AV GARNET	18.6	16.3	7.4	7.2	104.4	132.8	28.6	102.1	15.0	1.31	10.2
AV JADE	7.8	12.5	5.0	4.8	106.7	134.8	28.3	89.9	9.8	0.96	9.9
AV ZIRCON	19.0	15.7	7.3	7.0	104.4	132.0	27.6	98.7	22.5	1.31	9.5
Monola 515TT	20.3	18.5	7.5	5.8	108.6	136.1	27.3	87.9	12.3	1.24	12.4
LINE A	19.5	17.9	7.3	6.4	101.4	132.4	31.3	89.9	8.3	1.25	8.9
LINE B	20.8	19.7	7.4	6.4	101.1	132.4	31.4	88.6	10.4	0.84	9.9
LINE C	17.5	14.6	7.1	6.0	102.0	131.5	29.7	92.3	8.4	0.76	9.6
DHA canola	18.1	15.7	7.1	5.9	107.8	135.0	27.2	88.2	10.5	1.17	11.0
LINE D	22.5	20.3	7.2	5.9	106.6	134.4	27.9	76.4	10.3	0.95	10.8
LINE E	22.6	19.8	7.6	5.4	108.5	135.8	27.3	70.6	8.9	0.92	11.1
Min Value	7.8	11.8	5.0	4.8	100.9	129.7	27.3	82.7	9.8	0.96	8.2
Max Value	20.3	18.5	7.5	7.2	108.6	136.1	28.8	102.1	22.5	1.35	18.7
Mean	17.6	16.4	7.0	6.2	104.7	133.2	28.5	90.0	12.0	1.14	11.0
Variance	0.67	0.98	0.02	0.01	0.04	0.07	0.13	1.35	2.74	0.00	0.15
Standard error	0.81	0.98	0.14	0.11	0.21	0.27	0.35	1.15	1.65	0.06	0.39
LSD	1.62	1.95	0.28	0.21	0.41	0.54	0.71	2.30	3.30	0.11	0.78

[1]Emergence measured in plants per square meter. [2]Harvest plant count measured in plants per square meter. [3]Score, 1 low, 9 high. [4]Height in centimeters at maturity. [5]Number of shattered seeds at maturity. [6]Moisture level in grain at harvest

Table 57. Combined-site comparison of agronomic characteristics

Agronomic Characteristic	Mean		Reference Range[1]	
	DHA canola	AV Jade	Minimum	Maximum
Emergence (no./m^2)	23.6	20.3	7	24
Seedling Vigor (1-9 scale)	4.1	5.9	2.5	9.0
Plant Height (cm)	101.9	107	62	133
Lodging (1-9 scale)	1	1.2	0.9	2.8
Shattering (seeds/m^2)	6.5	6.2	0	40.6
Plant Survival (no./m^2)	15.6	18.8	7.9	21.4
Days to Flowering	49.3	47.3	43	52
Flowering Duration	27.9	29.6	25	31
Flowering End	76.6	76	71	79
Yield (t/ha)	2.4	2.5	0.6	6.1
[1]Reference range = minimum and maximum mean values among the reference varieties				

In each experiment, DHA canola was agronomically comparable to the control or commercial comparator lines and showed no biologically meaningful differences. These observations also tracked the presence of insect and disease stressors and the plant responses in the field. In each case, DHA canola responded similarly to the control plants in these trials. Based on the analyses, DHA canola is comparable to conventional canola and would not pose a greater plant pest risk or increased weed potential than does conventional canola. These data support the conclusion of

agronomic comparability of DHA canola with commercially available canola regarding lack of increased weediness and plant pest potential.

There are many similarities in agronomic practices used in canola production between the United States, Australia, and Canada, including maturity groups of canola cultivars as well as weed, insect and disease control practices. Accordingly, data generated in the field trials conducted in Australia and Canada are sufficient to support the environmental and food and feed safety of DHA canola for the U.S. environment.

2. Germination Evaluations

AV-Jade and two segregates, one with high DHA, and another one with medium DHA, grown simultaneously in a contained facility. Seeds were collected from glasshouse grown DHA canola plants at 25, 30, and 35 days after flowering. The storage potential of transgenic DHA canola seeds was subjected to natural aging for six months at three different temperatures, 4, 24 and 32 °C. They were also tested by accelerated aging at 42 °C with 100% relative humidity for up to four days. Storage conditions on the physiological quality of the seeds were evaluated by the standard germination test (SGT), germination at unfavorable temperatures (10, 16 and 32 °C), electrical conductivity and viability by tetrazolium chloride (TZ).

Standard germination test (SGT): Standard germination test was conducted on freshly harvested seed with three biological replicates of 35 seeds each at 24 °C on moist filter paper in dark conditions. Filter paper sheets were unrolled after eight days and seedlings counted and recorded, as described in Janmohammadi et al. (2008). The same germination test was also conducted at three other, less than ideal, temperatures of 10, 16 and 32 °C. The rate of radicle emergence was also observed.

Natural aging: To test responses after natural aging, seeds were stored for 6 months at three temperatures, 4 (cold room), 24 and 32 °C in a sealed bag and tested by SGT as described above.

Accelerated aging test (AAT): AAT was performed according to the method known as ‘gerbox’ with minor modification as described by Janmohammadi et al., 2008. Three grams of seeds were placed as a single layer on fine mesh suspended inside a plastic box (11.5 x 10 x 9 cm) containing 100 ml sterile distilled water. The boxes were covered with lids and incubated in Qualtex, thermostat oven at 42 °C for 24, 48 72 and 96 hrs. After the completion of AAT, seeds were subjected to germination according the methodology described for SGT. Following each aging treatment, the seeds were allowed to air dry at room temperature until their original weight was restored and seeds were sterilized using chlorine gas as described by Kereszt et al. (2007), sealed in polythene bags in a refrigerator until use. Germination of fresh, naturally aged seed and AAT seed was estimated by SGT. The results of the AAT seeds were compared with the germination percentage of freshly harvested seeds.

Electrical conductivity (EC) test: The EC quickly and objectively measures the condition of seed cell membranes, providing a strong indicator of seed quality and vigor. Low quality seeds have

poor membrane structure that allows electrolytes such as AAs and inorganic ions to leak through and show a lower speed of cell membrane repair during seed water uptake for germination. Leakage of these nutrients can stimulate the growth of pathogenic microorganisms and impair seedling emergence. Therefore, seeds with higher conductivity measurements reveal electrolyte leakage and lower quality. High quality seeds contain their nutrients within their membranes.

Solute leakage of 0-aged (control) and AAT canola seeds (three lines) was estimated by soaking 1 g seeds with three replicates in 50 ml of deionized water and incubated at 24 °C. The electrical conductivity of seed leachate was measured using a Lutron conductivity meter after 24-48 hrs of seed soaking and expressed in $\mu S.cm^{-1}g^{-1}$ as described by Miloševic et al. (2010).

Tetrazolium chloride test-viability: Freshly harvested seed and seed stored at 4 °C or 32 °C for 6 months were tested with tetrazolium chloride for viability. Three replicates of 100 seeds each were preconditioned to saturation in rolls of paper towels moistened with water. Paper towels were placed in petri plates and incubated at room temperature (22 °C) for 24 hours. After the preconditioning period, the seeds were immersed in tetrazolium solution 0.01% and incubated at 37 °C in the dark. After incubation, seeds were rinsed with running water and individually assessed using 40X magnification (microscope) and classified in to three categories as described by Santos et al. (2007).

Statistical analysis: SGT, tetrazolium chloride test and electrical conductivity tests were arranged in a completely randomized design. Results from these tests were analyzed by one way ANOVA using Sigmaplot (v13) by the Holm-Sidak method (Holm-Sidak, 2017). Differences were considered significant at the 4.8% level.

Results

Optimum germination conditions provided by SGT (constant temperature of 24 °C), resulted in equal germination of DHA canola and AV Jade seeds (Table 58). Rate of radicle emergence was also visually compared between all the three lines tested and no significant differences were observed (Figure 27). These results suggest that introduction and seed specific over-expression of LC-PUFA pathway genes has no obvious impact on seed germination under optimal conditions, and thus unlikely to exhibit any competitive disadvantage during seedling establishment when compared with its conventional parent.

Germination percentages were also tested at three other temperatures (10 °C, 16 °C and 32 °C). Germination percentages of high DHA canola and AV Jade at 16 °C were identical, no significant differences were observed ($P<0.05$) and comparable with SGT conducted at optimal temperature (22 °C). However, the germination percentages of high DHA canola was impacted at lower (10 °C) and higher (32 °C) temperatures when compared with AV Jade (Table 58). High DHA canola seeds germinated later than AV Jade at low temperature 10 °C and there were significant differences in their germination percentages compared to AV Jade ($P<0.05$). These differences in germination are not surprising since winter sowing can delay and reduce seedling emergence,

particularly in small seeded crops like canola. Delayed germination under sub-optimal conditions does not give the high DHA canola seeds any competitive advantage versus AV Jade.

Table 58. Germination percentages of freshly harvested AV Jade and high DHA canola

Seed type	Germination percentages (measured day 8)			
	10 °C	16 °C	24 °C	32 °C
AV Jade	100 ±0.0[a]	100±0.0 [a]	100±0.0 [a]	98.25±2.5[a]
High DHA canola	81.33±6.1[b]	100±0.0 [a]	100±0.0 [a]	86.8±6.3[b]

Values with in a column followed by the same letter are not significantly different at the 0.05 level of probability (P), based on the pair-wise multiple comparison (Holm-Sidak method).

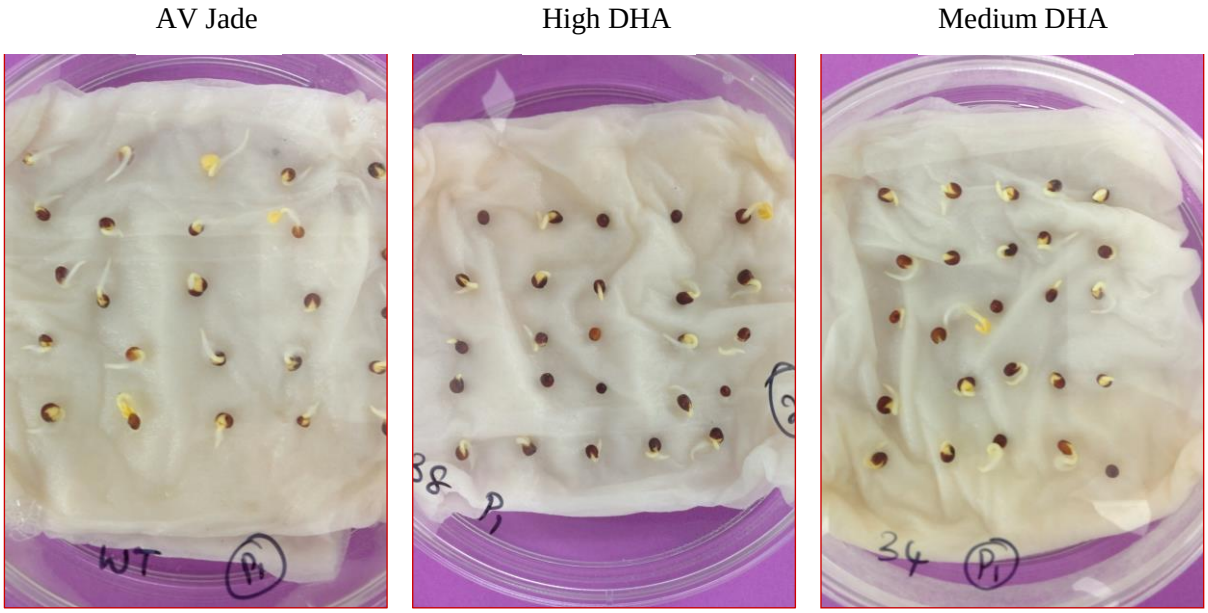

Figure 27. SGT of natural aging AV Jade, high DHA and medium DHA canola at 10 °C (3 days after sowing)

AV Jade, high DHA, and medium DHA canola seed stored at 4 °C (cold room), and 32 °C (constant) for 6 months were also tested for germination (SGT). The germination percentages of all the three lines stored at 4 and 32 °C for 6 months (relative humidity 60-70%, standard laboratory conditions) were comparable with the germination percentages of freshly harvested seed (0 aged) (Table 59).

Table 59. SGT of seed stored at 4 and 32 °C for 6 months

Seed type	SGT of seed stored for 6 months	
	4 °C for 6 months	32 °C for 6 months
AV Jade	98.7 ±2.2[a]	100 ±0.0[a]
B50-27-18-38 (high DHA)	97.4 ±2.2[a]	98.6 ±2.4[ab]
B50-27-116-34 (medium DHA)	96.4 ±0.0[a]	94.1 ±2.8[bc]

Values with in a column followed by the same letter are not significantly different at $P<0.05$.

Germination results shown in Table 59 reveal that DHA canola seed stored at 32 °C for six months has no impact on its germination and is very similar to AV Jade. These results were also comparable with freshly harvested (zero aging) seed and seed stored at 4 °C for 6 months. No significant differences were observed in viability percentages using TZ between AV Jade and DHA canola in any of the three different storage conditions

Freshly harvested seed (0 aged) showed no significant differences between AV Jade and DHA canola in electrical conductivity (Table 60). Significant differences were observed in seed stored at different temperatures for 6 months. As storage temperature increased, conductivity increased in either AV Jade or transgenic lines; however, the electrical conductivity is more than double in seed stored at 32 °C for six months when compared with 0 aged seed.

Table 60. Electrical conductivity of AV Jade and DHA canola seed

Type of seed	Electrical conductivity ($\mu S.cm^{-1}g^{-1}$)
Fresh Seed (0 aged)	
AV Jade	85.0±4.4[a]
High DHA	84.3±4.5[a]
Medium DHA	89.0±2.0[a]
Seed stored at 4 ºC for 6 months	
AV Jade	90.7±2.1[a]
High DHA	103.3±2.5[b]
Medium DHA	106.3±3.2[bc]
Seed stored at 24 ˚C for 6 months	
AV Jade	97.0±1.7[a]
High DHA	133.0±3.6[b]
Medium DHA	139.7±1.5[c]
Seed stored at 32 ˚C for 6 months	
AV Jade	109.0±2.6[a]
High DHA	187.0±6.7[b]
Medium DHA	205±5.0[c]

Values within a column of subgroup followed by the same letter are not significantly different at $P<0.05$.

Seed viability percentages of TZ staining along with SGT of high DHA, medium DHA and AV Jade canola results are presented in Table 61. In general, all the three lines stored at different conditions for six months, exhibited adequate TZ staining pattern after 24 hrs of hydration on paper towels/tissues as per SGT followed by 4 hrs TZ staining at 37 °C. No significant differences (P<0.05) observed in viability percentages between AV Jade and the high DHA line in any of the three different storage conditions. DHA canola seed stored at 32 °C for six months showed no obvious differences when compared with AV Jade seed. The EC test also detected significant differences in seed quality of three lines tested (AAT at 42 °C with 100% relative humidity) as the aging period increased, the conductivity also increased. After a 24 hour aging period, a 2.5-fold increase in EC was observed in both high and medium DHA canola when compared with AV Jade canola seed EC (Table 61).

Table 61. Comparison of SGT and TZ viability percentages of AV Jade and DHA canola

Type of seed	SGT	TZ viability (%) 4 hrs (0.01% TZ) at 37 °C
Freshly harvested seed (0 aged)		
AV Jade	100±0.0^{a}	100±0.0^{a}
High DHA	100±0.0^{ab}	100±0.0^{a}
Medium DHA	96.3±0.2^{c}	94.7±1.7^{c}
Seed stored at 4 °C for 6 months		
AV Jade	98.7 ±2.2^{a}	98.6±0.4^{a}
High DHA	97.4 ±2.2^{a}	96.8±0.9^{a}
Medium DHA	96.4 ±0.0^{a}	94.4±1.6^{bc}
Seed stored at 32 °C for 6 months		
AV Jade	100 ±0.0^{a}	99.0±1.0^{a}
High DHA	98.6 ±2.4^{ab}	98.5±0.9^{a}
Medium DHA	94.1 ±2.8^{bc}	91.7±1.4^{c}

Values with in a column of subgroup followed by the same letter are not significantly different at P<0.05.

AAT at 42 °C with 100% relative humidity for different periods impacted seed quality of AV Jade and transgenic lines differently. As the aging period increased, the germination of all the three lines tested decreased. In this study, after 48 hrs of AAT aging high DHA line germination dropped to 1.3% from 95.8% and medium DHA line germination dropped to 2.8% from 89.4%, whereas AV Jade seed germination was around 88%. These results clearly reveal that transgenic seed with altered LC-PUFA were susceptible to stress caused by high temp (42 °C) and humidity (Table 62).

Table 62. AAT on seed germination of AV Jade and DHA canola seed

AAT period	Seed germination percentage (SGT)		
	AV Jade	(High DHA)	(Medium DHA)
Control (0 hrs)	100±0.0[a]	100±0.0[a]	96.3±0.1[a]
24 hrs	96.8±0.1[ab]	95.8±0.41[b]	89.4±4.4[b]
48 hrs	88.7±10.2[bc]	1.3±2.3[c]	2.8±2.4[c]
72 hrs	53.2±3.5[d]	1.6±2.8[d]	0.0[d]
96 hrs	53.4±6.1[de]	0.0[c]	0.0[c]

Values within a column of subgroup followed by the same letter are not significantly different at $P<0.05$.

The EC test also detected significant differences in seed quality of three lines tested as the aging period increased, the conductivity also increased (Table 63). After 24 hours, around 2.5-fold increase in EC was observed in both high and medium DHA canola when compared with AV Jade canola seed EC. After 48 hrs of AAT period, 5 to 6-fold increase in EC was observed in DHA lines whereas a marginal increase in EC was observed in AV Jade canola seed.

Table 63. AAT on EC of AV Jade and transgenic canola seed

AAT period	Electrical conductivity ($\mu S.cm^{-1}g^{-1}$)		
	AV Jade	High DHA	Medium DHA
Control (0 hrs)	85.0±4.4[a]	84.3±4.5 [a]	89.0±2.0 [a]
24 hrs	123±3.0[b]	292±2.6 [b]	279±7.5 [b]
48 hrs	149±3.5[c]	838±6.7 [c]	817±6.7 [c]
72 hrs	588±6.2[d]	1590±9.0 [d]	1767±17.8 [d]

Conclusion

Under the multiple parameters tested, DHA canola generally showed similar germination and vigor results compared AV Jade. One important exception was observed during the TZ viability test at the more extreme temperatures of 10 and 32 °C. Overall, it is expected that DHA canola will exhibit equivalent seed viability and vigor as conventional canola under most conditions.

3. Ecological Observations and Disease Susceptibility

In 2015, field trial experiment sites were sown across eight locations (Table 64) in Western Victoria, Australia. Each trial was designed as a randomized complete block experiment consisting of 5 replicates (block) by 14 treatment entries (6 transgenic lines and 8 cultivars). In 2016, one site was planted in Alberta and another in Saskatchewan, Canada. Each of those two trials was also a randomized complete block design and had 5 replicates by 12 treatment entries (2 transgenic lines and 10 cultivars).

Blackleg leaf severity symptoms representative of *L. maculans* and *L. biglobosa* was recorded as a 1 to 9 score for one replicate across five sites. Not all plots were scored due lack of observable variation, while symptoms associated with cankering and stem breakage were not observed.

Blackleg disease leaf symptoms observed were at very low levels at all sites (Table 65). The NARBL site was sown using bare seed (untreated with fungicide) and showed no differential line

emergence which indicates sufficient or relevant race specific disease resistance were present in all lines. Leaf symptoms are not always predictive of the degree of stem cankering caused by *L. maculans* which is the main cause of yield loss and basis for resistance rating in Australia (Sosnowski et al., 2004). Given the lack of cankering and stem breakage, all lines can be considered resistant to the disease pressure present in this study.

Table 64. List of experimental trials, location and code names

Site Name	Location	Site synonym
1506_NAR	NURRABIEL, AUSTRALIA	NAR
1507_NARBL	NURRABIEL-BL, AUSTRALIA	NARBL
1508_DOU	DOUGLAS, AUSTRALIA	DOU
1509_GRN	GREEN LAKE, AUSTRALIA	GRN
1510_TOO	TOOLONDO, AUSTRALIA	TOO
1512_GYM	GYMBOWEN, AUSTRALIA	GYM
1513_KAN	KANIVA, AUSTRALIA	KAN
1514_ARA	ARARAT, AUSTRALIA	ARA
COALHURST	COALHURST, CANADA	CH
VANGUARD	VANGUARD, CANADA	VG

Table 65. Ecological observations of blackleg

Agronomic Characteristic	Mean*		Reference Range	
	DHA canola	AV Jade	Minimum	Maximum
Blackleg Disease Incidence	1.6	1.8	1.4	2

*Scoring: 1 = very low infection; 9 > 40% infection

A summary of blackleg observations for both Australia and Canada field trials is provided in Table 66. The commercial varieties tested are described in Tables 1 and 2. In addition, they were compared with various DHA events closely related to DHA canola, designated in Table 66 as Line A-E. In every case, the severity was not qualitatively different from various control lines growing at the same locations. These results support the conclusion that the ecological interactions for DHA canola were comparable to control canola lines with similar genetics or to conventional canola lines.

Table 66. Site by line treatment mean blackleg disease symptom score (1-9)

Site / Line name	NAR	NARBL	GYM	TOO	ARA	CH	VG
ATR Bonito	3	2	2	1	2	3	2
ATR Gem	3	2	1	2	2	3	3
ATR Stingray	1	1	3	1	2	1.6	2.8
ATR Wahoo	2	1	1	2	2	2.4	2.5
AV Garnet	2	2	2	1	2	2.4	3
AV Jade	3	1	2	2	2	1.6	1.8
AV Zircon	2	1	1	2	2	1.6	1
Monola 515TT	1	1	2	1	2	2.5	2.5
Line A	1	1	1	1	2	NR	NR
Line B	1	NR	NR	NR	2	NR	NR
Line C	2	1	1	1	2	NR	NR
DHA canola	1	1	1	1.5	3	1.2	2.2
Line D	1	1	1	2	2	NR	NR
Line E	2	2	2	3	2	NR	NR
DHA canola	NR	NR	NR	NR	NR	0.5	1.8
DK7444	NR	NR	NR	NR	NR	1.7	2.2
LL130	NR	NR	NR	NR	NR	1.6	2.4

NR = not rated

4. Conclusions from agronomic, phenotypic and ecological evaluations

DHA canola was field tested at ten sites in major canola growing regions of Australia and Canada. All field tests were conducted under field permits granted by the OGTR in Australia and CFIA in Canada. Agronomic performance assessments were conducted in multi-site field studies to measure characteristics such as emergence, seedling vigor, plant height, lodging, and yield. All field trials were also observed for opportunistic disease or insect stressors as well as normal phenotypic characteristics. Based on field observation at these sites, pathogen susceptibility or resistance characteristics of the DHA canola were unchanged when compared to those of the AV Jade. The DHA canola remains resistant or tolerant to blackleg. There were no meaningful differences observed between DHA canola and AV Jade for plant pest characteristics and no indication of a selective advantage that could result in increased weediness potential of DHA canola. It was also observed that DHA canola was not associated with reduced yield or agronomic performance.

Results of these studies demonstrated that, except for FA profile, DHA canola is agronomically, phenotypically, and phenologically equivalent to AV Jade and other conventional canola varieties. Further, these studies showed no different biological effect of DHA canola compared to AV Jade with respect to interaction with various diseases or insects. Therefore, these results reinforce the conclusion that the cultivation of DHA canola poses no different plant pest or weediness potential and will have no different environmental impact than the cultivation of commercial canola varieties.

IX. Environmental consequences and impact on agronomic practices

1. Overview

This section provides a brief review and assessment of the plant pest potential of DHA canola and its impact on agronomic practices as well as the environmental impact of the introduced proteins. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article. Typically, the definition of "plant pest" includes living organisms that can directly or indirectly injure, damage, or cause disease in any plant or plant product. Information in this petition related to plant pest risk characteristics includes: 1) mode of action and changes to plant metabolism; 2) composition; 3) expression and characteristics of the gene product; 4) potential for weediness of the regulated article; 5) impacts to non-target organisms; 6) disease and pest susceptibilities; 7) impacts on agronomic practices; and 8) impacts on the weediness of any other plant with which it can interbreed, as well as the potential for gene flow. The following lines of evidence form the basis for the plant pest risk assessment in this petition: 1) insertion of two functional copies of the expression cassettes; 2) characterization of the proteins expressed; 3) safety and mode of action of the proteins; 4) compositional equivalence of DHA canola seed to conventional canola; 5) phenotypic and agronomic characteristics demonstrating no increased plant pest potential including disease and pest susceptibilities; 6) no new observed risk to non-target organisms, threatened or endangered species; 7) familiarity with canola as a cultivated crop; and 8) no greater likelihood to significantly impact agronomic practices, including land use, cultivation practices, or the management of weeds, diseases, and insects compared to commercially grown canola.

Using the assessment above, the data and analysis presented in this petition leads to a conclusion that DHA canola is unlikely to be a plant pest, and therefore should no longer be subject to regulation under 7 CFR § 340. Because the data presented in this petition demonstrate that DHA canola has no potential to cause injury or damage to protected interests under the noxious weed authority, DHA canola would not be considered a "noxious weed" as defined by the Plant Protection Act.

2. Plant Pest Assessment

In two separate growing seasons in field trials in Australia and Canada, DHA canola was compared to the parent control and other conventional canola lines with respect to key vegetative and reproductive development characteristics associated with the competitiveness and survival of plant species. Also, DHA canola was assessed for disease susceptibility and interactions with insects. Data from these studies demonstrated that DHA canola has no different agronomic or phenotypic characteristics, disease or insect interactions. In combination, these studies support a conclusion that DHA canola poses no different plant pest or weediness risk or environmental impact compared to that from the cultivation of conventional canola lines. Also, extensive analyses of the nutrient and anti-nutrient content of the grain and forage produced from DHA canola confirmed that these products are as nutritious and safe as grain and forage produced by conventional canola lines.

3. Weediness Potential

B. napus is generally regarded as an opportunistic species, that is, a species adapted to take advantage of temporary conditions such as disturbed areas (CFIA, 1994), not as an invasive species in undisturbed natural ecosystems (Crawley et al., 2001). Although *B. napus* has some characteristics typical of weedy species such as a high reproductive capacity, rapid growth and multiple pollination mechanisms (self, wind, insect), it also has many characteristics typical of domesticated species including low genetic diversity, lack of long-distance seed dispersal mechanisms, limited population persistence, lack of primary seed dormancy and an inability to compete well with perennial species (Hall et al., 2005).

B. napus is not listed as an invasive weed in the Catalog of Invasive Plant Species of the United States, nor is it present on the lists of noxious weed species maintained by the federal government (7 CFR § 360). *B. napus* has been documented to be present in disturbed areas such as roadsides and railways used for transportation of seed and the margins of fields where it has been previously grown. However, populations of canola outside agricultural fields do not effectively compete with perennial vegetation, and usually persist only for a few years in the absence of ongoing seed introductions into areas from spillage during handling and transport or processing (Knispel et al., 2008). The viability of the large majority of canola seed in soil declines over time (Gulden et al., 2004). Biotechnology-derived canola seed has not been demonstrated to persist longer than conventional canola seed (Gruber et al., 2004).

Canola seed can persist in the soil for several years becoming secondarily dormant, and then germinate as volunteers (Gruber et al., 2004). Canola that has germinated and emerged unintentionally in a subsequent crop, also known as volunteer canola, may compete with the succeeding rotational crop. However, problems controlling volunteer canola are not common. Volunteers, including volunteers with herbicide-tolerant traits, can be managed with pre-plant or selective post-emergent herbicide applications or by mechanical means. In comparative studies between DHA canola and the conventional control, phenotypic, agronomic and environmental interaction data were evaluated for changes that would impact the plant pest potential, and, in particular, plant weediness potential. Results of these evaluations show that there are no biologically significant differences between DHA canola and the conventional control for traits potentially associated with weediness, responses to abiotic stressors and disease or pest susceptibilities. Collectively, these findings support the conclusion that DHA canola has no increased weed potential compared to conventional canola and it is no more likely to become a weed than conventional canola.

4. Gene Flow Assessment

In general, it is difficult for a transgene to become established in a wild relative or recipient population. In order for successful introgression of a transgene in a recipient population, several

conditions must exist and several steps must take place before this would occur (Devos et al., 2009). These are as follows:

- There must at least be a partial overlap in flowering periods between the transgenic canola and the wild relative.
- The wild relative must grow within the physical range of viable pollen dispersal of the transgenic canola, and viable pollen grains must reach the recipient stigma.
- Viable and fertile interspecific F1 hybrids must be produced, and the transgene successfully integrated into the recipient plant genome.
- The transgene must be transmitted through successive backcross generations.
- The transgene must be stabilized in the genome of the relative through backcrossing over multiple generations.
- The introgressed transgene must be either neutral or provide a fitness advantage such that it persists in the wild relative's populations.

When considering the likelihood of gene flow of DHA canola to other species, additional information related to canola flowering synchrony and flowering characteristics, pollen dispersal mechanisms and pollinator biology are discussed below in order to provide context for the gene flow assessment.

Flowering synchrony and flower characteristics

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

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

Pollen dispersal mechanisms

B. napus is primarily a self-pollinating crop, and the majority of large-scale outcrossing studies with fertile canola indicate outcrossing rates of <2% (Salisbury, 2002). Canola pollen, which is heavy and sticky, can also be transferred from plant to plant through physical contact with

neighboring plants, via pollinator insects and, to a smaller extent, by wind. In general, windborne pollen may make little to no contribution to long distance pollination (OGTR, 2017).

Approximately half of canola pollen travels less than three meters from the source; the vast majority of pollen travels less than 10 meters, with the amount of pollen decreasing as the distance from the pollen source increases (Thompson et al., 1999). Pollinating insects, in particular honeybees and bumblebees, play a major role in *B. napus* pollination and are believed to be involved in the transfer of pollen over long distances (OGTR, 2017).

Pollinators

Wind is the by far the most important component of pollen dispersal, and especially when insect pollinators are scarce (Hoyle et al, 2007). Insect foraging behavior is complex. The dynamics of bee-mediated pollen movement depend on a number of factors including spatial arrangement of plants, environmental conditions, plant density, availability of pollen, and the size and location of the receiving populations.

In situations with abundant flowers, such as in a cultivated field, individual honey bees generally collect nectar and pollen from flowers in the same or immediately adjacent plants. The majority of bee flights are less than one meter, thereby limiting the likelihood of long distance pollen dispersal via insect pollinators (OGTR, 2017).

Honeybees forage during daylight and are unlikely to carry viable pollen grains to impact fertilization beyond 12 hours. Honeybees are sensitive to weather events and barometric pressure, and respond to these events by decreasing foraging distances (USDA-APHIS, 1998). The distance and success of pollen-mediated gene flow is dependent on its dispersal in space by either wind or insects, and on the length of time the pollen grain remains viable (OGTR, 2017). Canola pollen viability gradually decreases after four to five days in natural circumstances depending on environmental conditions, particularly temperature and humidity (OGTR, 2002).

Likelihood of hybridization

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

Multiple studies have been conducted to gather experimental data to infer the likelihood of hybridization using a variety of techniques: experimental crosses (manual hand pollination), spontaneous crosses (non-assisted crosses under field conditions), and *in vitro* methods (e.g. embryo rescue). Cross-compatibility varies with the particular genotype used and with the polarity of the cross (i.e. dependent upon which species was the maternal parent). Most combinations are

unsuccessful and where crosses are successful, rates of hybrid production are typically very low (FitzJohn et al., 2007).

Hybridization of *B. napus* with cultivated and wild *Brassica* species is very unlikely. Even among the closely related *Brassica* species, hybridization with *B. napus* is highly unlikely. For example, although *B. napus* and *B. rapa* share the A genome, Scott and Wilkinson (1998) reported only 7% of *B. rapa* populations had any hybrids, indicating that 93% of *B. rapa* populations had no hybrids, and hybridization rates were low (0.4-1.5%) in field situations; further, less than 2% of hybrid seedlings survived. Other published hybridization rates are equally low, *e.g*. <3% for *B. juncea* (Jørgensen et al., 1996), <0.003% for *R. raphanistrum* (Chèvre et al., 2000), <0.000034% for *S. arvensis*. In many cases of attempted hybridizations with *B. napus* as male donor, no hybrids were produced (Salisbury, 2002).

Assessment of Gene Flow Potential from DHA Canola to Relevant Species

Gene flow assessments were conducted for a variety of species on the basis of overlapping geographic ranges with canola production and reports of at least one successful hybridization (by hand pollination or in the field) with *B. napus*. Where limited information was available about hybridization, the species was also examined further if present in primary canola growing-areas.

Crop Species

***B. carinata* (Ethiopian mustard)**

No distribution information is available for *B. carinata* in the U.S. (USDA Plants Database, 2017).

***B. juncea* (Indian or brown mustard)**

B. juncea can occur throughout the U.S. (USDA Plants Database, 2017) and is both a crop and a weed. *B. juncea* (AABB genome) has a common set of chromosomes with *B. napus* (AACC genome), although their A subgenomes arose independently (Yang et al, 2016). Spontaneous hybridization has been reported at <3% for *B. juncea*, although hybridization is less successful when *B. napus* is the female parent (Jørgensen et al., 1998). In general, F1 hybrids are rarely more fit than highly selected and well adapted counterparts, and low initial frequencies, reduced fitness and viability, and competitive disadvantage with respect to parents can lead to hybrid extinction. The pollen and seed fertility of the F1 hybrids is typically less than 30%, however spontaneous backcrossing progeny with improved fertility have been documented (Jørgensen et al., 1999).

***B. nigra* (Black mustard)**

B. nigra can grow throughout regions of the U.S. (USDA Plants Database, 2017) and is classified as both crop and weed. Hybrids between *B. nigra* and *B. napus* have been reported to occur at a low frequency when produced under controlled conditions. However, under field conditions no hybrids resulted from co-cultivation of *B. nigra* and *B. napus* (Bing et al., 1996).

***B. oleracea* (cauliflower, cabbage, broccoli)**

B. oleracea has a more limited distribution than other *Brassica* spp. (USDA Plants Database, 2017). Although *B. oleracea* and *B. napus* share a common set of chromosomes, the frequency of successful crosses is very low, and embryos often abort at early stages of development. Thus far, no viable hybrid seeds have been obtained from crosses between *B. napus* and *B. oleracea* without the assistance of embryo rescue or ovule culture (Myers, 2006).

***B. rapa* (Turnip, Chinese cabbage)**

B. rapa is a closely related species of canola (amphidiploid), which has a similar life history to canola, but with a shorter growing season. *B. napus* (AACC) and *B. rapa* (AA) have a common set of chromosomes, making interspecific outcrossing more common (Bing et al., 1996). Gene flow measurements reported only 7% of *B. rapa* populations had any hybrids, indicating that 93% of *B. rapa* populations had no hybrids, and hybridization rates were low (0.4-1.5%) in field situations. Importantly, less than 2% of hybrid seedlings survived (Scott and Wilkinson, 1998). Hybridization frequencies are higher when *B. rapa* occurs as a weed within canola crops, but varies significantly with experimental design. Reduced dormancy of *B. rapa* x canola hybrids relative to the persistent wild *B. rapa*, coupled with the reduced fertility of the inter-specific hybrid (Jørgensen et al., 1999) makes it very unlikely that populations of these hybrids will persist.

Weed Species:

B. tournefortii

B. tournefortii grows in the southwestern U.S. (USDA Plants Database, 2017), therefore geographic overlap is limited to winter canola production, however as previously discussed, asynchrony of flowering is probable in this case (Boyles, 2010).

Sinapis arvensis

S. arvensis occurs throughout the U.S. (USDA Plants Database, 2017) and is a weed frequently found in canola fields in weed surveys. However, greenhouse studies conducted by Moyes et al. (2002) have confirmed the low probability of hybridization between *B. napus* and *S. arvensis.*

Related Weeds Frequently Found in Agricultural Fields

In addition to examination of geographic range and likelihood of hybridization, it is important that abundance of weed species in agricultural fields is considered in the risk assessment. Weed abundance in agricultural fields is partially determined by crop production practices, and weed species prominence can be significantly impacted by cropping systems and cultivation practices (CFIA, 1994).

5. Conclusions on the Potential Plant Pest Risk of DHA Canola

DHA canola does not pose a plant pest risk. The proteins and their source organisms are familiar and have a history of safe use. The transgenic DNA in DHA canola is as safe for consumption as any other DNA. *B. napus* is not considered a noxious or invasive weed in the U.S. Although it does demonstrate some weedy characteristics, canola has undergone many years of selective breeding and domestication and is a poor competitor to other species. In addition, the agronomic characteristics and germination data for DHA canola demonstrated no change that would allow for development of weedy characteristics different from other canola varieties. None of the intentionally introduced genes provide a selective advantage in unmanaged ecosystems. Commercially available herbicides, such as glyphosate or imidazolinones, as well as mechanical means can be used in crop settings for volunteer control.

Gene flow from DHA canola was evaluated with respect to plant pest risk. Although crops and certain wild/weedy relatives have exchanged genes for centuries, the concern with genetically modified crops is that the acquisition of transgenes may increase the fitness of recipient plants (Ellstrand, 2003) and the potential weediness or invasiveness in the crop itself or in its wild or weedy relatives as a result of transgene movement (Warwick et al., 2009). Successful hybridization of *B. napus* and a wild/weedy relative is highly unlikely. The introduced genes did not change the ability of the plant to interbreed to other plant species. Furthermore, the evaluation 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. The consequences of gene flow and introgression of the ω3 trait from DHA canola to the same or sexually compatible species is anticipated to be the same as for existing commercial canola varieties.

Although gene flow from DHA canola to relatives is possible, it will not result in increased weediness or invasiveness of these relatives based on the agronomic and ecological assessments. Large-scale cultivation of transgenic canola has occurred for nearly 20 years in Canada and the United States. To date, there are no reports of problems with interspecific crosses and introgression of introduced genes into cultivated or wild relatives of canola. In fact, weed species diversity surveys in western Canada have shown no differences before and after the adoption of genetically modified canola (Beckie et al, 2011).

6. Potential Impact on Canola Agronomic Practices

An assessment of current canola agronomic practices was conducted to determine whether the cultivation of DHA canola has the potential to impact current canola and weed management practices. Canola fields are typically managed agricultural areas that are dedicated to crop production. DHA canola is likely to be used in common rotations on land previously used for agricultural purposes. Certified seed production will continue to use well-established industry practices to deliver high quality seed containing DHA canola to growers. Cultivation of DHA canola is not expected to differ from current canola cultivation. DHA canola is similar to

conventional canola in its agronomic, phenotypic, ecological and compositional characteristics and has levels of resistance to insects and diseases comparable to conventional canola. Therefore, no significant impacts on current cultivation and management practices for canola are expected following the introduction of DHA canola. Based on this assessment, introduction of DHA canola will not impact current U.S. canola cultivation practices or weed management practices.

X. Adverse Consequences of Introduction

DHA canola testing has demonstrated that it is substantially equivalent to AV Jade, apart from the intended changes in the production of PUFAs. Nuseed knows of no study results or other observations associated with DHA canola that indicate there would be anticipated to be adverse consequences from introduction.

It has also been demonstrated that the presence of the proteins introduced in DHA canola will not present adverse environmental effects. The phenotypic evaluations of DHA canola, including an assessment of seed germination and dormancy characteristics, plant growth and development characteristics, pollen characteristics and environmental interactions also indicated DHA canola is unchanged compared to conventional canola. The current practice to control volunteer canola plants will not be altered by DHA canola, nor will it adversely impact cultivation practices or weed management. It is expected that growers who choose not to grow DHA canola will not be impacted by the commercial use of this product. It is also not anticipated that the commercial use of DHA canola will have any potential impacts on non-target organisms or on threatened or endangered species.

Nuseed knows of no study results or observations associated with DHA canola, the improved FA profile or the DHA enzyme proteins, indicating that there would be an adverse environmental consequence from the introduction of DHA canola. DHA canola oil contains a reduced level of saturated fats and an increase in ω3 fish oils. The decrease in saturated fats and increase in ω3 oil provides important options for a more sustainable supply of fish oil. As demonstrated by field results and laboratory tests, the only phenotypic difference between DHA canola and conventional canola is the improved FA profile.

The data and information presented in this petition demonstrate that DHA canola is unlikely to pose an increased plant pest potential or to have an adverse environmental consequence compared to conventional canola. This conclusion is reached based on multiple lines of evidence developed from a detailed characterization of the product compared to conventional canola, followed by risk assessment on detected differences. The characterization evaluations included molecular and protein analyses, which confirmed the insertion of two T-DNA inserts. Extensive characterization of the plant phenotype, including compositional analysis of key nutrients and anti-nutrients also indicated DHA canola, except for the intended FA changes, was unchanged compared to conventional canola. The history of safe use is supported by the approved food and feed uses of fish, fish oil as well as canola oil. Therefore, the risks for humans, animals, and other non-targeted

species from DHA canola are negligible under the conditions of use. Additionally, the introduction of DHA canola will not adversely impact cultivation practices or the management of weeds, diseases, and insects in canola production systems. Successful adoption of DHA canola will provide growers with an opportunity to produce this value-added specialty canola that produces oil with a healthier profile to help meet higher demand for DHA fish oils in food, feed and nutraceutical markets.

References

Ahmad, M, Hirz, M, Pichler, H, Schwab, H. 2014. Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Applied Microbiology and Biotechnology 98: 5301-5317.

Arondel, V, Lemieux, B, Hwang, I, Gibson, S, Goodman, HM, Somerville, CR. 1992. Map-based cloning of a gene controlling omega-3 fatty acid desaturation in Arabidopsis. Science 258: 1353-1355.

Batt, C.A. 2014. *Pichia pastoris*, Encyclopedia of Food Microbiology (Second Edition). Academic Press, Oxford: 42-46.

Beckie, HJ, Harker, KN, Légère, A, Morrison, MJ, Séguin-Swartz, G, Falk, KC. 2011. GM Canola: The Canadian Experience. Farm Policy Journal 8(1): 43-49.

Belide, S, Zhou X.R., Kennedy Y, Lester, G, Shrestha, P, Petrie, J.R., Singh, S.P. 2013. Rapid expression and validation of seed-specific constructs in transgenic LEC2 induced somatic embryos of *Brassica napus*. Plant Cell, Tissue and Organ Culture 113:543-553. Doi:10.1007/s11230-013-0295-1.

Bevan, M. 1984. Binary Agrobacterium vectors for plant transformation. Nucleic Acids Research 12:8711–8721.

Bhalla, P.L., and Singh, M.B. 2008. Agrobacterium-mediated transformation of *Brassica napus* and *Brassica oleracea*. Nature Protocols 3, 181-189.

Bing, D.J., R.K. Downey G.F.W. Rakow. 1996. Hybridizations among *Brassica napus*, *B. rapa* and *B. juncea* and their two weedy relatives *B. nigra* and *Sinapis arvensis* under open pollination conditions in the field. Plant Breeding 115: 470-473.

Bourdichon, F, Casaregola, S, Farrokh, C, Frisvad, J.C., Gerds, ML, Hammes, W.P., Harnett, J, Huys, G, Laulund, S, Ouwehand, A, Powell, I.B., Prajapati, J.B., Seto, Y, Ter Schure, E, Van Boven, A, Vankerckhoven, V, Zgoda, A, Tuijtelaars, S, Hansen, EB. 2012. Food fermentations: Microorganisms with technological beneficial use. International Journal of Food Microbiology 154:87-97.

Bowie, JU. 2001. Stabilizing membrane proteins. Current Opinion in Structural Biology 11:397-402.

Boyles M. 2010. Common questions. Department of Plant and Soil Sciences Okanola, Oklahoma State University. http://www.canola.okstate.edu/commonquestions/index.htm.

Brown, J, David, JB, Lauver, M, Wysocki, D. 2008. U.S. Canola Association Canola Growers' Manual. U.S. Canola Association. University of Idaho and Oregon State University.

Brown, MR. 1991. The amino-acid and sugar composition of 16 species of microalgae used in mariculture. Journal of Experimental Marine Biology and Ecology 145:79-99.

Brown, MR, Jeffrey, SW, Volkman, JK, Dunstan, GA. 1997. Nutritional properties of microalgae for mariculture. Aquaculture 151: 315-331.

Buerth, C, Tielker, D, Ernst, JF. 2016. *Candida utilis* and *Cyberlindnera* (Pichia) *jadinii*: yeast relatives with expanding applications. Applied Microbiology Biotechnology 100: 6981-6990.

Canola Council, 2017. What is Canola? – Canola Council of Canada. http://www.canolacouncil.org/oil-and-meal/what-is-canola/.

CFIA, 1994. http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/directive-94-08/biology-documents/brassica-napus-l-/eng/1330729090093/1330729278970.

Chacón-Lee, TL and Gonzalez-Marino, GE. 2010. Microalgae for "Healthy" Foods-Possibilities and Challenges. Comprehensive Reviews in Food Science and Food Safety 9:655-675.

Chaudhary S, Van Rooijen G, Moloney M, Singh S. 2001. Flax seed specific promoters. International Patent Application WO 2001/16340.

Chen, HQ, Gu, ZN, Zhang, H, Wang, MX, Chen, W, Lowther, WT, Chen, YQ. 2013. Expression and purification of integral membrane fatty acid desaturases. PLoS ONE 8:e58139.

Chèvre AM, Eber F, Darmency H, Fleury A, Picault H, Letanneur JC, Renard M. 2000. Assessment of interspecific hybridization between transgenic oilseed rape and wild radish under normal agronomic conditions. Theoretical and Applied Genetics 100**:** 1233-1239.

Chèvre AM, Eber F, Renard M, Darmency H. 1999. Gene flow from oilseed rape to weeds. In Lutman, PJW, ed, Geneflow and Agriculture: Relevance for Transgenic Crops. British Crop Protection Council 1999 Symposium Proceedings No. 72, Farnham, UK, pp 125-130.

Cho, HP, Nakamura, MT, Clarke, SD. 1999. Cloning, expression, and nutritional regulation of the mammalian Δ-6 desaturase. The Journal of Biological Chemistry 274: 471-477.

Choi Y, Ahn JH, Choi YD, Lee JS. 1995. Tissue-specific and developmental regulation of a gene encoding a low molecular weight sulfur-rich protein in soybean seeds. Molecular and General Genetics. 246(2):266-268.

CODEX Alimentarius Commission. 2003. Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Twenty-Fifth Session, Rome, Italy 30 June-5 July, 2003. Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants and Appendix IV, Annex on the assessment of possible allergenicity: 47-60.

Coutu C, Brandle J, Brown D, Brown K, Miki B. 2007. pORE: a modular binary vector series suited for both monocot and dicot plant transformation. Transgenic Research 16: 771-781.

Crawley, M.J., S.L. Brown, R.S. Hails, D.D. Kohn M. Rees. 2001. Biotechnology - Transgenic crops in natural habitats. Nature 409: 682-683.

Devos, Y., A. De Schrijver D. Reheul. 2009. Quantifying the introgressive hybridisation propensity between transgenic oilseed rape and its wild/weedy relatives. Environmental Monitoring and Assessment 149: 303-322.

Domergue, F, Lerchi, J, Zähringer, U, Heinz, E. 2002. Cloning and functional characterization of *Phaeodactylum tricornutum* front-end desaturases involved in eicosapentaenoic acid biosynthesis. European Journal of Biochemistry 269:4105-4113.

Domergue, F, Spiekermann, P, Lerchl, J, Beckmann, C, Kilian, O, Kroth, PG, Boland, W, Zahringer, U, Heinz, E. 2003. New insight into *Phaeodactylum tricornutum* fatty acid metabolism. Cloning and functional characterization of plastidial and microsomal delta12-fatty acid desaturases. Plant Physiology 131:1648-1660.

Domergue, F, Abbadi, A, Zahringer, U, Moreau, H, Heinz, E. 2005. In vivo characterization of the first acyl-CoA Delta6-desaturase from a member of the plant kingdom, the microalga *Ostreococcus tauri*. Biochemical Journal 389:483-490.

Dröge, W, Broer, I, Pühler, A. 1992. Transgenic plants containing the phosphinothricin-N-acetyltransferase gene metabolize the herbicide L-phosphinothricin (glufosinate) differently from untransformed plants. Planta 187:142–151.

Dyall, SC. 2015. Long-chain omega-3 fatty acids and the brain: a review of the independent and shared effects of EPA, DPA and DHA, Frontiers in Aging Neuroscience. 7(52):1-15.

EFSA, 2014. Scientific Opinion on the extension of use for DHA and EPA-rich algal oil from Schizochytrium sp. EFSA J doi: 10.2903/j.efsa.2014.3843.

Eiamsa-ard, P, Kanjana-Opas, A, Cahoon, EB, Chodok, P, Kaewsuwan, S. 2013. Two novel *Physcomitrella patens* fatty acid elongases (ELOs): identification and functional characterization. Applied Microbiology and Biotechnology 97: 3485-3497.

Ellstrand NC. 2003. Current knowledge of gene flow in plants: implications for transgene flow. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 358: 1163–1170.

Fan, Y-Y and Chapkin, RS. 1998. Importance of dietary gamma-linolenic acid in human health and nutrition. The Journal of Nutrition 128:1411-1414.

FARRP, 2016. Allergen database, version 16. University of Nebraska, Food Allergy Research and Resource Program, Lincoln, Nebraska. http://www.allergenonline.org/.

FitzJohn, RG, Armstrong, TA, Newstrom, LE, Wilton, AD, Cochrane, M. 2007. Hybridisation within *Brassica* and allied genera: evaluation of potential for transgene escape. Euphytica on-line.

Gallie DR, Sleat DE, Watts JW, Turner PC, Wilson TMA. 1987. A comparison of eukaryotic viral 59-leader sequences as enhancers of mRNA expression in vivo. Nucleic Acids Research 15:8693–8711.

Gantar, M. and Svirčev, Z. 2008. Microalgae and cyanobacteria: Food for thought. Journal of Phycology 44:260-268.

Gillette, MA and Carr, SA. 2013. Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. Nature Methods 10: 28-34.

Gilmour, AR, Gogel, BJ, Cullis, BR and Thompson, R. 2009. ASREML user guide, release 3.0, Biometric bulletin, no 3, VSN International Ltd, The Waterhouse, Waterhouse St, Hemel Hempstead, UK HP1 1ES.

Girke, T, Schmidt, H, Zahringer, U, Reski, R, Heinz, E. 1998. Identification of a novel Δ6-acyl-group desaturase by targeted gene disruption in *Physcomitrella patens*. Plant Journal 15: 39-48.

Gogus, U and Smith, C. 2010. n-3 Omega fatty acids: a review of current knowledge, International Journal of Food Science and Technology 45:417-436.

Gouveia, L, Coutinho, C, Mendonça, E, Batista, AP, Sousa, I, Bandarra, NM, Raymundo, A. 2008. Functional biscuits with PUFA-ω3 from *Isochrysis galbana*. Journal of the Science of Food and Agriculture 88: 891-896.

Gruber, S, Pekrun, C, Claupein, W. 2004. Seed persistence of oilseed rape (*Brassica napus*): Variation in transgenic and conventionally bred cultivars. Journal of Agricultural Science 142: 29-40.

Gulden, RH, Thomas, AG, Shirtliffe, SJ. 2004. Secondary dormancy, temperature, and burial depth regulate seedbank dynamics in canola. Weed Science 52: 382-388.

Hall, G, Jr., Allen, GC, Loer, DS, Thompson, WF, Spiker, S. 1991. Nuclear scaffolds and scaffold-attachment regions in higher plants. Proceeding from the National Academy of Sciences U.S.A. 88:9320–9324.

Hall, LM, Rahman, MH, Gulden, RH, Thomas, AG. 2005. Volunteer oilseed rape – will herbicide resistance traits assist ferality? Chapter 5. In: Crop Ferality and Volunteerism. Gressel, J, ed. OECD: 59-79.

Halweg C, Thompson W.F., Spiker S. 2005. The Rb7 matrix attachment region increases the likelihood and magnitude of transgene expression in tobacco cells: a flow cytometric study. Plant Cell 17:418–429.

Heidari, MR, Azad, EM, Mehrabani, M. 2006. Evaluation of the analgesic effect of *Echium amoenum* Fisch and C.A. Mey. extract in mice: Possible mechanism involved. Journal of Ethnopharmacology 103:345-349.

Hérouet, C, Esdaile, D.J., Mallyon, B.A., Debruyne, E, Schulz, A, Currier, T, Hendrickx, K, van der Klis, R-J, Rouan, D. 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the pat and bar sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regulatory Toxicology and Pharmacology 41:134-149.

Holm-Šídák. 2017. The Holm-Šídák approach to multiple comparisons. GraphPad Statistics Guide; The Holm-Šídák test in Prism.

Hoyle, M, Hayter, K, Cresswell, JE. 2007. Effect of pollinator abundance on self-fertilization and gene flow: application to GM canola. Ecological Applications 17: 2123-2135.

Huang, YS, Chaudhary, S, Thurmond, JM, Bobik, EG, Jr., Yuan, L, Chan, GM, Kirchner, SJ, Mukerji, P, Knutzon, DS. 1999. Cloning of delta12- and delta6-desaturases from *Mortierella alpina* and recombinant production of gamma-linolenic acid in *Saccharomyces cerevisiae*. Lipids 34: 649-659.

Janmohammadi, M, Fallahnezhad, Y, Golshan, M Mohammadi, H. 2008. Controlled ageing for storability assessment and predicting seedling early growth of canola cultivars (*Brassica napus* L.) Journal of Agriculture and Biological Sciences 3:22-26.

Jørgensen RB, Andersen B, Hauser TP, Landbo L, Mikkelsen TR, Østergård H. 1998. Introgression of crop genes from oilseed rape (*Brassica napus*) to related wild species - an avenue for the escape of engineered genes. Acta Horticulturae 459: 211-217.

Jørgensen RB, Andersen B, Landbo L, Mikkelsen TR. 1996. Spontaneous hybridization between oilseed rape (*Brassica napus*) and weedy relatives. Acta Horticulturae 407: 193-200.

Jørgensen RB, Hauser T, Landbo L, Mikkelsen TR, Østergård H. 1999. *Brassica napus* and *B. campestris*: spontaneous hybridisation, back-crossing and fitness components of offspring plants. In Environmental Impact of Genetically Modified Crops, Research Report No. 10. Department of the Environment, Transport and the Regions, London, pp 20-27.

Kajikawa, M, Yamato, KT, Kohzu, Y, Nojiri, M, Sakuradani, E, Shimizu, S, Sakai, Y, Fukuzawa, H, Ohyama, K. 2004. Isolation and characterization of D6-desaturase, an ELO-like enzyme and D5-desaturase from the liverwort *Marchantia polymorpha* and production of arachidonic and eicosapentaenoic acids in the methylotrophic yeast *Pichia pastoris*. Plant Molecular Biology 4: 335-352.

Kajikawa, M, Yamato, KT, Sakai, Y, Fukuzawa, H, Ohyama, K, Kohchi, T. 2006. Isolation and functional characterization of fatty acid Δ5-elongase gene from the liverwort Marchantia polymorpha L. FEBS Microbiolology Letters 580: 149-154.

Kang, HK, Lee, JH, Kim, D, Day, DF, Robyt, JF, Park, K-H, Moon, T-W. 2004. Cloning and expression of *Lipomyces starkeyi* α-amylase in *Escherichia coli* and determination of some of its properties. FEMS Microbiology Letters 233: 53-64.

Kay, R, Chan, A, Daly, M McPherson, J. 1987. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236:1299-1302.

Kereszt A, Li D.X., Indrasumunar A, Nguyen C.D.T., Nontachaiyapoom S, Kinkema M, Gresshoff PM. 2007. Agrobacterium rhizogenes-mediated transformation of soybean to study root biology. Nature Protocols 2:948-952.

Knispel, A.L., S.M. McLachlan, R.C. Van Acker L.F. Friesen. 2008. Gene flow and multiple herbicide resistance in escaped canola populations. Weed Science 56: 72-80.

Kurtzman, C.P. 2003. Phylogenetic circumscription of Saccharomyces, Kluyveromyces and other member of the Saccharomycetacea, and the proposal of the new genera Lachancea, Nakaseomyces, Naumovia, Vanderwaltozyma and Zygotorulaspora. FEMS Yeast Research 4:233-245.

Lazo G.R., Stein PA, Ludwig R.A. 1991. A DNA transformation competent Arabidopsis genomic library in Agrobacterium. Biotechnology 9: 963-967.

Leonard, AE, Bobik, EG, Dorado, J, Kroeger, PE, Chuang, L-T, Thurmond, JM, Parker-Barnes, JM, Das, T, Huang, Y-S, Mukerji, P. 2000. Cloning of a human cDNA encoding a novel enzyme involved in the elongation of long-chain polyunsaturated fatty acids. Biochemistry Journal 350: 765-770.

Leonard, AE, Pereira, SL, Sprecher, H, Huang, YS. 2004. Elongation of long-chain fatty acids. Progress in Lipid Research 43: 36-54.

Liang, S, Liu, X, Chen, F, Chen, Z. 2004. Current microalgal health food R & D activities in China. Hydrobiology 512:45-48.

Liu, H, Guo, Z, Zheng, H, Wang, S, Wang, Y, Liu, W, Zhang, G. 2014. Functional characterization of a Δ5-like fatty acyl desaturase and its expression during early embryogenesis in the noble scallop *Chlamys nobilis* Reeve. Molecular Biology Reports 41: 74376-7445.

Livore, VI, Tripodi, KEJ, Uttaro, AD. 2007. Elongation of polyunsaturated fatty acids in trypanosomatids. FEBS J 274: 264-274.

Logan, AC. 2004. Omega-3 fatty acids and major depression: a primer for the mental health professional, Lipids in Health and Disease 3(1):25.

Matsuda, T, Sakaguchi, K, Hamaguchi, R, Kobayashi, T, Abe, E, Hama, Y, Hayashi, M, Honda, D, Okita, Y, Sugimoto, S, Okino, N, Ito, M. 2012. Analysis of Δ12-fatty acid desaturase function revealed that two distinct pathways are active for the synthesis of PUFAs in *T. aureum* ATCC 34304. The Journal of Lipid Research 53:1210-1222.

Meesapyodsuk, D, Qiu, X. 2016. Biosynthetic mechanism of very long chain polyunsaturated fatty acids in *Thraustochytrium* sp. 26185. The Journal of Lipid Research DOI:10.1194/jlr.M070136.

Meyer, A, Kirsch, H, Domergue, F, Abbadi, A, Sperling, P, Bauer, J, Cirpus, P, Zank, TK, Moreau, H, Roscoe, TJ, Zahringer, U, Heinz, E. 2004. Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. The Journal of Lipid Research 45: 1899-1909.

Louise V. Michaelson, L. Lazarus, CM, Griffiths, G, Napieri, JA, Stobart, AK. 1998. Isolation of a Δ5-Fatty Acid Desaturase Gene from *Mortierella alpine*. The Journal of Biological Chemistry 273(30): 19055-19059.

Miloševic M, Vujakovic, M, Karagic D. 2010. Vigour tests as indicators of seed viability. Genetika, Vol 42, No. 1: 103-118.

Moyes, CL, Lilley, JM, Casais, CA, Cole, SG, Haeger, PD, Dale, PJ. 2002. Barriers to gene flow from oilseed rape (*Brassica napus*) into populations of *Sinapis arvensis*. Molecular Ecology, 11: 103–112.

Myers, J. 2006. Outcrossing Potential for Brassica Species and Implications for Vegetable Crucifer Seed Crops of Growing Oilseed Brassicas in the Willamette Valley. Oregon State University Extension Service Special Report 1064.

Napier, JA, Hey, SJ, Lacey, DJ, Shewry, PR. 1998. Identification of a Caenorhabditis elegans Δ6-fatty-acid-desaturase by heterologous expression in Saccharomyces cerevisiae. Biochemistry Journal 330 (Pt 2): 611-614.

Nichols PD, Petrie J, Singh S. 2010. Long-chain omega-3 oils - An update on sustainable sources https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3257669/

OECD. 2011. Revised consensus document on compositional considerations for new varieties of low erucic acid rapeseed (canola): Key food and feed nutrients, anti-nutrients and toxicants. Series on the safety of novel foods and feeds No. 24. ENV/JM/MONO(2011)55. OECD, Paris.

OECD. 2012. Consensus document on the biology of the Brassica crops (Brassica spp.). Series on Harmonisation of Regulatory Oversight in Biotechnology No. 54.

OGTR. 2002. The biology and ecology of canola (*Brassica napus*). http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/canola-3/$FILE/brassica.pdf.

OGTR. 2017. The Biology of *Brassica napus* L. (canola) and *Brassica juncea* (L.) Czern. & Coss. (Indian mustard). Version 2.1: February 2017.

Okuley, J, Lightner, J, Feldmann, K, Yadav, N, Lark, E, Browse, J. 1994. Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell 6:147-158.

Pereira, SL, Huang, Y-S, Bobik, EG, Kinney, AJ, Stecca, KL, Packer, JCL, Mukerji, P. 2004. A novel omega3-fatty acid desaturase involved in the biosynthesis of eicosapentaenoic acid. Biochemistry Journal 378: 665-671.

Petrie, JR, Liu, Q, Mackenzie, AM, Shrestha, P, Mansour, MP, Robert, SS, Frampton, DF, Blackburn, SI, Nichols, PD, Singh, SP. 2010a. Isolation and characterisation of a high-efficiency desaturase and elongases from microalgae for transgenic LC-PUFA production. Marine Biotechnology 12:430-438.

Petrie, JR, Shrestha, P, Belide, S, Kennedy, Y, Lester, G, Liu, Q, Divi, UK, Mulder, R.J., Mansour, M.P., Nichols, P.D., Singh, S.P. 2014. Metabolic engineering *Camelina sativa* with fish oil-like levels of DHA. PLoS One 9:e85061.

Petrie, JR, Shrestha, P, Mansour, MP, Nichols, PD, Liu, Q, Singh, SP. 2010b. Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants using an acyl-CoA Delta 6-desaturase with omega 3-preference from the marine microalga *Micromonas pusilla*. Metabolic Engineering 12:233-240.

Petrie JR, Shrestha P, Zhou XR, Mansour MP, Liu Q, Belide S, Nichols PD, Singh SP. 2012. Metabolic Engineering Plant Seeds with Fish Oil-Like Levels of DHA. PLoS ONE 7:e49165. doi:10.1371/journal.pone.0049165

Peyou-Ndi, MM., Watts, JL, Browse, J. 2000. Identification and characterization of an animal Δ12 fatty acid desaturase gene by heterologous expression in *Saccharomyces cerevisiae*. Archives of Biochemistry and Biophysics 376:399-408.

Rakow, G, and Woods, DL. 1987. Outcrossing in rape and mustard under Saskatchewan prairie conditions. Canadian Journal of Plant Science 67: 147-151.

Rauh, M. 2012. LC–MS/MS for protein and peptide quantification in clinical chemistry. Journal of Chromatography B, 883–884: 59-67.

Reddy, AS, Nuccio, ML, Gross, LM, Thomas, TL. 1993. Isolation of a Δ6-desaturase gene from the cyanobacterium *Synechocystis* sp. strain PCC 6803 by gain-of-function expression in Anabaena sp. strain PCC 7120. Plant Molecular Biology 22: 293-300.

Robert, SS, Singh, SP, Zhou, X-R, Blackburn, SI, Mansour, PM, Nichols, PD, Liu, Q, Green, AG. 2005. Metabolic engineering of Arabidopsis to produce nutritionally important DHA in seed oil. Functional Plant Biology 32:473-479.

Rogers, SG, O'Connell, K, Horsch, RB, Fraley, RT. 1985. In: Biotechnology in Plant Science, eds, Zaitlin, M, Day, P, Hollaender, A, Wilson, CA, Academic Press, Inc., New York, NY: 219-226.

Rossak M, Smith M, Kunst L. 2001. Expression of the FAE1 gene and FAE1 promoter activity in developing seeds of Arabidopsis thaliana. Plant Molecular Biology 46:717-725.

Saito, T, Morio, T, Ochiai, H. 2000. A second functional Δ5 fatty acid desaturase in the cellular slime mould *Dictyostelium discoideum*. European Journal of Biochemistry 267: 1813-1818.

Sakamoto, T, Los, DA, Higashi, S, Wada, H, Nishida, I, Ohmori, M, Murata, N. 1994. Cloning of omega 3 desaturase from cyanobacteria and its use in altering the degree of membrane-lipid unsaturation. Plant Molecular Biology 26: 249-263.

Sakuradani, E, Kobayashi, M, Ashikari, T, Shimizu, S. 1999. Identification of delta12-fatty acid desaturase from arachidonic acid-producing mortierella fungus by heterologous expression in the yeast *Saccharomyces cerevisiae* and the fungus *Aspergillus oryzae*. European Journal of Biochemistry 261:812-820.

Sakuradani, E, Shimizu, S. 2009. Single cell oil production by *Mortierella alpina*. Journal of Biotechnology 144:31-36.

Salisbury, PA. 2002. Gene flow between *Brassica napus* and other Brassicaceae species. PAS0201, 1-45. Unpublished, Institute of Land and Food Resources, University of Melbourne.

Sanders, PR, Winter, JA, Bamason, AR, Rogers, SG, Fraley, RT. 1987. Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucleic Acids Research. 15:1543-1558.

Santos MAO, Novembre ADLC, Marcos-Filho J. 2007. Tetrazolium test to assess viability and vigour of tomato seeds. Seed Science and Technology 35:213-223.

SAS Institute. 2017. What's New in SAS® 9.4. SAS Documentation, June 2017.

Sayanova, O, Smith, MA, Lapinskas, P, Stobart, AK, Dobson, G, Christie, WW, Shewry, PR, Napier, JA. 1997. Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of delta-6 desaturated fatty acids in transgenic tobacco. Proceedings of the National Academy of Sciences U.S.A. 94: 4211-4216.

Sayanova, O, Haslam, R, Guschina, I, Lloyd, D, Christie, W.W., Harwood, J.L., Napier, J.A. 2006. A bifunctional Δ12, Δ15-desaturase from *Acanthamoeba castellanii* directs the synthesis of highly unusual n-1 series unsaturated fatty acids. Journal of Biological Chemistry 281:36533-36541.

Scott, SE, Wilkinson, MJ. 1998. Transgene risk is low. Nature 320.

Smith, A, Cullis, B, Thompson, R. 2001. Analysing variety by environment data using multiplicative missed modes and adjustments for spatial field trend, Biometrics 57:1138-1147.

Sosnowski, MR, Scott, ES, Ramsey, RD. 2004. Infection of Australian canola cultivars (*Brassica napus*) by *Leptosphaeria maculans* is influenced by cultivar and environmental conditions. Australasian Plant Pathology 33**:**401-411.

Spychalla, JP, Kinney, AJ, Browse, J. 1997. Identification of an animal ω-3 fatty acid desaturase by heterologous expression in Arabidopsis. Proc Natl Acad Sci U.S.A. 94: 1142-1147.

Stalberg K, Ellerström M, Josefsson L, Rask L. 1993. Deletion analysis of a 2S seed storage protein promoter of *Brassica napus* in transgenic tobacco. Plant Molecular Biology 23:671–683.

Stark, KD, Van Elswyk, ME, Higgins, MR, Weatherford, CA, Salem, N. 2016. Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults Progress in Lipid Res 63:132-152.

Tanomman, S, Ketudat-Cairns, M, Jangprai, A, Boonanuntanasarn, S. 2013. Characterization of fatty acid delta-6 desaturase gene in Nile tilapia and heterogenous expression in *Saccharomyces cerevisiae*. Comparative Biochemistry and Physiology - Part B: Biochemistry and Molecular Biology 166: 148-156.

Thomas, K, Aalbers, M, Bannon, GA, Bartels, M, Dearman, RJ, Esdaile, DJ, Fu, TJ, Glatt, CM, Hadeld, N, Hatzos, C, Hee, SL, Heylings, JR, Goodman, RE, Henry, B, Herouet, C, Holsapple, M, Ladics, GS, Landry, TD, MacIntosh, SC, Rice, EA, Privalle, LS, Steiner, HY, Teshima, R, van Ree, R, Woolhiser, M, Zawodny, J. 2004. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. Regulatory Toxicology and Pharmacology 39:87–98.

Thompson CE, Squire G, Mackay GR, Bradshaw JE, Crawford J, Ramsay G. 1999. Regional patterns of gene flow and its consequences for GM oilseed rape. Lutman, PJW, ed.

USDA-APHIS. 1998. Environmental Assessment and Finding of No Significant Impact: Response to AgroEvo. USA Company Petition 97-205-01p for Determination of Nonregulated Status for Glufosinate-ammonium Tolerant Canola Event T45.

USDA-FAS. 2016. Production, Supply, and Distribution Database. oilseeds, rapeseed: https://apps.fas.usda.gov/psdonline/app/index.html.

USDA-ERS. 2017. http://www.ers.usda.gov/data-products/oil-crops-yearbook/oil-crops-yearbook/#World Supply and Use of Oilseeds and Oilseed Products.

USDA-GIPSA, 1992. US Official Standards for Grain, Canola. https://www.gipsa.usda.gov/fgis/standards/810canola.pdf

USDA Plants Database. 2017. https://plants.usda.gov/core/profile?symbol=SIAR4.

Vagner, M, Santigosa, E. 2011. Characterization and modulation of gene expression and enzymatic activity of delta-6 desaturase in teleosts: A review. Aquaculture 315: 131-143.

Van De Wouw, A.P., Marcroft, S.J., Howlett, B.J. 2016. Blackleg Disease of Canola in Australia. Crop and Pasture Science 67(3-4): 273-283.

Vodkin L.O., Rhodes P.R., Goldberg R.B. 1983. cA lectin gene insertion has the structural features of a transposable element. Cell 34 (3):1023-1031.

Warwick, SI, Beckie, HJ, Hall, LM. 2009. Gene flow, invasiveness, and ecological impact of genetically modified crops. Annals of the New York Academy of Sciences 1168: 72–99.

Watanabe, K, Oura, T, Sakai, H, Kajiwara, S. 2004. Yeast Δ12 fatty acid desaturase: Gene cloning, expression, and function. Bioscience, Biotechnology, and Biochemistry 68:721-727.

Webb, E.C. 1992. Enzyme Nomenclature: 178-199. Academic Press, New York.

Wehrmann, A, Van Vliet, A, Opsomer, C, Botterman, J, Schulz, A. 1996. The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. Nature Biotechnology 14:1274-1278.

Welham, SJ, Gogel, BJ, Kelly, A, Cullis, BR, Butler, DG, Thompson, R. 2013. Analysis of linear missed models by AS Reml-R with Applications in plant Plant Breeding: Course Notes, VSN International.

Wood, C, Petrie, J, Shrestha, P, Mansour, M, Nichols, P, Green, A, Singh, S. 2009. A leaf-based assay using interchangeable design principles to rapidly assemble multistep recombinant pathways. Plant Biotechnology Journal 7: 914-924.

Yang, J, Liu, D. Wang, X, Ji, C, Cheng, F, Liu, B, Hu, Z, Chen, S, Pental, D, Ju, Y, Yao, P, Li, X, Xie, K, Zhang, J, Wang, J, Liu, F, Ma, W, Shopan, J, Zheng, H, Mackenzie, S, Zhang, M. 2016. The genome sequence of allopolyploid *Brassica juncea* and analysis of differential homoeolog gene expression influencing selection. Nature Genetics 48(10): 1225-1234.

Yurko-Mauro, K, Alexander, DD, Van Elswyk, ME. 2015. Docosahexaenoic acid and adult memory: a systematic review and meta-analysis. PLoS One doi:0.1371/journal.pone.0120391.

Zhang, X, Li, M, Wei, D, Xing, L. 2008. Identification and characterization of a novel yeast ω3-fatty acid desaturase acting on long-chain n-6 fatty acid substrates from *Pichia pastoris*. Yeast 25: 21-27.

Zhou, X-R, Green, AG, Singh, SP. 2011. *Caenorhabditis elegans* Δ12-desaturase FAT-2 is a bifunctional desaturase able to desaturate a diverse range of fatty acid substrates at the Δ12 and Δ15 positions. The Journal of Biological Chemistry 286:43644-43650.

Zhou, X-R, Robert, Singh, SP., Petrie, JR, Frampton, DMF, Mansour, MP, Blackburn, SI, Nichols, PD, Green, AG, Singh, SP. 2007. Isolation and characterization of genes from the marine microalga *Pavlova salina* encoding three front-end desaturases involved in docosahexaenoic acid biosynthesis. Phytochemistry 68:785-796.

Zhou, X-R, Robert, S, Singh, SP, Green, A. 2006. Heterologous production of GLA and SDA by expression of an *Echium plantagineum* Δ6-desaturase gene. *Plant Sci* 170:665-673.

Zhou, X-R, Horne, I, Damcevski, K, Haritos, V, Green, A, Singh, S. 2008. Isolation and functional characterization of two independently-evolved fatty acid Δ12-desaturase genes from insects. Insect Molecular Biology, 17: 667–676.

Appendix A: Materials and Methods for Protein Analyses

I. *LACHANCEA KLUYVERI* Δ12-DESATURASE

A. MATERIALS

(1) TARGET PROTEIN

The Δ12-desaturase gene used in DHA canola event was previously cloned from yeast *L. kluyveri* (Watanabe et al. 2004). The Lackl-Δ12D protein was expressed as native sequence in Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie, 2014), as well as in yeast *P. pastoris* cell as His-tag fusions with or without a secretion peptide. The His-tag fusion vectors contained a coding sequence encoding a His-tag (His_{10}) and a PreScission protease cleavage site (SLEVLFQ↓GP) fused to the codon optimized *Lackl-Δ12D* gene.

(2) OTHER MATERIALS

The Lackl-Δ12D gene was synthesized at GeneArt (Life Science Technologies, Germany), according to the sequence in NCBI database under accession AB115968 as a His-tag fusion with or without Pichia secretion peptide (SP), and cloned into the Pichia expression vector pPinkα-HC (Invitrogen, Carlsbad, CA, USA).

B. METHODS

(1) SEQUENCE COMPARISON

The Lackl-Δ12D gene was previously cloned from yeast *L. kluyveri* (Watanabe et al. 2004). The translated amino acid sequence was compared to other published Δ12-desaturases or related fatty acid desaturase presented in food or used in food production, with Vector NTI software (Version 11, Invitrogen).

(2) TRANSFORMATION OF PICHIA CELL

Pichia transformation was essentially done according to the published protocol (Chen et al., 2013). Pichia expression vector DNA containing *Lackl-Δ12D* gene was first linearized by single restriction enzyme digestion in vector backbone, and precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol overnight at -20ºC. The precipitated DNA was resuspended in 10 µL of Milli-Q™ (MQ) water for yeast transformation. The yeast PichiaPink™ strain 4 (Invitrogen) was first activated from the stab culture on a fresh Yeast extract-Peptone-Dextrose (YPD) plate at 28ºC for 3 to 4 days. Single colony was inoculated in 10 mL of YPD medium and cultured at 28ºC with shaking (200 rpm) for 24 hours, followed by inoculating 100 mL of culture to OD_{600}=0.2 from the previous 10 mL culture and allowed to grow at 28ºC for 8 to 10 hrs until OD_{600}=1.0 to 1.5. The cells were spun down at 3000 rpm for 10 min and washed with 100 mL chilled MQ water, followed by washing with 50 mL of chilled MQ water then by 10 mL of cold

1 M sorbitol. The cells were resuspended in 300 µL of 1 M sorbitol and dispensed into 80 uL aliquots in Eppendorf tubes. The prepared Pichia competent cells were mixed with 10 µL of linearized DNA, incubated on ice for 5 min and electroporated. After electroporation, the cells were recovered from electroporation cuvettes with 1 mL of YPD with sorbitol (Invitrogen), incubated at 28ºC without shaking for 4 hrs, then plated out on Pichia Adenine Dropout Dextrose (Invitrogen) plate and incubate at 28ºC for 2-4 days. White yeast colonies were selected for further analysis.

(3) ENZYME ACTIVITY ANALYSIS IN PICHIA CELL

Individual white colonies of Pichia were inoculated into 10 mL Buffered Glycerol-complex Medium (BMGY, 1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% Yeast Nitrogen Base; 0.0004% biotin; 1% glycerol) in 50 ml Falcon tubes and cultured for 48 hrs at 28ºC with shaking at 250 rpm. From each sample, 2.5 mL of the above culture were inoculated into 50 mL of BMGY medium in 250 mL flasks and grown at 28ºC for 24 hrs at 250 rpm. Yeast cells were collected by centrifugation at 3000 rpm for 10 min and resuspended in 5 mL induction medium (BMMY, 1% yeast extract; 2% peptone; 100 mM potassium phosphate, pH 6.0; 1.34% Yeast Nitrogen Base; 0.0004% biotin; 0.5% methanol) at 28ºC for 3 days, by adding 50 uL of methanol to the culture every 24 hrs. In the case of fatty acid substrate feeding was needed, the fatty acid was added to the final concentration of 0.5 mM with 1% of detergent NP-40 in BMMY medium. The cells were harvested by centrifuging at 3000 rpm for 5 min, and washed with 1% NP-40, 0.5% NP-40 then water as described (Zhou et al. 2006).

(4) FATTY ACID ANALYSIS

For fatty acid analysis, cells were dried overnight with a freezing-vacuum dryer. Fatty acid methyl esters (FAME) were prepared with 0.75 mL of 1N methanolic-HCl at 80ºC for 3 hrs, and extracted with 0.5 mL 0.9% NaCl followed by 0.3 mL of hexane after cooling down to room temperature. The hexane phase containing FAMEs were recovered after centrifuging at 3000 rpm for 5 min, transferred to GC vials, dried down to 30 µL with nitrogen. GC analysis was done according to previously described (Zhou et al. 2011).

C. RESULTS AND DISCUSSION

This section includes additional information to the main text of this petition.

(1) PHYLOGENETIC TREE

The fatty acid Δ12-desaturases have been cloned from a wide range of organisms, including traustochytrid (Matsuda et al. 2012), diatom (Domergue et al. 2003), fungus (Sakuradani et al. 1999), plant (Okuley et al. 1994), nematode (Peyou-Ndi et al. 2000), insect (Zhou et al. 2008). The Lackl-Δ12D shared high homology to other Δ12-desaturase proteins as shown in Figure A-1.

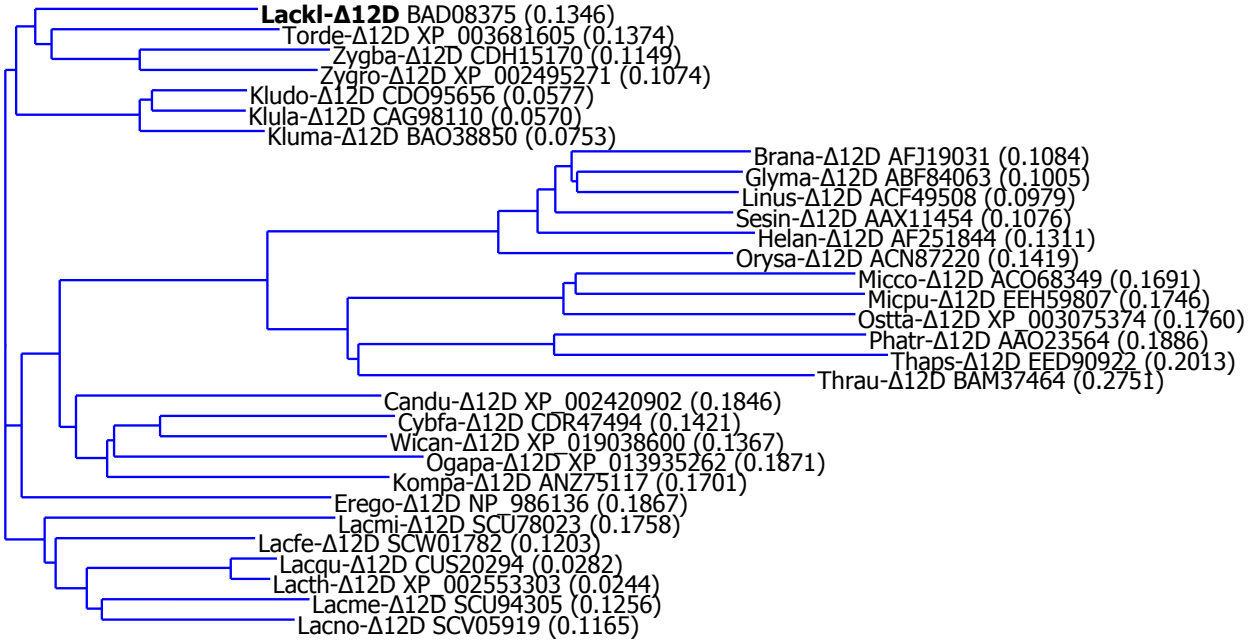

Figure A-1. Phylogenetic tree for sequence comparison of Lackl-Δ12D with representative Δ12-desaturases

The phylogenetic tree was generated with Vector NTI software (Invitrogen, Carlsbad, USA). The protein sequences were named as 5-letter initial of species name, followed by NCBI accession numbers. Brana, *Brassica napus* (canola); Candu, *Candida dubliniensis* (fungus); Cybfa, *Cyberlindnera fabianii* (fungus); Erego, *Eremothecium gossypii* (fungus); Glyma, *Glycine max* (soy bean); Helan, *Helianthus annuus* (sunflower); Linus, *Linum usitatissimum* (flax); Kludo, *Kluyveromyces dobzhanskii* (fungus); Klula, *K. lactis* (fungus); Kluma, *K. marxianus* (fungus); Kompa, *Komagataella pastoris* (fungus); Lacfe, *Lachancea fermentati* (fungus); Lackl, *Lachancea kluyveri* (fungus); Lacme, *L. meyersii* (fungus); Lacmi, *L. mirantina* (fungus); Lacno, *L. nothofagi* (fungus); Lacqu, *L. quebecensis* (fungus); Lacth, *L. thermotolerans* (fungus); Micco, *Micromonas commode* (alga); Micpu, *M. pusilla* (alga); Ogapa, *Ogataea parapolymorpha* (fungus); Orysa, *O. sativa* (rice); Ostta, *Ostreococcus tauri* (alga); Phatr, *P. tricornutum* (diatom); Sesin, *S. indicum* (sesame); Thaps, *T. pseudonana* (alga); Thrau, *T. aureum* (protist); Torde, *Torulaspora delbrueckii* (fungus); Wican, *Wickerhamomyces anomalus* (fungus); Zybga, *Z. bailii* (fungus); Zygro, *Z. rouxii* (fungus).

(2) HETEROLOGOUS EXPRESSION

The enzyme functionality of Lackl-Δ12D has been confirmed in different heterologous expression systems, including yeast (Watanabe et al. 2004), Arabidopsis seed (Petrie et al., 2012) and Camelina seed (Petrie et al., 2014). In this study, Lackl-Δ12D was expressed in *P. pastoris*, as fusion proteins designated as SP::His_{10}::Lackl-Δ12D or His_{10}::Lackl-Δ12D. In SP::His_{10}::Lackl-Δ12D, the Lackl-Δ12D sequence was fused to *Saccharomyces cerevisiae α*-mating type signal peptide as secretion peptide, followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure A-2). In His_{10}::Lackl-Δ12D, the Lackl-Δ12D sequence was fused to His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure A-3). No SP was used in His_{10}::Lackl-Δ12D.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSN
STNNGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMSAVTVTGS
DPKNRGSSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAIPHECYERRLATSLYYV
FRDIFCMLTTGYLTHKILYPLLISYTSNSIIKFTFWALYTYVQGLFGTGIWVLAHEC
GHQAFSDYGIVNDFVGWTLHSYLMVPYFSWKYSHGKHHKATGHMTRDMVFVPATKEE
FKKSRNFFGNLAEYSEDSPLRTLYELLVQQLGGWIAYLFVNVTGQPYPDVPSWKWNH
FWLTSPLFEQRDALYIFLSDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWVNHWL
VFITFLQHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFHDIIETHVLHHYCS
RIPFYNARPASEAIKKVMGKHYRSSDENMWKSLWKSFRSCQYVDGDNGVLMFRNINN
CGVGAAEK

Figure A-2. Amino acid sequence of SP::His10::Lackl-Δ12D

Lackl-Δ12D was expressed in *P. pastoris*, fused to secretion peptide (SP, underlined), followed by His-tag (His_{10}, double underlined) and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

MRPHHHHHHHHHHSLEVLFQGPMSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNV
FSVPDFTIKDILGAIPHECYERRLATSLYYVFRDIFCMLTTGYLTHKILYPLLISYT
SNSIIKFTFWALYTYVQGLFGTGIWVLAHECGHQAFSDYGIVNDFVGWTLHSYLMVP
YFSWKYSHGKHHKATGHMTRDMVFVPATKEEFKKSRNFFGNLAEYSEDSPLRTLYEL
LVQQLGGWIAYLFVNVTGQPYPDVPSWKWNHFWLTSPLFEQRDALYIFLSDLGILTQ
GIVLTLWYKKFGGWSLFINWFVPYIWVNHWLVFITFLQHTDPTMPHYNAEEWTFAKG
AAATIDRKFGFIGPHIFHDIIETHVLHHYCSRIPFYNARPASEAIKKVMGKHYRSSD
ENMWKSLWKSFRSCQYVDGDNGVLMFRNINNCGVGAAEK

Figure A-3. Amino acid sequence of His10::Lackl-Δ12D

Lackl-Δ12D was expressed in *P. pastoris*, fused to His-tag (His10, double underlined), and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

II. *Pichia pastora* ω3-Desaturase

A. MATERIALS

(1) TARGET PROTEIN

The ω3-desaturase gene used in DHA canola event was previously cloned from yeast *P. pastoris* (Zhang et al. 2008). The Picpa-ω3D protein was expressed as native sequence in Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie, 2014), as well as in yeast *P. pastoris* cell as His-tag fusions with or without *Saccharomyces cerevisiae* α-mating type signal peptide as secretion peptide. The His-tag fusion vectors contained a coding sequences encoding a His-tag (His_{10}) and a PreScission protease cleavage site (SLEVLFQ↓GP) fused to the codon optimized *Picpa-ω3D* gene.

(2) OTHER MATERIALS

The *Picpa-ω3D* gene was synthesized at GeneArt (Life Science Technologies, Germany), according to sequence in NCBI database under accession EF116884 as a His-tag fusion with or without Pichia secretion peptide, and cloned into the Pichia expression vector pPinkα-HC (Invitrogen, Carlsbad, CA, USA).

B. METHODS

(1) SEQUENCE COMPARISON

The *Picpa-ω3D* gene was previously cloned from yeast *P. pastoris* (Zhang et al. 2008). The translated amino acid sequence was compared to other published ω3/Δ15-desaturases or related fatty acid desaturase presented in food or used in food production, with Vector NTI software (Version 11, Invitrogen).

(2) TRANSFORMATION OF PICHIA CELL

Pichia transformation was essentially done according to published protocol (Chen et al., 2013). Pichia expression vector DNA containing *Picpa-ω3D* gene was first linearized by single restriction enzyme digestion in vector backbone, and precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol overnight at -20°C. The precipitated DNA was resuspended in 10 µL of MQ water for yeast transformation. The yeast PichiaPink™ strain 4 (Invitrogen) was first activated from the stab culture on a fresh YPD plate at 28 °C for 3 to 4 days. Single colony was inoculated in 10 mL of YPD medium and cultured at 28°C with shaking (200 rpm) for 24 hours, followed by inoculating 100 mL of culture to OD_{600}=0.2 from the previous 10 mL culture and allowed to grow at 28°C for 8 to 10 hrs until OD_{600}=1.0 to 1.5. The cells were spun down at 3000 rpm for 10 min and washed with 100 mL chilled MQ water, followed by washing with 50 mL of chilled MQ water then by 10 mL of cold 1 M sorbitol. The cells were resuspended in 300 µL of 1 M sorbitol and dispensed into 80 uL aliquots in Eppendorf tubes. The prepared Pichia competent cells were mixed with 10 µL of linearized DNA, incubated on ice for 5 min and electroporated. After electroporation, the cells were recovered from electroporation cuvettes with 1 mL of YPD with sorbitol (Invitrogen), incubated at 28°C without shaking for 4 hrs, then plated out on Pichia Adenine Dropout Dextrose (Invitrogen) plate and incubate at 28°C for 2-4 days. White yeast colonies were selected for further analysis.

(3) ENZYME ACTIVITY ANALYSIS IN PICHIA CELL

Individual white colonies of Pichia were inoculated into 10 mL BMGY in 50 ml Falcon tubes and cultured for 48 hrs at 28ºC with shaking at 250 rpm. From each sample, 2.5 mL of the above culture were inoculated into 50 mL of BMGY medium in 250 mL flasks and grown at 28°C for 24 hrs at 250 rpm. Yeast cells were collected by centrifugation at 3000 rpm for 10 min and resuspended in 5 mL induction medium BMMY at 28°C for 3 days, by adding 50 uL of methanol to the culture

every 24 hrs. In the case of fatty acid substrate feeding was needed, the fatty acid was added to the final concentration of 0.5 mM with 1% of detergent NP-40 in BMMY medium. The cells were harvested by centrifuging at 3000 rpm for 5 min, and washed with 1% NP-40, 0.5% NP-40 then water as described (Zhou et al. 2006).

(4) FATTY ACID ANALYSIS

For fatty acid analysis, cells were dried overnight with freezing-vacuum dryer. FAMEs were prepared with 0.75 mL of 1N methanolic-HCl at 80°C for 3 hrs, and extracted with 0.5 mL 0.9% NaCl followed by 0.3 mL of hexane after cooling down to room temperature. The hexane phase containing FAMEs were recovered after centrifuging at 3000 rpm for 5 min, transferred to GC vials, dried down to 30 µL with nitrogen. GC analysis was done according to previously described (Zhou et al. 2011).

C. RESULTS AND DISCUSSION

(1) PHYLOGENETIC TREE

The fatty acid ω3/Δ15-desaturases have been cloned from a wide range of organisms, including cyanobacteria (Sakamoto et al. 1994), protozoon (Sayanova et al. 2006), thraustochytrid (Meesapyodsuk and Qiu 2016), nematode (Spychalla et al. 1997), plant (Arondel et al. 1992) and fungus (Pereira et al. 2004). The Picpa-ω3D shared high homology to other Δ15-desaturase proteins as shown in Figure A-4.

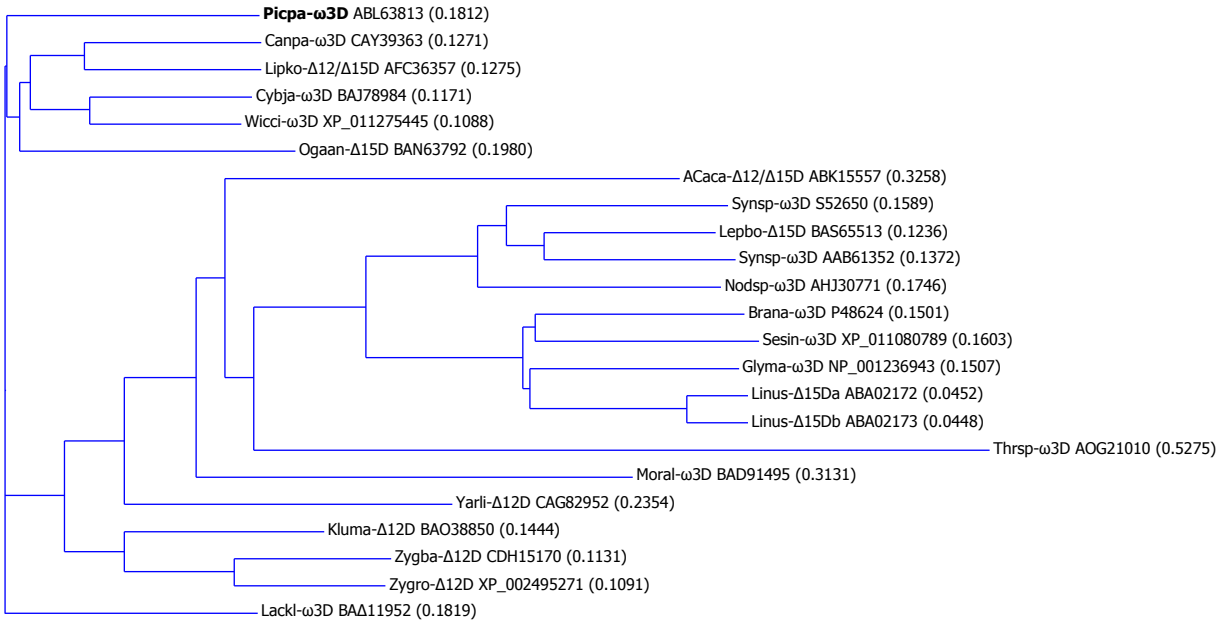

Figure A-4. Phylogenetic tree for sequence comparison of Picpa-ω3D with other FA desaturases

The phylogenetic tree was generated with Vector NTI software (Invitrogen, Carlsbad, USA). The protein sequences were named as 5-letter initial of species name, followed by NCBI accession numbers. Acaca, *Acathamoeba castellanii* (protozoon); Brana, *Brassica napus* (canola); Canpa, *Candida parapsilosis* (fungus); Cybja, *Cyberlindernera jadinii* (fungus); Glyma, *Glycine max* (soybean); Kluma, *Kluyveromyces marxianus* (fungus); Lackl, *Lachancea kluyveri* (fungus); Lepbo, *Leptolyngbya boryana* (cyanobacteria); Linus, *Linum usitatissmum* (flax); Lipko, *Lipomyces kononenkoae* (fungus); Moral, *M. alpina* (fungus); Nodsp, *Nodularia spunigena* (cyanobacteria); Ogaan, *O. angusta* (fungus); Picpa, *Pichia pastoris* (fungus); Sesin, *S. indicum* (sesame); Synsp, *Synechococcus* sp. (cyanobacteria); Thrsp, *T.* sp. (Thraustochytrid); Wicci, *Wickerhamomyces ciferrii* (fungus); Yarli, *Yarrowia lipolytica* (fungus); Zygba, *Z. bailii* (fungus);. Zygro, *Z. rouxii* (fungus). Δ12D, Δ12-desaturase; Δ15D, Δ15-desaturase; ω3D, ω3-desaturase.

(2) HETEROLOGOUS EXPRESSION

The enzyme functionality of Picpa-ω3D have been confirmed in different heterologous expression systems, including yeast (Zhang et al. 2008), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie et al. 2014). In this study, Picpa-ω3D was expressed in *P. pastoris*, as fusion proteins designated as SP::His_{10}::Picpa-ω3D or His_{10}::Picpa-ω3D. In SP::His_{10}::Picpa-ω3D, the Picpa-ω3D sequence was fused to *Saccharomyces cerevisiae α*-mating type signal peptide, followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure A-5). In His_{10}::Picpa-ω3D, the Picpa-ω3D sequence was fused to His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure A-6). No signal peptide was used in His_{10}::Picpa-ω3D.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSN
STNNGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMSKVTVSGS
EILEGSTKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIKDILDAIPKHCYERSLV
KSMSYVVRDIVAISAIAYVGLTYIPLLPNEFLRFAAWSAYVFSISCFGFGIWILGHE
CGHSAFSNYGWVNDTVGWVLHSLVMVPYFSWKFSHAKHHKATGHMTRDMVFVPYTAE
EFKEKHQVTSLHDIAEETPIYSVFALLFQQLGGLSLYLATNATGQPYPGVSKFFKSH
YWPSSPVFDKKDYWYIVLSDLGILATLTSVYTAYKVFGFWPTFITWFCPWILVNHWL
VFVTFLQHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIFHDIIETHVLHHYVSR
IPFYHAREATECIKKVMGEHYRHTDENMWVSLWKTWRSCQFVENHDGVYMFRNCNNV
GVKPKDT

Figure A-5. Amino acid sequence of SP::His10::Picpa- ω3D

Picpa-ω3D was expressed in *P. pastoris*, fused to mating type alpha signal peptide as secretion peptide (SP, underlined), followed by His-tag (His_{10}, double underlined) and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

MRPHHHHHHHHHHSLEVLFQGPMSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAI
DTFGNVFKVPDYTIKDILDAIPKHCYERSLVKSMSYVVRDIVAISAIAYVGLTYIPL
LPNEFLRFAAWSAYVFSISCFGFGIWILGHECGHSAFSNYGWVNDTVGWVLHSLVMV
PYFSWKFSHAKHHKATGHMTRDMVFVPYTAEEFKEKHQVTSLHDIAEETPIYSVFAL
LFQQLGGLSLYLATNATGQPYPGVSKFFKSHYWPSSPVFDKKDYWYIVLSDLGILAT
LTSVYTAYKVFGFWPTFITWFCPWILVNHWLVFVTFLQHTDSSMPHYDAQEWTFAKG
AAATIDREFGILGIIFHDIIETHVLHHYVSRIPFYHAREATECIKKVMGEHYRHTDE
NMWVSLWKTWRSCQFVENHDGVYMFRNCNNVGVKPKDT

Figure A-6. Amino acid sequence of His10::Picpa- ω3D

Picpa-ω3D was expressed in *P. pastoris,* fused to His-tag (His_{10}, double underlined), and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

III. *MICROMONAS PUSILLA* Δ6-DESATURASE

A. MATERIALS

(1) TARGET PROTEIN

The Δ6-desaturase gene used in DHA canola event was previously cloned from microalga *M. pusilla* (Petrie et al., 2010b). The Micpu-Δ6D protein was expressed as native sequence in yeast cell and *N. benthamiana* leaf (Petrie et al. 2010b), Arabidopsis seed (Petrie et al., 2012) and Camelina seed (Petrie, 2014), as well as in yeast *P. pastoris* cell as His-tag fusions with or without *Saccharomyces cerevisiae α*-mating type signal peptide as secretion peptide. The His-tag fusion vectors contained a coding sequence encoding a His-tag (His_{10}) and a PreScission protease cleavage site (SLEVLFQ↓GP) fused to the codon optimized *Micpu-Δ6D* gene.

(2) OTHER MATERIALS

The *Micpu-Δ6D* gene was synthesized at GeneArt (Life Science Technologies, Germany), according to sequence in NCBI database under accession XM_003056946 as a His-tag fusion with or without Pichia secretion peptide, and cloned into the Pichia expression vector pPinkα-HC (Invitrogen, Carlsbad, CA, USA).

B. METHODS

(1) SEQUENCE COMPARISON

The *Micpu-Δ6D* gene was previously cloned from microalga *M. pusilla* CCMP1545 (Petrie et al. 2010b). The translated amino acid sequence was compared to other published Δ6-desaturases or related fatty acid desaturase presented in food or used in food production, with Vector NTI software (Version 11, Invitrogen).

(2) TRANSFORMATION OF PICHIA CELL

Pichia transformation was essentially done according to published protocol (Chen et al., 2013). Pichia expression vector DNA containing *Micpu-Δ6D* gene was first linearized by single restriction enzyme digestion in vector backbone, and precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol overnight at -20ºC. The precipitated DNA was resuspended in 10 µL of MQ water for yeast transformation. The yeast PichiaPink™ strain 4 (Invitrogen) was first activated from the stab culture on a fresh YPD plate at 28ºC for 3 to 4 days. Single colony was inoculated in 10 mL of YPD medium and cultured at 28ºC with shaking (200 rpm) for 24 hours,

followed by inoculating 100 mL of culture to OD_{600}=0.2 from the previous 10 mL culture and allowed to grow at 28ºC for 8 to 10 hrs until OD_{600}=1.0 to 1.5. The cells were spun down at 3000 rpm for 10 min and washed with 100 mL chilled MQ water, followed by washing with 50 mL of chilled MQ water then by 10 mL of cold 1 M sorbitol. The cells were resuspended in 300 µL of 1 M sorbitol and dispensed into 80 uL aliquots in Eppendorf tubes. The prepared Pichia competent cells were mixed with 10 µL of linearized DNA, incubated on ice for 5 min and electroporated. After electroporation, the cells were recovered from electroporation cuvettes with 1 mL of YPD with sorbitol (Invitrogen), incubated at 28ºC without shaking for 4 hrs, then plated out on Pichia Adenine Dropout Dextrose (Invitrogen) plate and incubate at 28ºC for 2-4 days. White yeast colonies were selected for further analysis.

(3) Enzyme Activity Analysis in Pichia Cell

Individual white colonies of Pichia were inoculated into 10 mL BMGY in 50 ml Falcon tubes and cultured for 48 hrs at 28ºC with shaking at 250 rpm. From each sample, 2.5 mL of the above culture were inoculated into 50 mL of BMGY medium in 250 mL flasks and grown at 28ºC for 24 hrs at 250 rpm. Yeast cells were collected by centrifugation at 3000 rpm for 10 min and resuspended in 5 mL BMMY at 28ºC for 3 days, by adding 50 uL of methanol to the culture every 24 hrs. In the case of fatty acid substrate feeding was needed, the fatty acid was added to the final concentration of 0.5 mM with 1% of detergent NP-40 in BMMY medium. The cells were harvested by centrifuging at 3000 rpm for 5 min, and washed with 1% NP-40, 0.5% NP-40 then water as described (Zhou et al. 2006).

(4) Fatty Acid Analysis

For fatty acid analysis, cells were dried overnight with freezing-vacuum dryer. FAMEs were prepared with 0.75 mL of 1N methanolic-HCl at 80ºC for 3 hrs, and extracted with 0.5 mL 0.9% NaCl followed by 0.3 mL of hexane after cooling down to room temperature. The hexane phase containing FAMEs were recovered after centrifuging at 3000 rpm for 5 min, transferred to GC vials, dried down to 30 µL with nitrogen. GC analysis was done according to previously described (Zhou et al. 2011).

C. Results and Discussion

(1) Phylogenetic Tree

The fatty acid Δ6-desaturases have been cloned from bacteria (Reddy et al. 1993), alga (Domergue et al. 2005), diatom (Domergue et al. 2002), fungus (Huang et al. 1999), nematode (Napier et al. 1998), moss (Girke et al. 1998), plant (Sayanova et al. 1997), mouse and human (Cho et al. 1999). The Δ6-desaturases have been widely studied in vertebrates, including many fish species (Vagner and Santigosa 2011, Tanomman et al. 2013). The Δ6-desaturase enzymes can desaturate both ω3 ALA ($18:3^{\Delta 9,12,15}$) and ω6 LA ($18:3^{\Delta 9,12}$) at Δ6 position producing ω3 SDA ($18:4^{\Delta 6,9,12,15}$) and ω6

GLA (18:3$^{\Delta 6,9,12}$). In human and mice, Δ6-desaturases also involve in the desaturation of ω3 24:5$^{\Delta 9,12,15,18,21}$ to 24:6$^{\Delta 6,9,12,15,18,21}$ then converted to DHA (22:6$^{\Delta 4,7,10,13,16,19}$) by ß-oxidation. For DHA canola, marine microalga Micpu-Δ6D with ω3-preference was used (Petrie et al. 2010b). The Micpu-Δ6D protein shared high homology to other Δ6-desaturase proteins as shown in Figure A-7, especially to Δ6-desaturases from other algae, *Mantoniella squamata*, *Ostreococcus lucimarinus* and *O. tauri*.

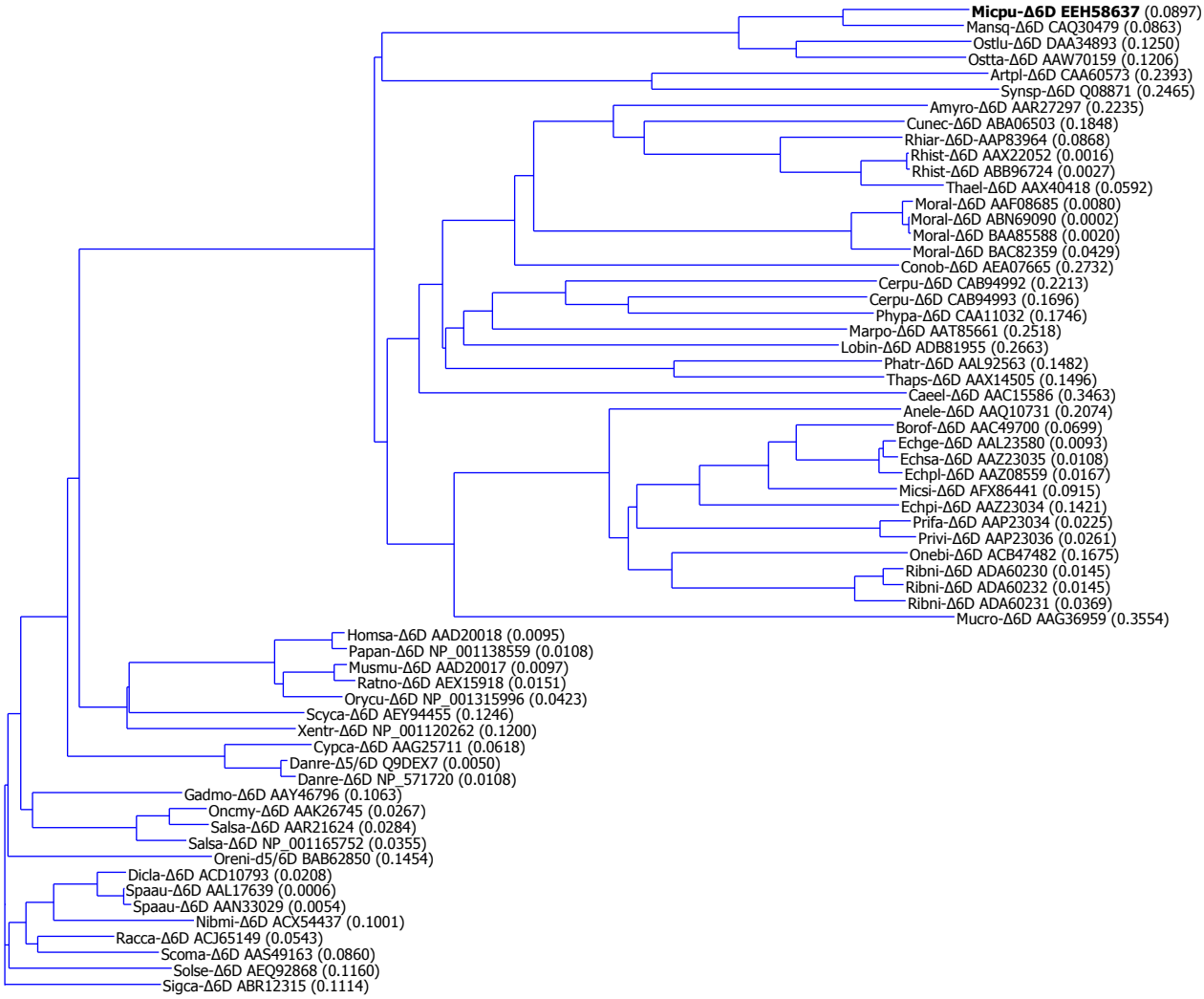

Figure A-7. Phylogenetic tree for sequence comparison of Micpu-Δ6D with representative Δ6-desaturases

The phylogenetic tree was generated with Vector NTI software (Invitrogen, Carlsbad, USA). The protein sequences were named as 5-letter initial of species name, followed by NCBI accession numbers. Amyro, *Amylomyces rouxii* (fungus); Anele, *Anemone leveillei* (plant); Artpl, *A. platensis* (bacterium); Borof, *B. officianalis* (plant); Caeel, *Caenorhabditis elegans* (nematode); Cerpu, *Ceratodon purpureus* (moss); Conob, *Conidiobolus obscurus* (fungus); Cunec, *Cunninghamella echinulate* (fungus); Cypca, *Cyprinus carpio* (carp); Danre, *Danio rerio* (zebrafish); Dicla, *Dicentrarchus labrax* (sea bass); Echge, *Echium gentianoides* (plant); Echpi, *E. pitardii* (plant); Echpl, *E. plantagineum* (plant); Echsa, *E. sabulicola* (plant); Gadmo, *Gadus morhua* (cod); Homsa, *H. sapiens* (human); Lobin, *Lobosphaera incisa* (alga); Mansq, *Mantoniella squamata* (alga); Marpo, *Marchantia polymorpha* (liverwort); Micpu, *Micromonas pusilla* CCMP1545 (alga); Micsi, *Microula*

sikkimensis (plant); Moral, *M. alpina* (fungus); Mucro, *Mucor rouxii* (fungus); Musmu, *Mus musculus* (mouse); Nibmi, *Nibea mitsukurii* (croaker); Oncmy, *Oncorhynchus mykiss* (trout); Onebi, *Oenothera biennis* (plant); Oreni, *Oreochromis niloticus* (nile tilapia); Orycu, *Oryctolagus cuniculus* (rabbit); Ostlu, *Ostreococcus lucimarinus* CCE9901 (alga); Ostta, *O. tauri* (alga); Papan, *Papio anubis* (baboon); Phatr, *P. tricornutum* (diatom); Phypa, *Physcomitrella patens* (moss); Prifa, *Primula farinose* (plant); Privi, *P. vialii* (plant); Racca, *Rachycentron canadum* (cobia); Ratno, *Rattus norvegicus* (rat); Rhiar, *Rhizopus arrhizus* (fungus); Rhist, *R. stolonifera* (fungus); Ribni, *Ribes nigrum* (plant); Salsa, *Salmo salar* (salmon); Scoma, Scophthalmus maximus (turbot); Scyca, Scyliorhinus canicula (catshark); Sigca, Siganus canaliculatus (Siganus oramin); Solse, Solea senegalensis (sole); Spaau, Sparus aurata (bream); Synsp, Synechocystis sp. (bacterium); Thael, Thamnidium elegans (fungus); Thaps, *T. pseudonana*; Xentr, Xenopus tropicalis (frog).

(2) HETEROLOGOUS EXPRESSION

The enzyme functionality of Micpu-Δ6D have been confirmed in different heterologous expression systems, including yeast cell and *N. benthamiana* leaf (Petrie et al. 2010b), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie et al. 2014). In this study, Micpu-Δ6D was expressed in *P. pastoris*, as fusion proteins designated as SP::His_{10}::Micpu-Δ6D or His_{10}::Micpu-Δ6D. In SP::His_{10}::Micpu-Δ6D, the Micpu-Δ6D sequence was fused to *Saccharomyces cerevisiae* α-mating type signal peptide, followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure A-8). In His_{10}::Micpu-Δ6D, the Micpu-Δ6D sequence was fused to His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure A-9). No secretion peptide was used in His_{10}::Micpu-Δ6D.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFS
NSTNNGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMCPPKTD
GRSSPRSPLTRSKSSAEALDAKDASTAPVDLKTLEPHELAATFETRWVRVEDVEYD
VTNFKHPGGSVIFYMLANTGADATEAFKEFHMRSLKAWKMLRALPSRPAEIKRSES
EDAPMLEDFARWRAELERDGFFKPSITHVAYRLLELLATFALGTALMYAGYPIIAS
VVYGAFFGARCGWVQHEGGHNSLTGSVYVDKRLQAMTCGFGLSTSGEMWNQMHNKH
HATPQKVRHDMDLDTTPAVAFFNTAVEDNRPRGFSRAWARLQAWTFVPVTSGLLVQ
AFWIYVLHPRQVLRKKNYEEASWMLVSHVVRTAVIKLATGYSWPVAYWWFTFGNWI
AYMYLFAHFSTSHTHLPVVPSDKHLSWVNYAVDHTVDIDPSRGYVNWLMGYLNCQV
IHHLFPDMPQFRQPEVSRRFVPFAKKWGLNYKVLSYYGAWKATFSNLDKVGQHYYV
NGKAEKAH

Figure A-8. Amino acid sequence of SP::His10::Micpu-Δ6D

Micpu-Δ6D was expressed in P. pastoris, fused to mating type alpha signal peptide as secretion peptide (underlined), followed by His-tag (His10, double underlined) and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

MRPHHHHHHHHHHSLEVLFQGPMCPPKTDGRSSPRSPLTRSKSSAEALDAKDASTA
PVDLKTLEPHELAATFETRWVRVEDVEYDVTNFKHPGGSVIFYMLANTGADATEAF
KEFHMRSLKAWKMLRALPSRPAEIKRSESEDAPMLEDFARWRAELERDGFFKPSIT
HVAYRLLELLATFALGTALMYAGYPIIASVVYGAFFGARCGWVQHEGGHNSLTGSV
YVDKRLQAMTCGFGLSTSGEMWNQMHNKHHATPQKVRHDMDLDTTPAVAFFNTAVE
DNRPRGFSRAWARLQAWTFVPVTSGLLVQAFWIYVLHPRQVLRKKNYEEASWMLVS
HVVRTAVIKLATGYSWPVAYWWFTFGNWIAYMYLFAHFSTSHTHLPVVPSDKHLSW
VNYAVDHTVDIDPSRGYVNWLMGYLNCQVIHHLFPDMPQFRQPEVSRRFVPFAKKW
GLNYKVLSYYGAWKATFSNLDKVGQHYYVNGKAEKAH

Figure A-9. Amino acid sequence of His10::Micpu-Δ6D

Micpu-Δ6D was expressed in *P. pastoris*, fused to His-tag (His_{10}, double underlined), and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

IV. *PYRAMIMONAS CORDATA* Δ6-ELONGASE

A. MATERIALS

(1) TARGET PROTEIN

The Δ6-elongase gene used in DHA canola event was previously cloned from the microalga *P. cordata* (Petrie et al. 2010a). The Pyrco-Δ6E protein was expressed as native sequence in yeast cell (Petrie et al. 2010a), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie, 2014), as well as in yeast *P. pastoris* cell as His-tag fusions with or without *Saccharomyces cerevisiae α*-mating type signal peptide as secretion peptide. The His-tag fusion vectors contained a coding sequence encoding a His-tag (His_{10}) and a PreScission protease cleavage site (SLEVLFQ↓GP) fused to the codon optimized *Pyrco-Δ6E* gene.

(2) OTHER MATERIALS

The *Pyrco-Δ6E* gene was synthesized at GeneArt (Life Science Technologies, Germany), according to sequence GQ202034 as a His-tag fusion with or without Pichia secretion peptide, and cloned into the Pichia expression vector pPinkα-HC (Invitrogen, Carlsbad, CA, USA).

B. METHODS

(1) SEQUENCE COMPARISON

The *Pyrco-Δ6E* gene was previously cloned from microalga *P. cordata* CS-140 (Petrie et al. 2010a). The translated amino acid sequence was compared to other published Δ6-elongases or related fatty acid desaturase presented in food or used in food production, with Vector NTI software (Version 11, Invitrogen).

(2) TRANSFORMATION OF PICHIA CELL

Pichia transformation was essentially done according to published protocol (Chen et al., 2013). Pichia expression vector DNA containing *Pyrco-Δ6E* gene was first linearized by single restriction enzyme digestion in vector backbone, and precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol overnight at -20ºC. The precipitated DNA was resuspended in 10 µL of MQ water for yeast transformation. The yeast PichiaPink^{TM} strain 4 (Invitrogen) was first activated from the stab culture on a fresh YPD plate at 28ºC for 3 to 4 days. Single colony was inoculated in 10 mL of YPD medium and cultured at 28ºC with shaking (200 rpm) for 24 hours, followed by inoculating 100 mL of culture to OD_{600}=0.2 from the previous 10 mL culture and allowed to grow at 28ºC for 8 to 10 hrs until OD_{600}=1.0 to 1.5. The cells were spun down at 3000 rpm for 10 min and washed with 100 mL chilled MQ water, followed by washing with 50 mL of chilled MQ water then by 10 mL of cold 1 M sorbitol. The cells were resuspended in 300 µL of 1 M sorbitol and dispensed into 80 uL aliquots in Eppendorf tubes. The prepared Pichia competent cells were mixed with 10 µL of linearized DNA, incubated on ice for 5 min and electroporated. After electroporation, the cells were recovered from electroporation cuvettes with 1 mL of YPD with sorbitol (Invitrogen), incubated at 28ºC without shaking for 4 hrs, then plated out on Pichia Adenine Dropout Dextrose (Invitrogen) plate and incubate at 28ºC for 2-4 days. White yeast colonies were selected for further analysis.

(3) ENZYME ACTIVITY ANALYSIS IN PICHIA CELL

Individual white colonies of Pichia were inoculated into 10 mL BMGY in 50 ml Falcon tubes and cultured for 48 hrs at 28ºC with shaking at 250 rpm. From each sample, 2.5 mL of the above culture were inoculated into 50 mL of BMGY medium in 250 mL flasks and grown at 28ºC for 24 hrs at 250 rpm. Yeast cells were collected by centrifugation at 3000 rpm for 10 min and resuspended in 5 mL BMMY at 28ºC for 3 days, by adding 50 uL of methanol to the culture every 24 hrs. In the case of fatty acid substrate feeding was needed, the fatty acid was added to the final concentration of 0.5 mM with 1% of detergent NP-40 in BMMY medium. The cells were harvested by centrifuging at 3000 rpm for 5 min, and washed with 1% NP-40, 0.5% NP-40 then water as described (Zhou et al. 2006).

(4) FATTY ACID ANALYSIS

For fatty acid analysis, cells were dried overnight with freezing-vacuum dryer. AMEs were prepared with 0.75 mL of 1N methanolic-HCl at 80ºC for 3 hrs, and extracted with 0.5 mL 0.9% NaCl followed by 0.3 mL of hexane after cooling down to room temperature. The hexane phase containing FAMEs were recovered after centrifuging at 3000 rpm for 5 min, transferred to GC vials, dried down to 30 µL with nitrogen. GC analysis was done according to previously described (Zhou et al. 2011).

C. RESULTS AND DISCUSSION

(1) PHYLOGENETIC TREE

The fatty acid Δ6-elongases have been cloned from nematode (Leonard et al. 2004), plant (Kajikawa et al., 2004) and alga (Meyer et al. 2004). In addition, fatty acid elongases (Elo) involved in the polyunsaturated fatty acid (PUFA) with similar function of Δ6-elongases are also isolated from many animals like frog, fish, sea squirt (Meyer et al. 2004) and human (Leonard et al. 2004). The Pyrco-Δ6E shared high homology to other Δ6-elongase or PUFA Elo proteins as shown in Figure A-10.

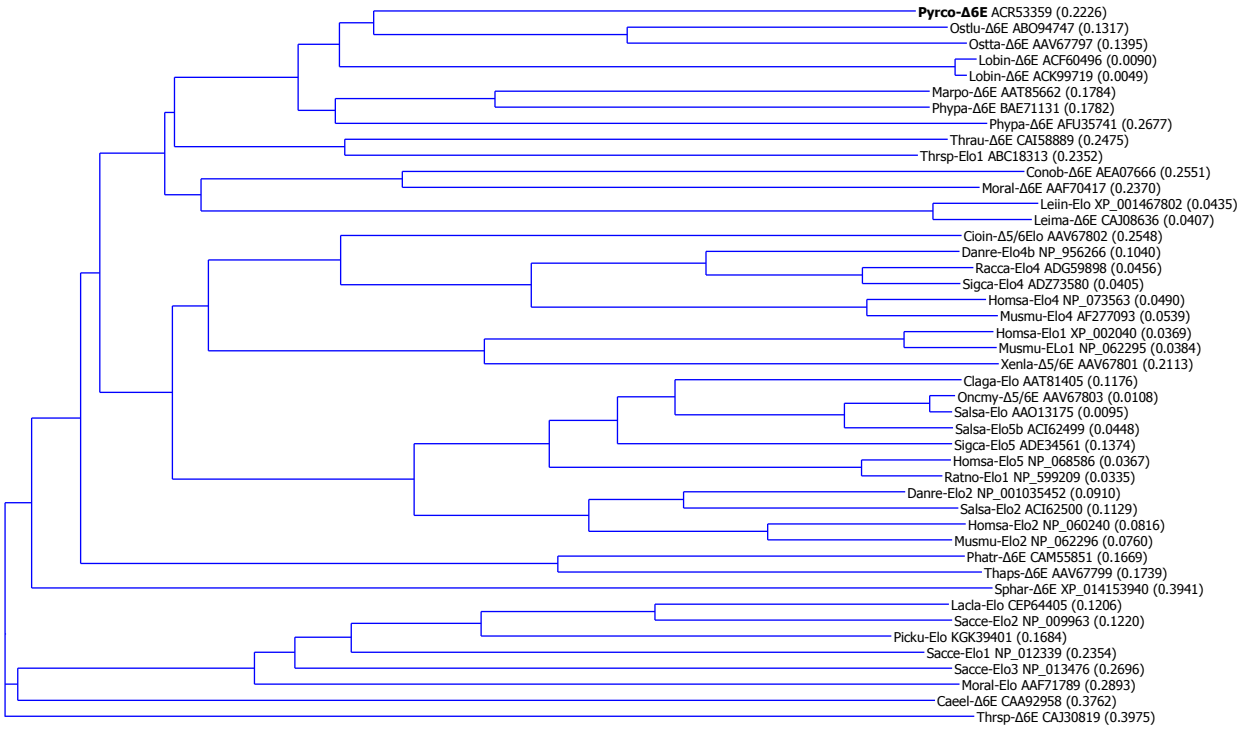

Figure A-10. Phylogenetic tree for sequence comparison of Pyrco-Δ6E with representative Δ6-elongases or other PUFA elongases

The phylogenetic tree was generated with Vector NTI software (Invitrogen, Carlsbad, USA). The protein sequences were named as 5-letter initial of species name, followed by NCBI accession numbers. Caeel, *Caenorhabditis elegans* (nematode); Cioin, *Ciona intestinalis* (sea squirt); Claga, *Clarias gariepinus* (catfish); Conob, *Conidiobolus obscurus* (fungus); Danre, *Danio rerio* (zebrafish); Euggr, *Euglena gracilis* (alga); Homsa, *H. sapiens* (human); Lacla, *Lachancea lanzarotensis* (fungus); Leiin, *Leishmania infantum* JPCM5 (kinetoplastid parasite); Leima, *L. major* strain Friedlin; lobin, *Lobosphaera incisa* (alga); Marpo, *Marchantia polymorpha* (liverwort); Moral, *M. alpina* (fungus); Musmu, *Mus musculus* (mouse); Oncmy, *Oncorhynchus mykiss* (trout); Ostlu, *Ostreococcus lucimarinus* CCE9901 (alga) ; Ostta, *O. tauri* (alga); Pavsa, *Pavlova salina* (alga); Pavsp, *P. sp*. CCMP459 (alga); Pavvi, *P. viridis* (alga); Phatr, *P. tricornutum* (diatom); Phypa, *Physcomitrella patens* (moss); Picku, *Pichia kudriavzevii* (fungus); Pyrco, *Pyramimonas cordata* (alga); Racca, *Rachycentron canadum* (cobia); Ratno, *Rattus norvegicus* (rat); Sacce, *Saccharomyces cerevisiae* (yeast); Salsa, *S. salar* (salmon); Sigca, *Siganus canaliculatus* (rabbitfish); Sphar, *Sphaeroforma arctica* (protist); Thaps, *T. pseudonana* (diatom); Thrau, *T. aureum* (protist);

Thrsp, *T. sp.*; Xenla, *Xenopus laevis* (frog). Δ6E, Δ6-elongase; Δ5/6E, bifunctional Δ5- and Δ6-elongase; Elo, PUFA elongase.

(2) HETEROLOGOUS EXPRESSION

The enzyme functionality of Pyrco-Δ6E have been confirmed in different heterologous expression systems, including yeast cell (Petrie et al. 2010a), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie et al. 2014). In this study, Pyrco-Δ6E was expressed in *P. pastoris*, as fusion proteins designated as SP::His_{10}::Pyrco-Δ6E or His_{10}::Pyrco-Δ6E. In SP::His_{10}::Pyrco-Δ6E, the Pyrco-Δ6E sequence was fused to *Saccharomyces cerevisiae* α-mating type signal peptide, followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure A-11). In His_{10}::Pyrco-Δ6E, the Pyrco-Δ6E sequence was fused to His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure A-12). No secretion peptide was used in His_{10}::Pyrco-Δ6E.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSN
STNNGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMEFAQPLVA
MAQEQYAAIDAVVAPAIFSATDSIGWGLKPISSATKDLPLVESPTPLILSLLAYFAI
VGSGLVYRKVFPRTVKGQDPFLLKALMLAHNVFLIGLSLYMCLKLVYEAYVNKYSFW
GNAYNPAQTEMAKVIWIFYVSKIYEFMDTFIMLLKGNVNQVSFLHVYHHGSISGIWW
MITYAAPGGDAYFSAALNSWVHVCMYTYYFMAAVLPKDEKTKRKYLWWGRYLTQMQM
FQFFMNLLQAVYLLYSSSPYPKFIAQLLVVYMVTLLMLFGNFYYMKHHASK

Figure A-11. Amino acid sequence of SP::His10::Pyrco-Δ6E

Pyrco-Δ6E was expressed in *P. pastoris*, fused to mating type alpha signal peptide as secretion peptide (underlined), followed by His-tag (His_{10}, double underlined) and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

MRPHHHHHHHHHHSLEVLFQGPMEFAQPLVAMAQEQYAAIDAVVAPAIFSATDSIGW
GLKPISSATKDLPLVESPTPLILSLLAYFAIVGSGLVYRKVFPRTVKGQDPFLLKAL
MLAHNVFLIGLSLYMCLKLVYEAYVNKYSFWGNAYNPAQTEMAKVIWIFYVSKIYEF
MDTFIMLLKGNVNQVSFLHVYHHGSISGIWWMITYAAPGGDAYFSAALNSWVHVCMY
TYYFMAAVLPKDEKTKRKYLWWGRYLTQMQMFQFFMNLLQAVYLLYSSSPYPKFIAQ
LLVVYMVTLLMLFGNFYYMKHHASK

Figure A-12. Amino acid sequence of His10::Pyrco-Δ6E

Pyrco-Δ6E was expressed in P. *pastoris*, fused to His-tag (His_{10}, double underlined), and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

V. PAVLOVA SALINA Δ5-DESATURASE

A. MATERIALS

(1) TARGET PROTEIN

The Δ6-desaturase gene used in DHA canola event was previously cloned from microalga *P. salina* (Zhou et al. 2007). The Pavsa-Δ5D protein was expressed as native sequence in yeast cells (Zhou et al. 2007), *N. benthamiana* leaf (Wood et al. 2009), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie, 2014), as well as in yeast *P. pastoris* cell as His-tag fusions with or without *Saccharomyces cerevisiae α*-mating type signal peptide as secretion peptide. The His-tag fusion vectors contained a coding sequence encoding a His-tag (His_{10}) and a PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) fused to the codon optimized *Pavsa-Δ5D* gene.

(2) OTHER MATERIALS

The *Pavsa-Δ5D* gene was synthesized at GeneArt (Life Science Technologies, Germany), based on the sequence (DQ995517, Zhou et al. 2007) as a His-tag fusion with or without Pichia secretion peptide, and cloned into the Pichia expression vector pPinkα-HC (Invitrogen, Carlsbad, CA, USA).

B. METHODS

(1) SEQUENCE COMPARISON

The *Pavsa-Δ5D* gene was previously cloned from microalga *P. salina* (Zhou et al. 2007). The translated amino acid sequence was compared to other published Δ5-desaturases or related fatty acid desaturase presented in food or used in food production, with Vector NTI software (Version 11, Invitrogen).

(2) Transformation of *Pichia* Cell

Pichia expression vector DNA containing *Pavsa-Δ5D* gene was first linearized by single restriction enzyme digestion in vector backbone, and precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol overnight at -20ºC. The precipitated DNA was resuspended in 10 µL of MQ water for yeast transformation. The yeast PichiaPink™ strain 4 (Invitrogen) was first activated from the stab culture on a fresh YPD plate at 28ºC for 3 to 4 days. Single colony was inoculated in 10 mL of YPD medium and cultured at 28ºC with shaking (200 rpm) for 24 hours, followed by inoculating 100 mL of culture to OD_{600}=0.2 from the previous 10 mL culture and allowed to grow at 28ºC for 8 to 10 hrs until OD_{600}=1.0 to 1.5. The cells were spun down at 3000 rpm for 10 min and washed with 100 mL chilled MQ water, followed by washing with 50 mL of chilled MQ water then by 10 mL of cold 1 M sorbitol. The cells were resuspended in 300 µL of 1 M sorbitol and dispensed into 80 uL aliquots in Eppendorf tubes. The prepared Pichia competent cells were mixed with 10 µL of linearized DNA, incubated on ice for 5 min and electroporated.

After electroporation, the cells were recovered from electroporation cuvettes with 1 mL of YPD with sorbitol (Invitrogen), incubated at 28ºC without shaking for 4 hrs, then plated out on Pichia Adenine Dropout Dextrose (Invitrogen) plate and incubate at 28ºC for 2-4 days. White yeast colonies were selected for further analysis.

(3) Enzyme Activity Analysis in *Pichia* Cell

Individual white colonies of *Pichia* were inoculated into 10 mL BMGY in 50 ml Falcon tubes and cultured for 48 hrs at 28ºC with shaking at 250 rpm. From each sample, 2.5 mL of the above culture were inoculated into 50 mL of BMGY medium in 250 mL flasks and grown at 28ºC for 24 hrs at 250 rpm. Yeast cells were collected by centrifugation at 3000 rpm for 10 min and resuspended in 5 mL BMMY at 28ºC for 3 days, by adding 50 uL of methanol to the culture every 24 hrs. In the case of fatty acid substrate feeding was needed, the fatty acid was added to the final concentration of 0.5 mM with 1% of detergent NP-40 in BMMY medium. The cells were harvested by centrifuging at 3000 rpm for 5 min, and washed with 1% NP-40, 0.5% NP-40 then water as described (Zhou et al. 2006).

(4) Fatty Acid Analysis

For fatty acid analysis, cells were dried overnight with freezing-vacuum dryer. FAMEs were prepared with 0.75 mL of 1N methanolic-HCl at 80ºC for 3 hrs, and extracted with 0.5 mL 0.9% NaCl followed by 0.3 mL of hexane after cooling down to room temperature. The hexane phase containing FAMEs were recovered after centrifuging at 3000 rpm for 5 min, transferred to GC vials, dried down to 30 µL with nitrogen. GC analysis was done according to previously described (Zhou et al. 2011).

C. Results and Discussion

(1) Phylogenetic Tree

The fatty acid Δ5-desaturases have been cloned from alga (Zhou et al., 2007), amoeba (Saito et al., 2000), liverwort (Kajikawa et al., 2004), fungus (Michaelson et al., 1998), scallop (Liu et al., 2014) and mammals (Leonard et al., 2000). The Pavsa-Δ5D shared high homology to other Δ5-desaturase proteins as shown in Figure A-13.

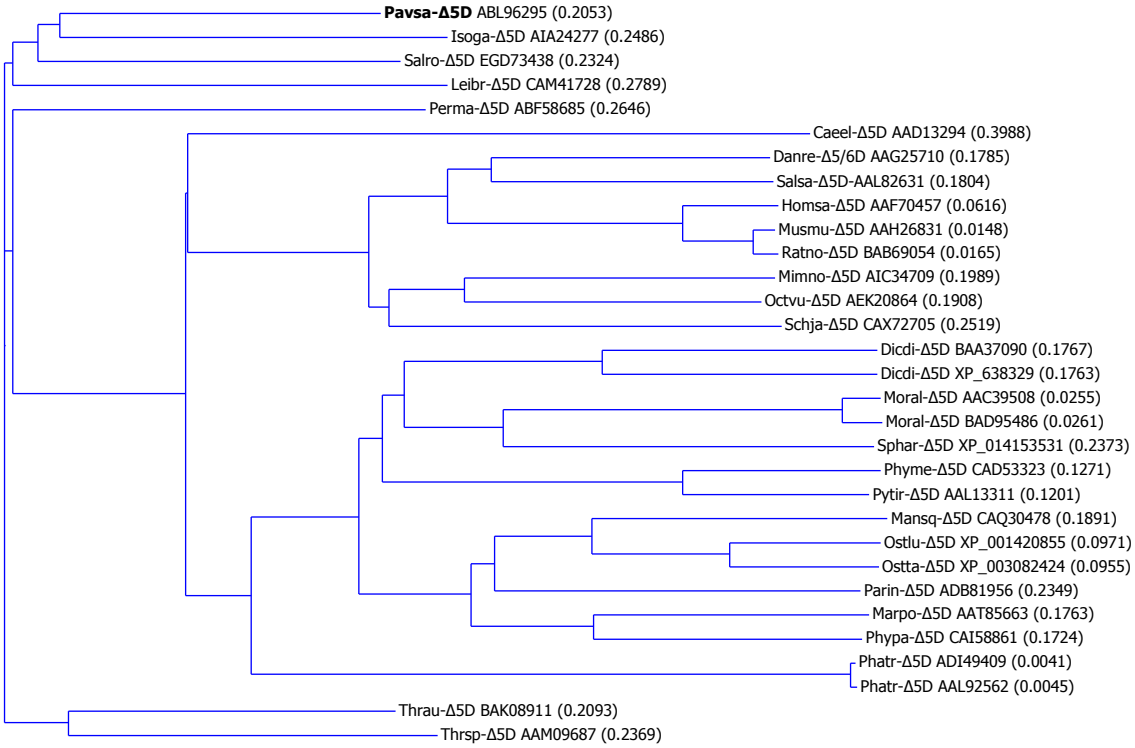

Figure A-13. Phylogenetic tree for sequence comparison of Pavsa-Δ5D with representative Δ5-desaturases

The phylogenetic tree was generated with Vector NTI software (Invitrogen, Carlsbad, USA). The protein sequences were named as 5-letter initial of species name, followed by NCBI accession numbers. Caeel, *Caenorhabditis elegans* (nematode); Danre, *Danio rerio* (zebrafish); Dicdi, *Dictyostelium discoideum* (amoeba); Homsa, *H. sapiens* (human); Isoga, *I. galbana* (alga); Leibr, *Leishmania braziliensis* (protozoa); Mansq, *Mantoniella squamata* (alga); Marpo, *Marchantia polymorpha* (liverwort); Mimno, *Mimachlamys nobilis* (scallop); Moral, *M. alpina* (fungus); Musmu, *Mus musculus* (mouse); Octvu, *O. vulgaris* (octopus); Ostlu, *Ostreococcus lucimarinus* CCE9901 (alga); Ostta, *O. tauri* (alga); Parin, *Parietochloris incisa* (alga); Pavsa, *P. salina* (alga); Perma, *Perkinsus marinus* (protozoan); Phatr, *P. tricornutum* (diatom); Phyme, *Phytophthora megasperma* (fungus); Phypa, *Physcomitrella patens* (moss); Pytir, *Pythium irregulare* (fungus); Ratno, *Rattus norvegicus* (rat); Salro, *Salpingoeca rosetta*; Salsa, *Salmo salar* (salmon); Schja, *Schistosoma japonicum* (parasite); Sphar, *Sphaeroforma arctica* (protist); Thrau, *T. aureum* (protist); Thasp, *T. sp.* ATCC21685 (protist). Δ5D, Δ5-desaturase; Δ5/6D, bifunctional Δ5- and Δ6-desaturase.

(2) HETEROLOGOUS EXPRESSION

The enzyme functionality of Pavsa-Δ5D has been confirmed in different heterologous expression systems, including yeast cell (Zhou et al. 2007), *N. benthamiana* leaf (Wood et al. 2009), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie et al. 2014). In this study, Pavsa-Δ5D was expressed in *P. pastoris*, as fusion proteins designated as SP::His_{10}::Pavsa-Δ5D or His_{10}::Pavsa-Δ5D. In SP::His_{10}::Pavsa-Δ5D, the Pavsa-Δ5D sequence was fused to *Saccharomyces cerevisiae* α-mating type signal peptide, followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal (Figure A-14). In His_{10}::Pavsa-Δ5D, the Pavsa-Δ5D sequence was fused to His-tag (His_{10}) and PreScission protease

cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure A-15). No secretion peptide was used in His_{10}::Pavsa-Δ5D.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSN
STNNGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMPPRDSYSY
AAPPSAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGGKIIAYQVGTDATDAYK
QFHVRSAKADKMLKSLPSRPVHKGYSPRRADLIADFQEFTKQLEAEGMFEPSLPHVA
YRLAEVIAMHVAGAALIWHGYTFAGIAMLGVVQGRCGWLMHEGGHYSLTGNIAFDRA
IQVACYGLGCGMSGAWWRNQHNKHHATPQKLQHDVDLDTLPLVAFHERIAAKVKSPA
MKAWLSMQAKLFAPVTTLLVALGWQLYLHPRHMLRTKHYDELAMLGIRYGLVGYLAA
NYGAGYVLACYLLYVQLGAMYIFCNFAVSHTHLPVVEPNEHATWVEYAANHTTNCSP
SWWCDWWMSYLNYQIEHHLYPSMPQFRHPKIAPRVKQLFEKHGLHYDVRGYFEAMAD
TFANLDNVAHAPEKKMQ

Figure A-14. Amino acid sequence of SP::His10::Pavsa-Δ5D

Pavsa-Δ5D was expressed in *P. pastoris,* fused to mating type alpha signal peptide as secretion peptide (underlined), followed by His-tag (His_{10}, double underlined) and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

MRPHHHHHHHHHHSLEVLFQGPMPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGD
RAYDVTNFVKRHPGGKIIAYQVGTDATDAYKQFHVRSAKADKMLKSLPSRPVHKGYS
PRRADLIADFQEFTKQLEAEGMFEPSLPHVAYRLAEVIAMHVAGAALIWHGYTFAGI
AMLGVVQGRCGWLMHEGGHYSLTGNIAFDRAIQVACYGLGCGMSGAWWRNQHNKHHA
TPQKLQHDVDLDTLPLVAFHERIAAKVKSPAMKAWLSMQAKLFAPVTTLLVALGWQL
YLHPRHMLRTKHYDELAMLGIRYGLVGYLAANYGAGYVLACYLLYVQLGAMYIFCNF
AVSHTHLPVVEPNEHATWVEYAANHTTNCSPSWWCDWWMSYLNYQIEHHLYPSMPQF
RHPKIAPRVKQLFEKHGLHYDVRGYFEAMADTFANLDNVAHAPEKKMQ

Figure A-15. Amino acid sequence of His10::Pavsa-Δ5D

Pavsa-Δ5D was expressed in *P. pastoris,* fused to His-tag (His_{10}, double underlined), and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

VI. PYRAMIMONAS CORDATA Δ5-ELONGASE

A. MATERIALS

(1) TARGET PROTEIN

The Δ5-elongase gene used in DHA canola event was previously cloned from microalga *P. cordata* (Petrie et al. 2010a). The Pyrco-Δ5E protein was expressed as native sequence in yeast cell (Petrie et al. 2010a), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie, 2014), as well as in yeast *P. pastoris* cell as His-tag fusions with or without *Saccharomyces cerevisiae α*-mating type signal peptide as secretion peptide. The His-tag fusion vectors contained a coding sequence

encoding a His-tag (His_{10}) and a PreScission protease cleavage site (SLEVLFQ↓GP) fused to the codon optimized *Pyrco-Δ5E* gene.

(2) OTHER MATERIALS

The *Pyrco-Δ5E* gene was synthesized at GeneArt (Life Science Technologies, Germany), according to sequence GQ202035 as a His-tag fusion with or without Pichia secretion peptide, and cloned into the Pichia expression vector pPinkα-HC (Invitrogen, Carlsbad, CA, USA).

B. METHODS

(1) SEQUENCE COMPARISON

The *Pyrco-Δ5E* gene was previously cloned from microalga *P. cordata* CS-140 (Petrie et al. 2010a). The translated amino acid sequence was compared to other published Δ5-elongases or related fatty acid elongases presented in food or used in food production, with Vector NTI software (Version 11, Invitrogen).

(2) TRANSFORMATION OF PICHIA CELL

Pichia transformation was essentially done according to published protocol (Chen et al., 2013). Pichia expression vector DNA containing *Pyrco-Δ5E* gene was first linearized by single restriction enzyme digestion in vector backbone, and precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol overnight at -20ºC. The precipitated DNA was resuspended in 10 µL of MQ water for yeast transformation. The yeast PichiaPink™ strain 4 (Invitrogen) was first activated from the stab culture on a fresh YPD plate at 28ºC for 3 to 4 days. Single colony was inoculated in 10 mL of YPD medium and cultured at 28ºC with shaking (200 rpm) for 24 hours, followed by inoculating 100 mL of culture to OD_{600}=0.2 from the previous 10 mL culture and allowed to grow at 28ºC for 8 to 10 hrs until OD_{600}=1.0 to 1.5. The cells were spun down at 3000 rpm for 10 min and washed with 100 mL chilled MQ water, followed by washing with 50 mL of chilled MQ water then by 10 mL of cold 1 M sorbitol. The cells were resuspended in 300 µL of 1 M sorbitol and dispensed into 80 uL aliquots in Eppendorf tubes. The prepared Pichia competent cells were mixed with 10 µL of linearized DNA, incubated on ice for 5 min and electroporated. After electroporation, the cells were recovered from electroporation cuvettes with 1 mL of YPD with sorbitol (Invitrogen), incubated at 28ºC without shaking for 4 hrs, then plated out on Pichia Adenine Dropout Dextrose (Invitrogen) plate and incubate at 28ºC for 2-4 days. White yeast colonies were selected for further analysis.

(3) ENZYME ACTIVITY ANALYSIS IN PICHIA CELL

Individual white colonies of Pichia were inoculated into 10 mL BMGY in 50 ml Falcon tubes and cultured for 48 hrs at 28ºC with shaking at 250 rpm. From each sample, 2.5 mL of the above culture were inoculated into 50 mL of BMGY medium in 250 mL flasks and grown at 28ºC for 24 hrs at

250 rpm. Yeast cells were collected by centrifugation at 3000 rpm for 10 min and resuspended in 5 mL BMMY at 28ºC for 3 days, by adding 50 uL of methanol to the culture every 24 hrs. In the case of fatty acid substrate feeding was needed, the fatty acid was added to the final concentration of 0.5 mM with 1% of detergent NP-40 in BMMY medium. The cells were harvested by centrifuging at 3000 rpm for 5 min, and washed with 1% NP-40, 0.5% NP-40 then water as described (Zhou et al. 2006).

(4) FATTY ACID ANALYSIS

For fatty acid analysis, cells were dried overnight with a freezing-vacuum dryer. FAMEs were prepared with 0.75 mL of 1N methanolic-HCl at 80ºC for 3 hrs, and extracted with 0.5 mL 0.9% NaCl followed by 0.3 mL of hexane after cooling down to room temperature. The hexane phase containing FAMEs were recovered after centrifuging at 3000 rpm for 5 min, transferred to GC vials, dried down to 30 µL with nitrogen. GC analysis was done according to previously described (Zhou et al. 2011).

C. RESULTS AND DISCUSSION

(1) PHYLOGENETIC TREE

The fatty acid Δ5-elongases have been cloned from a wide range of organisms, including moss (Eiamsa-ard et al. 2013), alga (Robert et al. 2005), marine protist thraustochytrid, kinetoplastid parasite **(**Livore et al. 2007) and liverwort (Kajikawa et al., 2006). In addition, fatty acid elongases (Elo) involved in the polyunsaturated fatty acid (PUFA) with similar function of Δ6-elongases are also isolated from many animals like frog, fish, sea squirt (Meyer et al. 2004) and human (Leonard et al. 2004). Human PUFA elongase, Elo5, converted a wide range of exogenously added LC-PUFA substrates into their respective elongated fatty acid products, including SDA into ETA (Δ6-elongation) and EPA into DPA (Δ5-elongation) (Leonard et al. 2000, 2004). The Pyrco-Δ5E shared high homology to other Δ5-elongase, Δ6-elongase or PUFA Elo proteins as shown in Figure A-16.

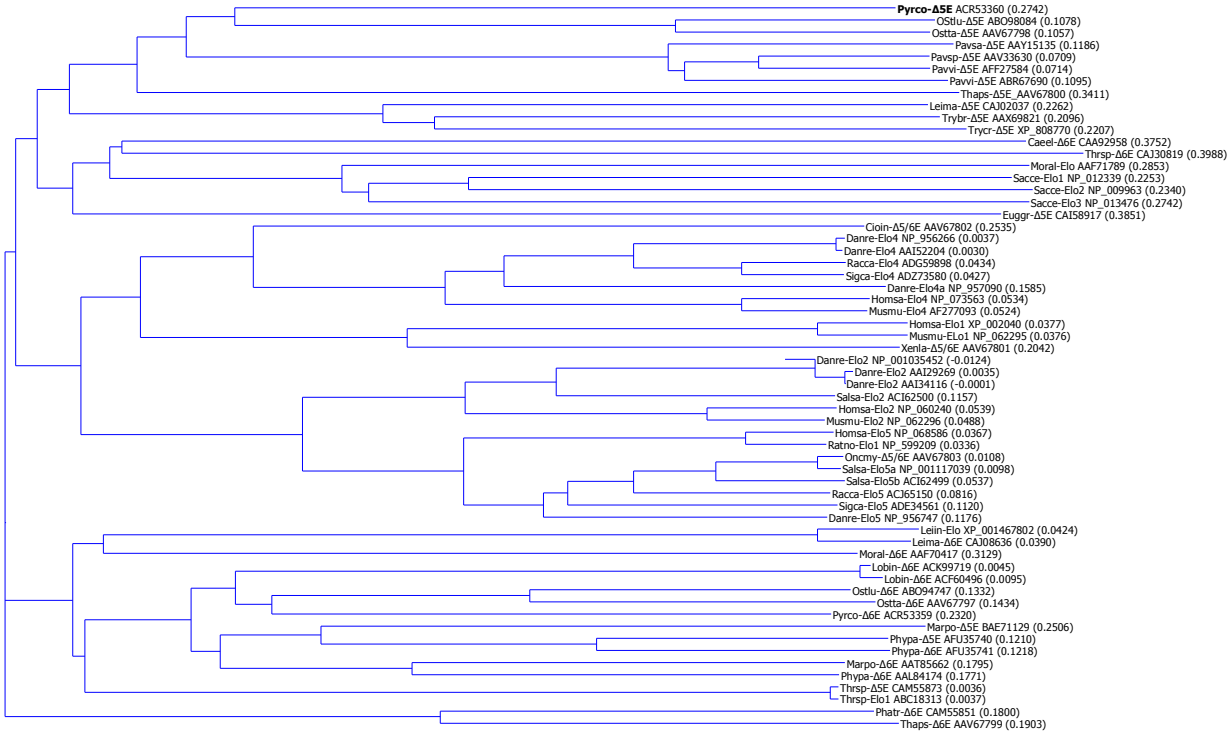

Figure A-16. Phylogenetic tree for sequence comparison of Pyrco-Δ5E with representative Δ5-elongases and other PUFA elongases

The phylogenetic tree was generated with Vector NTI software (Invitrogen, Carlsbad, USA). The protein sequences were named as 5-letter initial of species name, followed by NCBI accession numbers.

Caeel, *Caenorhabditis elegans* (nematode); Cioin, *Ciona intestinalis* (sea squirt); Danre, *Danio rerio* (zebrafish); Euggr, *Euglena gracilis* (alga); Homsa, *H. sapiens* (human); Leiin, *Leishmania infantum* JPCM5 (kinetoplastid parasite); Leima, *L. major* strain Friedlin; lobin, *Lobosphaera incisa* (alga); Marpo, *Marchantia polymorpha* (liverwort); Moral, *M. alpina* (fungus); Musmu, *Mus musculus* (mouse); Oncmy, *Oncorhynchus mykiss* (trout); Ostlu, *Ostreococcus lucimarinus* CCE9901 (alga); Ostta, *O. tauri* (alga); Pavsa, *P. salina* (alga); Pavsp, *P. sp*. CCMP459 (alga); Pavvi, *P. viridis* (alga); Phatr, *P. tricornutum* (diatom); Phypa, *Physcomitrella patens* (moss); Pyrco, *Pyramimonas cordata* (alga); Racca, *Rachycentron canadum* (cobia); Ratno, *Rattus norvegicus* (rat); Sacce, *Saccharomyces cerevisiae* (yeast); Salsa, *Salmo salar* (salmon); Sigca, *Siganus canaliculatus* (rabbitfish); Thaps, *T. pseudonana* (diatom); Thrau, *T. aureum* (protist); Thrsp, *T. sp*.; Trybr, *Trypanosoma brucei* (kinetoplastid parasite); Trycr, *T. cruzi strain* CL Brener; Xenla, *Xenopus laevis* (frog). Δ5E, Δ5-elongase; Δ6E, Δ6-elongase; Δ5/6E, bifunctional Δ5- and Δ6-elongase; Elo, PUFA elongase.

(2) HETEROLOGOUS EXPRESSION

The enzyme functionality of Pyrco-Δ5E have been confirmed in different heterologous expression systems, including yeast cell (Petrie et al. 2010a), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie et al. 2014). In this study, Pyrco-Δ5E was expressed in *P. pastoris*, as fusion proteins designated as SP::His_{10}::Pyrco-Δ5E or His_{10}::Pyrco-Δ5E. In SP::His_{10}::Pyrco-Δ5E, the Pyrco-Δ5E sequence was fused to *Saccharomyces cerevisiae* *α*-mating type signal peptide, followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal (Figure A-17). In His_{10}::Pyrco-Δ5E, the Pyrco-Δ5E sequence was fused to His-tag (His_{10})

and PreScission protease cleavage site (SLEVLFQ↓GP) at its N-terminal (Figure A-18). No secretion peptide was used in His_{10}::Pyrco-Δ5E.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSN
STNNGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMASIAIPAA
LAGTLGYVTYNVANPDIPASEKVPAYFMQVEYWGPTIGTIGYLLFIYFGKRIMQNRS
QPFGLKNAMLVYNFYQTFFNSYCIYLFVTSHRAQGLKVWGNIPDMTANSWGISQVIW
LHYNNKYVELLDTFFMVMRKKFDQLSFLHIYHHTLLIWSWFVVMKLEPVGDCYFGSS
VNTFVHVIMYSYYGLAALGVNCFWKKYITQIQMLQFCICASHSIYTAYVQNTAFWLP
YLQLWVMVNMFVLFANFYRKRYKSKGAKKQ

Figure A-17. Amino acid sequence of SP::His10::Pyrco-Δ5E

Pyrco-Δ5E was expressed in *P. pastoris*, fused to mating type alpha signal peptide as secretion peptide (underlined), followed by His-tag (His_{10}, double underlined) and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

MRPHHHHHHHHHHSLEVLFQGPMASIAIPAALAGTLGYVTYNVANPDIPASEKVPAY
FMQVEYWGPTIGTIGYLLFIYFGKRIMQNRSQPFGLKNAMLVYNFYQTFFNSYCIYL
FVTSHRAQGLKVWGNIPDMTANSWGISQVIWLHYNNKYVELLDTFFMVMRKKFDQLS
FLHIYHHTLLIWSWFVVMKLEPVGDCYFGSSVNTFVHVIMYSYYGLAALGVNCFWKK
YITQIQMLQFCICASHSIYTAYVQNTAFWLPYLQLWVMVNMFVLFANFYRKRYKSKG
AKKQ

Figure A-18. Amino acid sequence of His10::Pyrco-Δ5E

Pyrco-Δ5E was expressed in *P. pastoris*, fused to His-tag (His_{10}, double underlined), and PreScission protease cleavage site (SLEVLFQ↓GP, dotted underlined) at its N-terminal.

VII. *PAVLOVA SALINA* Δ4-DESATURASE

A. MATERIALS

(1) TARGET PROTEIN

The Δ4-desaturase gene used in the DHA canola event was previously cloned from the alga *P. salina* (Zhou et al., 2007). The Pavsa-Δ4D protein was expressed as a native sequence in yeast S288C cells (Zhou et al., 2007), Arabidopsis seed (Robert et al., 2005; Petrie et al., 2012), Camelina seed (Petrie et al., 2014) and *N. benthamiana* leaf (Petrie et al., 2010b), as well as a His-tag fusion in insect cell lines (*Sf9*) infected with *baculovirus* pFastBac vector (Life Science Technologies, Germany). The His-tag fusion vector contains a coding sequence that encods a His-tag (His_{10}) and a PreScission protease cleavage site (SLEVLFQ↓GP) fused to the codon optimized *Pavsa-Δ4D* gene.

(2) Other Materials

P. salina strain CS-49 was obtained from the CSIRO Collection of Living Microalgae[1].

For *baculovirus* expression, the codon-optimized *Pavsa-Δ4D* gene was synthesized at GeneArt (Life Science Technologies, Germany) as a His-tag fusion, and cloned into the pFastBac vector.

B. Methods

(1) Sequence Comparison

The *Pavsa-Δ4D* gene was previously cloned from the alga *P. salina* (Zhou et al., 2007). The translated amino acid sequence homology was compared to other published Δ4-desaturases or related front-end desaturases presented in food or used in food production, with Vector NTI software (Version 11, Invitrogen, Germany).

C. Results and Discussion

(1) Phylogenetic Tree

The Pavsa-Δ4D protein shares high homology to Δ4-desaturase proteins isolated from other organisms. Figure A-19 shows the phylogenetic tree of amino acid sequence comparison.

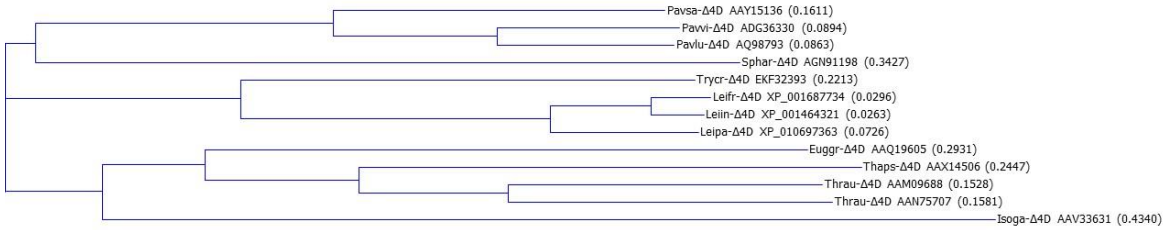

Figure A-19. Phylogenetic tree for sequence comparison of Pavsa-Δ4D

The phylogenetic tree was generated with Vector NTI software (Invitrogen, Carlsbad, USA). The protein sequences were named as 5-letter initial of species name, followed by NCBI accession numbers. Euggr, *Euglena gracilis* (Eukaryote algae); Isoga, *I. galbana* (microalga); Leifr, *Leishmania* major strain Friedlin (trypanosome); Leiin, *L. infantum* JPCM5 (trypanosome); Leipa, *L. panamensis* (trypanosome); Pavlu, *P. luthri* (microalga); Pavsa, *P. salina* (microalga); Pavvi, *P. viridis* (microalga); Sphar, *Sphaeroforma arctica* (protozoan); Thaps, *T. pseudonana* (marine phytoplankton); Thrau, *T. aureum* (moncentric fungus); Trycr, *Trypanosoma cruzi* (protozoan).

(2) Heterologous Expression

[1] https://www.csiro.au/en/Research/Collections/ANACC

The enzyme functionality of Pavsa-Δ4D has been confirmed in different heterologous expression systems, including yeast (Zhou et al., 2007), Arabidopsis seed (Robert et al., 2005; Petrie et al., 2012), Camelina seed (Petrie et al., 2014) and *N. benthamiana* leaf (Petrie et al., 2010a).

(3) EXPRESSION OF FUSION PROTEIN

In order to purify the target protein, the target enzyme was expressed as a His-tag fusion protein in *baculovirus.* The functionality in baculovirus was not tested. Nevertheless, the Pavsa-Δ4D has shown activity in different expression systems. The DHA canola expressing DHA synthesis pathway including Pavsa-Δ4D has resulted in approximately 10% of DHA in seed oil.

For *baculovirus* expression, the codon optimized *Pavsa-Δ4D* gene was fused to the coding sequence for a His-tag (His_{10}) and a PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its 5′-end, as shown in Figure A-20. The expressed fusion protein from insect cells was used for protein stability assay.

ATGCATCATCATCATCATCACCATCACCACCACTCCCTGGAAGTGCTGTTCCAGGGT
CCC**ATG**CCCCCATCCGCTGCTAAGCAGATGGGCGCTTCCACCGGTGTCCACGCTGGT
GTCACCGACTCCTCCGCTTTCACCCGCAAGGACGTGGCCGACCGTCCCGACCTGACC
ATCGTGGGCGACTCCGTGTACGACGCTAAGGCTTTCCGTTCCGAGCACCCTGGTGGT
GCTCACTTCGTGTCCCTGTTCGGTGGTCGTGACGCTACCGAGGCTTTCATGGAATAC
CACCGTCGTGCTTGGCCCAAGTCCCGTATGTCCCGTTTCCACGTGGGCTCCCTGGCT
TCCACCGAGGAACCCGTGGCTGCTGACGAGGGTTACCTGCAGCTGTGCGCTCGTATC
GCTAAGATGGTGCCCTCCGTGTCCTCCGGTTTCGCTCCCGCTTCCTACTGGGTCAAG
GCTGGCCTGATCCTGGGTTCCGCTATCGCTCTGGAAGCTTACATGCTGTACGCTGGC
AAGCGTCTGCTGCCCTCCATCGTGCTGGGCTGGCTGTTCGCTCTGATCGGCCTGAAC
ATCCAGCACGACGCTAACCACGGTGCTCTGTCCAAGTCCGCTTCCGTGAACCTGGCT
CTGGGCCTGTGCCAGGACTGGATCGGTGGTTCCATGATCCTGTGGCTGCAAGAGCAC
GTGGTCATGCACCACCTCCACACCAACGACGTGGACAAGGACCCCGACCAGAAGGCT
CACGGCGCTCTGCGTCTGAAGCCCACCGACGCTTGGTCCCCCATGCACTGGCTGCAG
CACCTGTACCTGCTGCCCGGCGAGACTATGTACGCTTTCAAGCTGCTGTTCCTGGAC
ATCTCCGAGCTGGTCATGTGGCGTTGGGAGGGCGAGCCCATCTCCAAGCTGGCTGGT
TACCTGTTCATGCCCTCCCTGCTGCTGAAGCTGACCTTCTGGGCTCGTTTCGTGGCT
CTGCCCCTGTACCTGGCTCCCTCCGTGCACACCGCTGTGTGTATCGCTGCTACCGTG
ATGACCGGTTCCTTCTACCTGGCTTTCTTCTTCTTCATCTCCCACAACTTCGAGGGT
GTCGCTTCCGTGGGTCCCGACGGTTCCATCACCTCCATGACCCGTGGTGCTAGCTTC
CTGAAGCGTCAGGCTGAGACTTCCTCCAACGTGGGCGGTCCCCTGCTGGCTACCCTG
AACGGTGGCCTGAACTACCAGATCGAGCACCACCTGTTCCCCCGTGTGCACCACGGT
TTCTACCCCCGTCTGGCTCCCCTGGTCAAGGCCGAGCTGGAAGCTCGTGGTATCGAG
TACAAGCACTACCCCACCATCTGGTCCAACCTGGCCTCCACCCTGCGTCACATGTAC
GCTCTGGGTCGTCGTCCCCGTTCCAAGGCTGAG**TAA**

Figure A-20. Nucleotide sequence of codon optimized Pavsa-Δ4D gene for baculovirus expression

The start codon (ATG) and stop codon (TGA) are shown in bold. The start codon (ATG) for the His-tag fusion coding sequence is underlined.

The total protein extracted from insect cells expressing His-tag::Pavsa-Δ4D was confirmed by Western blot against anti His-tag antibody. The expected MW of His-tag::Pavsa-Δ4D is approximately 51 kDa. A specific protein band close to 50 kDa was detected in the protein pellet (Figure A-21).

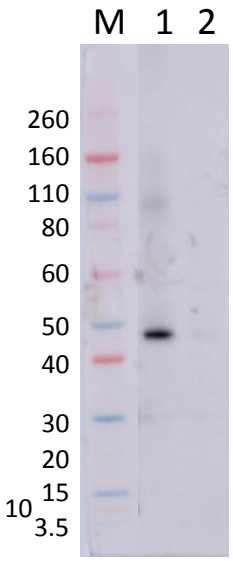

Figure A-21. Western blot analysis of His-tag::Pavsa-Δ4D protein expressed in baculovirus-infected insect cells

M, protein markers with molecular weight (in kDa) indicated to the left; lane 1, total protein in pellet; lane 2, total protein in supernatant.

Supplemental Information to USDA APHIS BRS in support of Petition 17-236-01p for Determination of Nonregulated Status for DHA Canola

OECD Unique Identifier: NS-B5ØØ27-4

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This information is provided to USDA APHIS BRS to supplement petition 17-236-01p. Nuseed conducted agronomic field trials in Canada and Australia, and stated that there are many similarities in agronomic practices used in canola production between North America and Australia, including weed, insect and disease control practices. Appearance of weeds, insects and diseases led to mitigation as needed, per typical commercial or research practices, to optimize conduct of trials in all regions. No important biological differences were observed in DHA canola and other reference lines. The experiments were selected across a range of agroecological environments to comprehensively compare agronomic traits. The Australian and North American experiments are directly comparable, in part due to common methodology and cultivar entries. In addition to the studies presented in the petition, Nuseed conducted six more studies in 2017 in North America and bulked up DHA canola in three other states. The information presented herein supports the assertion of similarity between the growing regions outlined in the petition, as well as presents supportive data from North America in 2017.

Canola cultivars are highly diverse and grown in multiple regions and countries representing a wide variety of climates, soils and hardiness zones around the world. Common in much of the world, notably in the US, Canada, Australia, Europe, Ukraine, Russia, China, India and Chile (Figure 1).

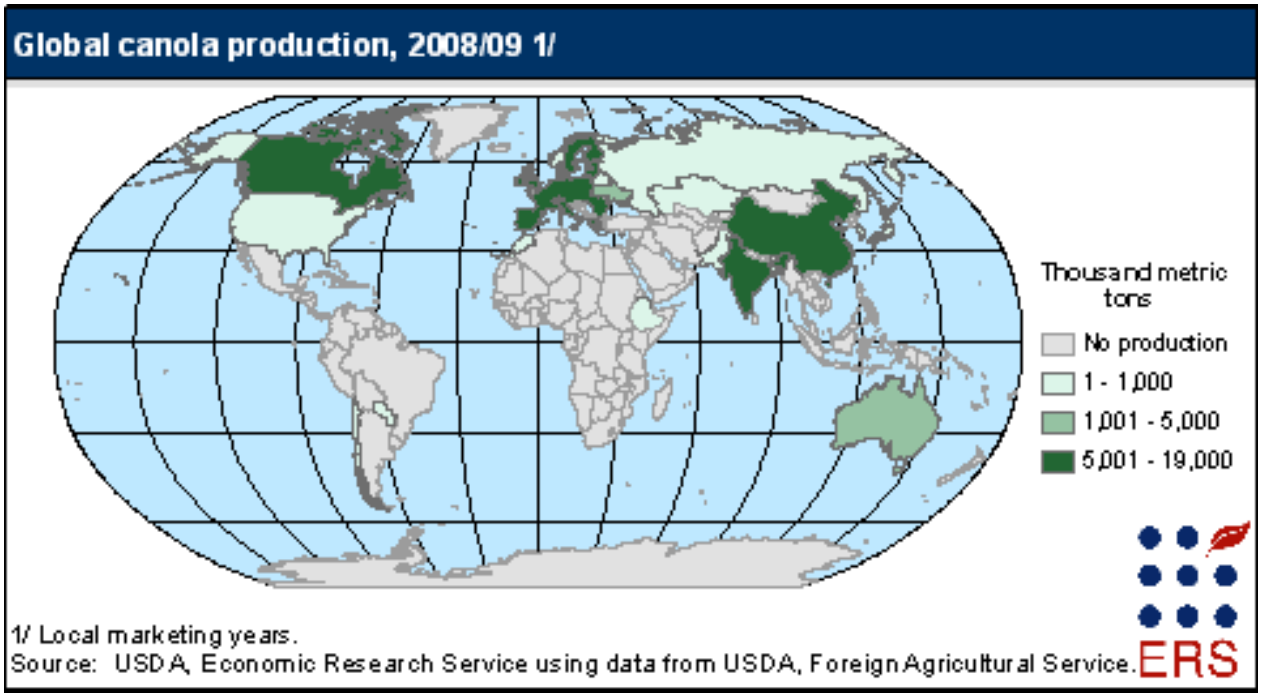

Figure 1. Global canola production. https://www.ers.usda.gov/topics/crops/soybeans-oil-crops/canola.aspx

In fact, rapeseed varieties are planted in many more countries throughout the world, as shown in Figure 2, with production estimated each year by the Food and Agricultural Organization of the United Nations. http://www.fao.org/faostat/en/#data/QC.

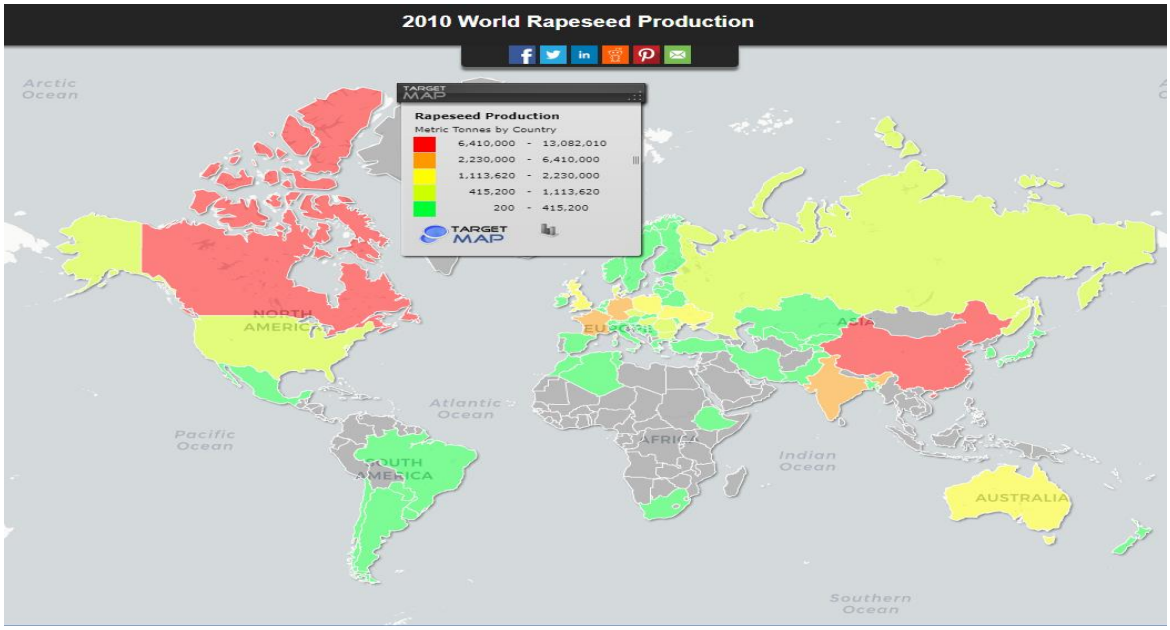

Figure 2. 2010 World Rapeseed Production

https://www.targetmap.com/viewer.aspx?reportId=17615 (accessed 13 February 2018)

Brassica napus is considered naturalized in much of the world, including throughout Canada, the US, Mexico, Central America and South America all the way to Chile. http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/directive-94-08/biology-documents/brassica-napus-l-/eng/1330729090093/1330729278970.

Hardiness zones were developed primarily for making planting recommendations for perennial plants but have also been used for phytosanitary risk analysis as an indicator of establishment potential. In 2008, a study was published that proposed an updated, uniform global plant hardiness map which could be used as a phytosanitary risk analysis tool (Magarey et al., 2008). Figure 3 shows that much of the Australian-grown canola is located in a hardiness zone similar to canola grown in the southern US, a zone where Nuseed grew a trial in 2016/17.

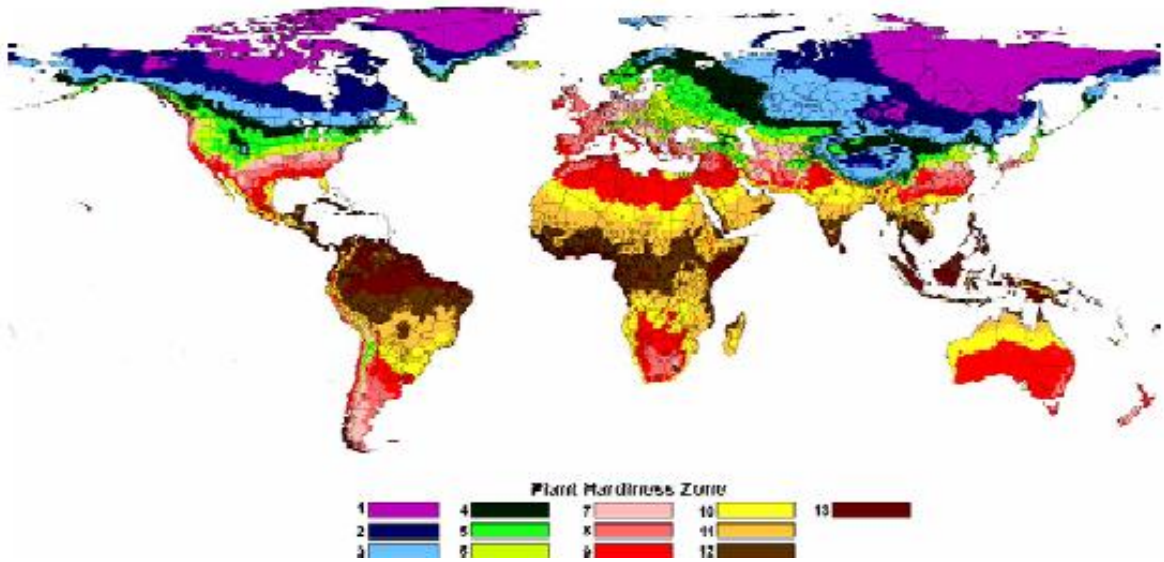

Figure 3. Thirty-year global plant hardiness zone map for the period 1978–2007

Although canola is grown in a wide range of soils, climates and environments, extensive research is performed every year which contributes to further understanding the phenological and agronomic yield drivers, leading to improved cultivars while extending the potential commercialization regions.

In Australia, canola is grown across the southern dryland cropping zone and mostly within winter-dominant rainfall environments with spring type cultivars that have low vernalization requirements, with yield primarily determined by available water during the growing season and water use efficiency of the cultivar, much as you would expect in dryer regions of the US and Canada. In general, Australian cultivars are considered to have high resistance to blackleg, which is also important to North America canola producers.

In Canada, spring canola is grown in the western provinces of Alberta, Saskatchewan and Manitoba. British Columbia, Ontario and Quebec also grow a substantial amount. New varieties are pushing the boundaries of where canola is being successfully grown, including the US Pacific Northwest states and across other northern states, mostly North Dakota (Brown et al., 2008). Canola is already cultivated in many other states besides, and has a wide range of potential geographic distribution. For example, much research has been done to improve varieties in Arkansas, Tennessee, North Carolina, South Carolina, Louisiana, Mississippi, Alabama, Georgia and Florida, representative of plant hardiness zones 3-8, as well as zones 1 and 2 in Canada. Acreage in the US is published by the USDA, and highlights production in Idaho, Minnesota, Montana, North Dakota, Oklahoma, Oregon and Washington, as well as Colorado and Kansas. https://www.usda.gov/nass/PUBS/TODAYRPT/cropan16.pdf.

Winter and spring canola have been produced on limited acreages in Washington State for many years. (Hang et al, 2009). Winter canola is planted in the fall from late August to mid-September in central Washington. Spring canola is planted from early April to early May depending on the soil moisture and temperature and type of canola. Ideally, canola needs to set blooms before temperatures reach 90 °F. It requires a similar amount of irrigation water as winter canola, about 10–12 inches.

The University of California, Davis, has been investigating canola since 2006, planting extensive variety trials and agronomic research at locations throughout California. (George et al., 2015). In fact, in their research they acknowledged that southern and western Australia canola growing regions are climatically comparable to much of California, and as such, refer California growers to information from those Australian regions. Recent trials in Australia have shown that spring sowing of winter canola can provide increased returns compared to a summer fallow. (GRDC, 2015). The advantages of a canola rotation have led to increased adoption especially for grazing options in recent years in Victoria's Western Districts.

SOILS

Canola grows on soil pH ranging from 5.0 to 8.0, yielding best when grown on deep, well-drained, silt loam soils; however, it has been including the loam soils commonly found throughout the Central Valley, Central Coast and Imperial Valley of California. Several cultivars have shown good yield potential and adaptability to growing conditions in the Great Plains region (Colorado, Kansas, Missouri, Nebraska, Oklahoma, Texas and Wyoming).

INSECTS

Several similar insect pests visit canola fields in both North America and Australia, including mites, aphids, moths, caterpillars and flea beetles.

DISEASES

The most important pathogen is blackleg (*Leptosphaeria maculans*), which can be carried over on infected stubble. Resistance genes were bred into canola to mitigate this important pest. Sclerotinia is also an important disease, and occasionally alternaria and mildews.

All field trials of DHA canola were observed for naturally occurring disease or insect biotic stressors, and an agronomic analysis was provided across ten different growing locations in Australia and Canada from 2015 and 2016. DHA canola was compared to AV Jade and at least 6 different commercial reference standard varieties. Across all measured parameters the DHA canola values fell within the range of the commercial reference standard varieties and were comparable to AV Jade. Another study was completed in 2016 in the Imperial valley of southern California. Three agronomic evaluation studies were performed in 2017 in Canada, and another three in the US. Observations of these studies are reported in the tables below. Finally, around 3,000 acres of DHA canola was grown for oil production for research testing in Washington and Oregon, and no unexpected diseases or insect pressure was observed. Although those acres were not analyzed in detail for agronomic comparisons, the growth rate, flowering, and harvest times produced yields within the ranges observed in other regions.

Ecological Observations for DHA Canola

Table 1. Canada: Insect Stressor Comparison between DHA Canola and Control

Year	Location	Insect Stressors	Severity Range*	DHA canola vs AV Jade
2016	Coalhurst, AB	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild-moderate	Same
2016	Vanguard, SK	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	Same
2017	Minto, MB	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	Same
2017	Vanguard, SK	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	Same
2017	Saskatoon, SK	Flea beetle (*Phyllothreta cruciferae* or *Phyllothreta striolata*)	Mild	Same

*Range of severity scores: Mild – very little insect injury (<10%) visible; moderate – noticeable plant tissue damage (10% to 30%)

Table 2. Australia: Insect Stressor Comparison between DHA Canola and Control

Year	Location	Insect Stressors	Severity Range*	DHA canola vs AV Jade
2015	All 8	Earth mites, *Halotydeus destructor*; Penthaleus spp; Lucerne flea, *Sminturus viridis*; False wireworms (*Isopteron punctatissimus*, Adelium spp.) Turnip aphid (*Lipaphis erysimi*), Cabbage aphid (*Brevicoryne brassicae*), Green peach aphid (*Myzus persicae*) Native budworm (*Helicoverpa punctigera*) Rutherglen bug (*Nysius vinitor*)	Mild	Same
2016	All 8	European earwig (*Foricula auricularia*) *Plutella xylostella*	Mild Mild	Same
2016	All 8	CROP ESTABLISHMENT Earth mites (*Halotydeus destructor*, Penthaleus spp.) Lucerne flea (*Sminthurus viridis*)	Mild Mild Mild	Same

		False wireworms (*Isopteron punctatissimus*, Adelium spp.)	Mild Mild	
2016	All 8	CROP FLOWERING AND PODDING Turnip aphid (*Lipaphis erysimi*), Cabbage aphid (*Brevicoryne brassicae*), Green peach aphid (*Myzus persicae*) Native budworm (*Helicoverpa punctigera*) Rutherglen bug (*Nysius vinitor*)	Mild Mild Mild Mild Mild Mild	Same

*Range of severity scores: Mild – very little insect injury (<10%) visible

Table 3. Canada: Disease Stressor Comparison between DHA Canola and Control

Yeara	Location	Disease Stressors	Severity Range*	DHA canola vs AV Jade
2016	Coalhurst, AB	Blackleg (*Leptosphaeria maculans*)	Mild	Same
2016	Vanguard, SK	Alternaria (*Alternaria brassicae*)	Mild-moderate	Same
2017	Minto, MB	None	NA	Same
2017	Vanguard, SK	None	NA	Same
2017	Saskatoon, SK	None	NA	Same

*Range of severity scores: Mild – very little disease injury (<10%) visible; Moderate – noticeable plant tissue damage (10% to 30%)

Table 4. Australia: Disease Stressor Comparison between DHA Canola and Control

Year	Location	Disease Stressors	Severity Range*	DHA canola vs AV Jade
2016	All 8	Blackleg (*Leptosphaeria maculans*); Alternaria (*Alternaria brassicae*)	Moderate; low	Same
2017	Ararat	Blackleg (*Leptosphaeria maculans*); Alternaria (*Alternaria brassicae*)	Moderate; low	Same

*Range of severity scores: Mild – very little disease injury (<10%) visible; Moderate – noticeable plant tissue damage (10% to 30%)

Table 5. USA: Insect Stressor Comparison between DHA Canola and Control

Year	Location	Insect Stressors	Severity Range*	DHA canola vs AV Jade
2016	CA	Aphids (*Myzus persicae*)	Mild	N/A
2017	SD	Flea beetle (*Phyllotreta cruciferae* or *Phyllothreta striolata*)	Mild	Same
2017	MN	Flea beetle (*Phyllotreta cruciferae* or *Phyllothreta striolata*)	Mild	Same
2017	ND	Flea beetle (*Phyllotreta cruciferae* or *Phyllothreta striolata*)	Mild	Same

*Range of severity scores: Mild – very little insect injury (<10%) visible

Table 6. USA: Disease Stressor Comparison between DHA Canola and Control

Year[a]	Location	Disease Stressors	Severity Range*	DHA canola vs AV Jade
2016	CA	None observed	N/A	N/A
2017	SD	Blackleg (*Leptosphaeria maculans* and/or *Leptosphaeria biglobosa*) Blackspot (*Alternaria brassicae* and/or other A. spp.)	Mild-moderate	Same
2017	MN	Blackleg (*Leptosphaeria maculans* and/or *Leptosphaeria biglobosa*)	Mild	Same
2017	ND	Blackleg (*Leptosphaeria maculans* and/or *Leptosphaeria biglobosa*)	Mild	Same

*Range of severity scores: Mild – very little disease injury (<10%) visible; Moderate – noticeable plant tissue damage (10% to 30%)

References

Roger D. Magarey, RD, Borchert,. DM, Schlegel, JW. 2008. Global plant hardiness zones for phytosanitary risk analysis. Scientia Agricola (Piracicaba, Brazil), v.65, special issue: 54-59.

Brown, J, David, JB, Lauver, M, Wysocki, D. 2008. U.S. Canola Association Canola growers' manual. U.S. Canola Association. University of Idaho and Oregon State University.

George, N, Hollingsworth, J, Kaffka, S. 2015. A guide for canola and camelina research in California, Department of Plant Sciences, University of California, Davis

Grain Research & Development Corporation. 2015. Spring-sown winter canola fact sheet. www.grdc.com.au.

Hang, AN, Collins, HP, Sowers, KE. 2009. Irrigated Spring and Winter Canola Production in Washington. WSU Extension Manual EM006E. Washington State University and USDA ARS Prosser, Washington.

Table 1. FAs in DHA canola

Common Name	Lipid Name	Δ^x Name	Chemical Name	Amount (%)*
OA	C18:1 n-9	$18:1^{\Delta 9}$	9-octadecenoic acid	35 – 50
LA	C18:2 n-6	$18:2^{\Delta 9,12}$	9,12-octadecadienoic acid	2 – 12
Gamma linolenic acid (GLA)	C18:3 n-6	$18:3^{\Delta 6,9,12}$	6,9,12-octadecatrienoic acid	0 – 7
ALA	C18:3 n-3	$18:3^{\Delta 9,12,15}$	9,12,15-octadecatrienoic acid	4 – 25
Stearidonic acid (SDA)	C18:4 n-3	$18:4^{\Delta 6,9,12,15}$	6,9,12,15-octadecatetraenoic acid	0 – 4
Eicosatetraenoic acid (ETA)	C20:4 n-3	$20:4^{\Delta 8,11,14,17}$	8,11,14,17-eicosatetraenoic acid	0 – 4
EPA	C20:5 n-3	$20:5^{\Delta 5,8,11,14,17}$	5,8,11,14,17-eicosapentaenoic acid	0 – 4
Docosapentaenoic acid (DPA)	C22:5 n-3	$22:6^{\Delta 7,10,13,16,19}$	7,10,13,16,19-docosapentaenoic acid	0 – 4
DHA	C22:6 n-3	$22:6^{\Delta 4,7,10,13,16,19}$	4,7,10,13,16,19-docosahexaenoic acid	6 – 15
Total monounsaturated FA (MUFAs)				4 – 40
Total PUFAs				20 – 75

*0 indicates < LOQ

Statistical analysis could not be conducted on C18:1 *trans*, C18:2 n-9, C18:2 *trans*, C18:3 n-6, C18:4 n-3, C18 total, C20:3 n-3, C20:3 total, C20:4 n-3, C20:4 total, C20:5 n-3, C22:1 n-9, C22:1 total, C22:4 n-3, C22:5 n-3, C22:5 n-6 and C22:5 total, because more than 30% of the values were <LOQ. And for FA profile analysis, data for C16:1 *trans*, C16:3 n-3, C20:2 n-9, C20:3 n-6, C20:3 n-9, C20:4 n-6, C22:2 n-6, and C22:4 n-6 were omitted as ALL results were below the LOQ.

Table 45. Fatty acid composition in seed

Analyte	Test Material*	Nº	Mean (%)	Std Dev	Range	p-value
C20:5 n-3	CMP	39	0.010	0.02	0.00-0.08	NA
	GMO	40	0.430	0.04	0.32-0.52	
	REF	279	0.00	0.00	0.00-0.40	NA
C22:5 n-3	CMP	39	0.03	0.05	0.00-0.20	NA
	GMO	40	1.05	0.09	0.80-1.23	
	REF	279	0.01	0.07	0.00-1.04	NA

*DHA canola (GMO), AV Jade (CMP) and commercial references (REF)