Author/s (year)	[redacted]
Title (version Nº)	**Protein characterization and safety of the proteins expressed in DHA canola (OECD ID NS-B50027-4)**
Owner	**Nuseed Pty Ltd**
Date	**January 17, 2017**
Project	**Omega 3 Canola**
Report Number	**2016-016**
Testing Facility	**CSIRO** **Black Mountain Laboratories** **Canberra, ACT** **Australia CSIRO Agriculture & Food** **Queensland Biosciences Precinct** **St Lucia, Brisbane, Queensland 4067** **Australia**
Test method	**LC-MRM-MS**
GLP	**No GLP**
Confidentiality	**None**

TITLE:

PROTEIN CHARACTERIZATION / SAFETY OF THE PROTEINS EXPRESSED IN DHA CANOLA (OECD ID NS-BÅØ27-4)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ALA	α-Linolenic acid, $18:3^{\Delta 9,12,15}$ (ω3)
CoA	Coenzyme A
DHA	Docosahexaenoic acid, $22:6^{\Delta 4,7,10,13,16,19}$ (ω3)
DHA canola	Genetically modified canola, event NS-B50027-4
DPA	Docosapentaenoic acid, $22:5^{\Delta 7,10,13,16,19}$ (ω3)
Elo	Fatty acid elongase
EPA	Eicosapentaenoic acid, $20:5^{\Delta 5,8,11,14,17}$ (ω3)
ETA	Eicosatetraenoic acid, $20:4^{\Delta 8,11,14,17}$ (ω3)
GLA	γ-linolenic acid, $C18:3^{\Delta 6,9,12}$ (ω6)
kDa	Kilo dalton
LA	Linoleic acid, $18:2^{\Delta 9,12}$ (ω6)
Lackl-Δ12D	*Lachancea kluyveri* Δ12-desaturase
LC-MS	Liquid chromatography-Mass Spectrometry
Micpu-Δ6D	*Micromonas pusilla* Δ6-desaturase
MMT	Million metric ton
OA	Oleic acid, $18:1^{\Delta 9}$
ω3 LC-PUFA	Omega-3 long-chain (≥C20) polyunsaturated fatty acids
ORF	Open reading frame
PAT	phosphinothricin N-acetyltransferase
Pavsa-Δ4D	*Pavlova salina* Δ4-desaturase
Pavsa-Δ5D	*Pavlova salina* Δ5-desaturase
pI	Theoretical isoelectric point
Picpa-ω3D	*Pichia pastoris* Δ15-/ω3-desaturase
Pyrco-Δ5E	*Pyramimonas cordata* Δ5-elongase
Pyrco-Δ6E	*Pyramimonas cordata* Δ6-elongase
PUFA	Polyunsaturated fatty acid
SDA	Stearidonic acid, $18:4^{\Delta 6,9,12,15}$ (ω3)
SGF	Simulated gastric fluid
SP	Secretion peptide
X:Y	A fatty acid containing X carbons with Y double bonds

EXECUTIVE SUMMARY

In collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO), Nuseed Pty Ltd has developed genetically modified canola event NS-B50027-4 which accumulates significant amounts of docosahexaenoic acid (DHA, 22 : 6ω3) in the seed oil (DHA canola). The purpose of this report is to summarize all of the current safety information regarding the newly expressed proteins and the elite event itself, DHA canola.

The results from these studies demonstrated that the genes, gene sources and proteins raise no safety concerns for DHA canola. The vector used to produce DHA canola was specifically designed to convert oleic acid (OA) to DHA in canola seed, and contained expression cassettes of seven microalgae and yeast genes (*Lachancea kluyveri* Δ12-desaturase, Lackl-Δ12D; *Pichia pastoris* ω3-/Δ15-desaturase, Picpa-ω3D: *Micromonas pusilla* Δ6-desaturase, Micpu-Δ6D; *Pyramimonas cordata* Δ6-elongase, Pyrco-Δ6E; *Pavlova salina* Δ5-desaturase, Pavsa-Δ5D; *P. cordata* Δ5-elongase, Pyrco-Δ5E; *P. salina* Δ4-desaturase (Pavsa-Δ4D) in the DHA biosynthetic pathway and the herbicide selection marker phosphinothricin N-acetyltransferase (PAT) gene. The elite event contains two T-DNA inserts that are required to produce the desired trait. DHA canola is a stable event as measured across 5 generations by both genetic and phenotypic analysis. The potential open reading frame (ORF) analysis of these two T-DNA inserts did not find any similarities to known toxins or allergens.

The safety of the introduced proteins is supported by: 1) the history of safe use of proteins similar to those in DHA canola that have been routinely consumed for many years; 2) their quick digestion in pepsin and/or trypsin; and 3) 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.

Agronomic and compositional analyses were conducted across 10 and 8 sites, respectively. The DHA canola values fell within the range of non-transgenic commercial varieties and were comparable to the parental canola variety in both agronomy and composition measurements, aside from the intended changes to the fatty acid pathway (e.g., high DHA).

In conclusions, 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.

TITLE:

PROTEIN CHARACTERIZATION / SAFETY OF THE PROTEINS EXPRESSED IN DHA CANOLA (OECD ID NS-B5ØØ27-4)

I. INTRODUCTION

The omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA) eicosapentaenoic acid (EPA, 20:5ω3), docosapentaenoic acid (DPA, 22:5ω3) and DHA are widely recognised for their beneficial roles in human health, particularly those related to cardiovascular and inflammatory health. EPA, DPA and DHA are primarily sourced from wild-caught fish oils and algal oils, with algae being the primary producer in the marine food web. These sources are under pressure due to increasing demand for ω3 LC-PUFA by aquaculture, nutraceutical and pharmaceutical applications. Additional sources of these fatty acids can be produced by engineering land-based oilseed crops to convert native fatty acids to marine-type ω3 LC-PUFA which are then accumulated in seed oil. Canola is a commonly grown oilseed with 67 million metric tons (MMT) of rapeseed produced globally in 2015/16[1].

In collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO), Nuseed Pty Ltd has developed genetically modified canola event NS-B50027-4, DHA canola, which accumulates significant amounts of DHA in the seed oil.

In this DHA canola, seven fatty acid desaturases and elongases were introduced to convert OA to DHA in a single pathway expression vector. The pathway (Figure 1) was comprised of the Lackl-Δ12D (Watanabe et al. 2004), Picpa-ω3D (Zhang et al. 2008), Micpu-Δ6D (Petrie et al. 2010b), Pyrco-Δ6E (Petrie et al. 2010a), Pavsa-Δ5D (Zhou et al. 2007), Pyrco-Δ5E (Petrie et al. 2010a) and Pavsa-Δ4D (Zhou et al. 2007). The functionalities and activities of these enzymes have been demonstrated in different heterologous expression systems (see Report Nºs 2016-005, 2016-006, 2016-007, 2016-008, 2016-009, 2016-010, 2016-011) and in transgenic Arabidopsis or Camelina seeds (Petrie et al. 2012; Petrie et al. 2014). Based on the sequence similarity and functionality, these seven proteins can be classified into three groups: (1) yeast acyl-CoA type fatty acid desaturases including Lackl-Δ12D and Picpa-ω3D (Figure 1, blue) that introduce a double bond at the Δ12 and Δ15 positions, respectively; (2) algae

[1] http://www.ers.usda.gov/data-products/oil-crops-yearbook/oil-crops-yearbook/#World Supply and Use of Oilseeds and Oilseed Products

fatty acid elongases including Pyrco-Δ6E and Pyrco-Δ5E (Figure 1, purple) that add two carbons to the carboxyl end of fatty acids; and (3) algae front-end fatty acid desaturases that introduce a double bond between an existing double bond and the carboxyl end of fatty acids (Zhou et al. 2007) including Micpu-Δ6D, Pavsa-Δ5D and Pavsa-Δ4D (Figure 1, green).

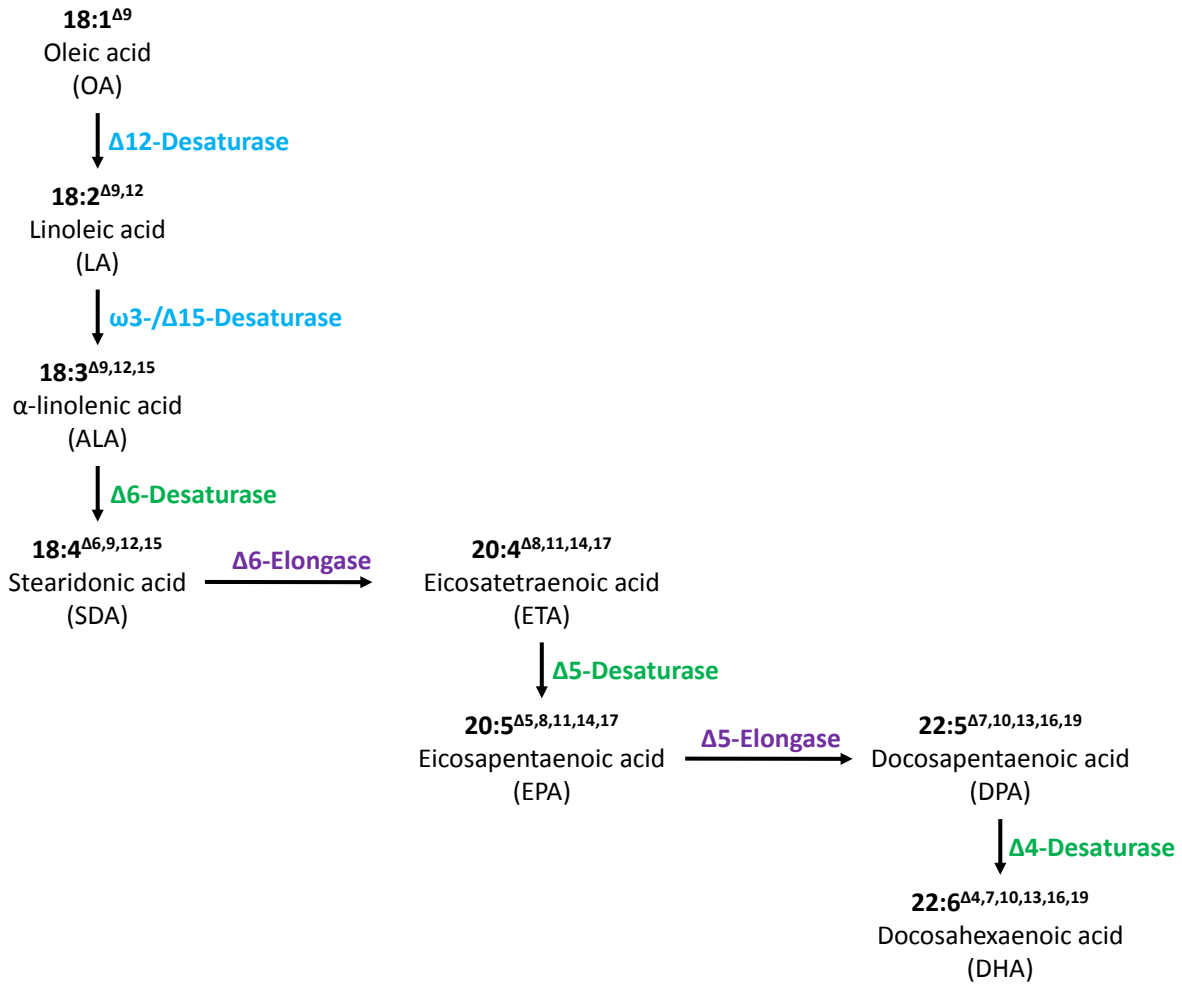

Figure 1. DHA biosynthesis pathway engineered into DHA canola event NS-B50027-4. Seven enzymes introduced in canola to convert oleic acid to final product docosahexaenoic acid were grouped into 3 classes, two fatty acid desaturases from yeast in blue, two elongases from microalgae in purple, and three front-end desaturases from microalgae in green (see text for detail).

II. PURPOSE

The purpose of this report is to summarize all of the current information regarding the newly expressed proteins in DHA canola. These elongases and desaturases are all enzymes in the fatty acid pathway that have been engineered into canola to convert OA to DHA. Each of the enzymes has been characterized and the stability/digestibility of three representative enzymes from each group, Picpa-ω3D, Pyrco-Δ5E and Pavsa-Δ4D, have been determined. Safety data from the elite event DHA canola is also presented, including a brief summary of the agronomic and composition results. Additionally, the protein content of each introduced protein was quantified in DHA canola tissues collected over a growing season.

III. DHA CANOLA PATHWAY GENES

A. GENE SOURCES

The *Lackl-Δ12D* gene used in DHA canola was previously cloned from yeast *L. kluyveri* (Watanabe et al. 2004). *L. kluyveri* was formerly called as *Saccharomyces kluyveri,* and was reclassified (Kurtzman, 2003). The open reading frame (ORF) of *Lackl-Δ12D* gene in DHA canola consisted of 1251 bp, and is shown in Figure 2 (Report Nº 2016-005).

The ω3-desaturase gene used in DHA canola event was previously cloned from yeast *P. pastoris* (Zhang et al. 2008). *P. pastoris* was also called as *Komagataella pastoris.* The ORF of *Picpa-ω3D* gene consisted of 1248 bp, and is shown in Figure 2 (Report Nº 2016-006).

The *Micpu-Δ6D* gene was previously cloned from microalga *M. pusilla* (Petrie et al. 2010b). The ORF of *Micpu-Δ6D* gene consisted of 1392 bp, and is shown in Figure 2 (Report Nº 2016-007).

The *Pyrco-Δ6E* gene was previously cloned from microalga *P. cordata* (Petrie et al. 2010a). The ORF of *Pyrco-Δ6E* gene consisted of 867 bp, and is shown in Figure 2 (Report Nº 2016-008).

The *Pavsa-Δ5D* gene was previously cloned from microalga *P. salina* (Zhou et al. 2007). The ORF of *Pavsa-Δ5D* gene consisted of 1278 bp, and is shown in Figure 2 (Report Nº 2016-009).

The *Pyrco-Δ5E* gene was previously cloned from microalga *P. cordata* (Petrie et al. 2010a). The ORF of *Pyrco-Δ5E* gene consisted of 804 bp, and is shown in Figure 2 (Report Nº 2016-010).

The Δ4-desaturase gene used in DHA canola was previously cloned from the microalga *P. salina* (Zhou et al., 2007). The ORF of the *Pavsa-Δ4D* gene consisted of 1344 bp, is shown in Figure 2 (Report Nº 2016-011).

B. DNA CONSUMPTION SAFETY

DNA is the basis for all life with the same nucleic acids and structure, and as such is generally recognized as safe. Humans and animals are exposed to DNA in everything they consume. The addition of transgenes to canola, such as those from the fatty acid pathway in DHA canola, is indistinguishable from any other DNA that is present in their diet.

It is important to note that foreign DNA represents a substantial part of regular food and feed and typically several grams of DNA are consumed each and every day. Decades of research have indicated that dietary DNA poses no direct toxicity on human health. On the contrary, exogenous nucleotides have been shown to play important beneficial roles in gut function and the immune system (Carver et al. 1999).

Nucleic acids are split into nucleotides in the intestine by the pancreatic nucleases, and the nucleotides are further digested into nucleosides and phosphoric acid by surface bound enzymes located on mucosal cells. The nucleosides are finally digested into their constituents: sugars, purine and pyrimidine bases.

Transgenes are composed of the same nucleic acids and they are handled by the digestive system in the same manner as typical food genes. Furthermore, a transgene is usually present in extremely small amounts. Finally, the EU-sponsored research project ENTRANSFOOD and GMOBILITY, related to the safe use of GM food and GM feed has confirmed that introduced DNA is indistinguishable from the host DNA in its physical and chemical properties and behaves identically (Van den Eede et al. 2004).

C. POTENTIAL HORIZONTAL GENE TRANSFER

In vivo gene transfer of DNA from GM plants to gastrointestinal bacteria or mammalian cells (horizontal gene transfer, HGT), while theoretically possible, is a very remote possibility. DNA from transgenes is no different from DNA that has been consumed throughout history without any adverse effects. The ENTRANSFOOD working group, the European network on safety assessment of genetically modified food crops, confirmed that consumption of transgenic food or feed does not pose any additional generalized risk (Van den Eede et al. 2004). This project delivered an excellent review publication that supports the lack of HGT.

Although it can occur theoretically, DNA transfer from plant to eukaryotic cells would have to be under extremely stringent laboratory conditions, which cannot be realistically extrapolated to food or feed consumption. Even under such conditions, the potential transfer of DNA remains very improbable with estimates of $2x10^{-11}$ to $1.3x10^{-21}$ per bacterium (Van den Eede et al. 2004). The presence of ingested DNA sequences in immune and gut cells makes adverse consequences unlikely. Somatic cells of the gut lining have a rapid turnover, such that the most likely fate of modified cells is to be lost in the faeces (Van den Eede et al. 2004). In addition, one of the functions of the immune cells is to eliminate foreign DNA.

D. DHA CANOLA GENE SAFETY

The genes introduced into DHA canola are no different from the genetic material that humans and animals consume on a routine basis in food and feeds. Furthermore, as outlined in the characterization reports (2016-005, 2016-006, 2016-007, 2016-008, 2016-009, 2016-010, 2016-011), none of the sources of these genes 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.

IV. CHARACTERIZATION OF THE DHA PATHWAY ENZYMES

A. *LACHANCEA KLUYVERI* Δ12-DESATURASE

The translated *L. kluyveri* Δ12-desaturase (Lackl-Δ12D) contained 416 amino acid residues (Figure 2). The molecular weight of Lackl-Δ12D is predicted as 48.2 kDa, with an estimated isoelectric point (pI) of 7.84. The *L. kluyveri* Δ12-desaturase (Lackl-Δ12D) protein catalyses the desaturation of OA at Δ12 position to linoleic acid (LA) ($18:1^{\Delta 9} \rightarrow 18:2^{\Delta 9,12}$). For the Lackl-Δ12D protein, there is one potential glycosylation site within this amino acid sequence derived from the nucleotide sequence of the inserted DNA (indicated in green).

MSAVTVTGSDPKNRGSSSNTEQEVPKVAIDTNGNVFSVPDFTIKDILGAIPHECYERR
LATSLYYVFRDIFCMLTTGYLTHKILYPLLISYTSNSIIKFTFWALYTYVQGLFGTGI
WVLAHECGHQAFSDYGIVNDFVGWTLHSYLMVPYFSWKYSHGKHHKATGHMTRDMVFV
PATKEEFKKSRNFFGNLAEYSEDSPLRTLYELLVQQLGGWIAYLFV**NVT**GQPYPDVPS
WKWNHFWLTSPLFEQRDALYIFLSDLGILTQGIVLTLWYKKFGGWSLFINWFVPYIWV
NHWLVFITFLQHTDPTMPHYNAEEWTFAKGAAATIDRKFGFIGPHIFHDIIETHVLHH
YCSRIPFYNARPASEAIKKVMGKHYRSSDENMWKSLWKSFRSCQYVDGDNGVLMFRNI
NNCGVGAAEK

Figure 2. Amino acid sequence of Lackl-Δ12D.

Similarity to other proteins in consumed foods, used in food production or in animal feeds
The fatty acid Δ12-desaturases have been cloned from a wide range of organisms and share high homology to other Δ12-desaturase proteins, 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).

The Lackl-Δ12D shared high homology (33-79%) to other Δ12-desaturase proteins from yeasts, fungi and various crop plants, such as canola, rice, soybean, flax, sunflower and sesame (see Table 1, Report Nº 2016-005). Sesame, linseed, soybean, sunflower and canola are typical oil crops for food application. Specifically, the introduced Lackl-Δ12D protein in DHA canola shared 36% of sequence identity with the endogenous canola Δ12-desaturase.

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 where the gene was cloned is widely used 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.

Microbial food cultures (MFC) 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 present "Inventory of MFC", including many isolates that express Δ12-desaturase proteins. These microbial cultures are important in the production of cheeses, cocoa beans, tea, beer and wine, which are widely consumed. A number of food proteins and enzymes have also been expressed in *P. pastoris* (Batt, 2014).

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 1 shows the enzyme activity of Lackl-Δ12D expressed as fusion proteins in *P. pastoris* with or without secretion peptide. Overexpression of Lackl-Δ12D fusion protein in *P. pastoris* substantially increased the desaturation activity of 18:1 to 18:2 compared to vector alone (Table 1). In addition, the His_{10}::Lackl-Δ12D had higher activity than SP::His_{10}::Lackl-Δ12D.

Table 1. 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 fatty acid in cells. Conversion rate is based on all the products (including part of 18:2 that have been further desaturated by *P. pastoris* host cell ω3-desaturase) compare to the remaining substrate 18:1. SP, secretion peptide. n = repeats with individual colonies.

The genome sequence including the T-DNA insertions in DHA canola was analysed. The translated amino acid sequence of Lackl-Δ12D in the insert was confirmed to be identical to the original sequence (see Figure 8, Report Nº 2016-005).

B. *PICHIA PASTORA* ω3-DESATURASE

The translated, *P. pastoris* ω3/Δ15-desaturase (Picpa–ω3D) contained 415 amino acid residues (Figure 3). The molecular weight of Lackl-Δ12D is predicted as 47.8 kDa, with estimated pI as 7.67. For the Picpa-ω3D protein, there are two potential glycosylation sites within this amino acid sequence derived from the nucleotide sequence of the inserted DNA (indicated in green).

MSKVTVSGSEILEGSTKTVRRSGNVASFKQQKTAIDTFGNVFKVPDYTIKDILDAIPK
HCYERSLVKSMSYVVRDIVAISAIAYVGLTYIPLLPNEFLRFAAWSAYVFSISCFGFG
IWILGHECGHSAFSNYGWV**NDT**VGWVLHSLVMVPYFSWKFSHAKHHKATGHMTRDMVF
VPYTAEEFKEKHQVTSLHDIAEETPIYSVFALLFQQLGGLSLYLAT**NAT**GQPYPGVSK
FFKSHYWPSSPVFDKKDYWYIVLSDLGILATLTSVYTAYKVFGFWPTFITWFCPWILV
NHWLVFVTFLQHTDSSMPHYDAQEWTFAKGAAATIDREFGILGIIFHDIIETHVLHHY
VSRIPFYHAREATECIKKVMGEHYRHTDENMWVSLWKTWRSCQFVENHDGVYMFRNCN
NVGVKPKDT

Figure 3. Amino acid sequence of Picpa-ω3D.

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

The fatty acid ω3/Δ15-desaturases have been cloned from a wide range of organisms and share high homology to other Δ15-desaturase proteins, including cynobacteria (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 (22-72%) to other ω3/Δ15-desaturase proteins from yeasts, fungi and various crop plants, such as canola, soybean, flax and sesame (see Table 1, Report Nº 2016-006). Sesame, soybean, flax and canola are typical oil crops for food application. Specifically, the introduced Picpa-ω3D protein in DHA canola shared 28% of 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 misnomer *Lindnera jadinii*) is a yeast stain 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 generally recognized as safe (GRAS) by the US Food and Drug Administration.

The yeast *Lipomyces starkeyi* and *L. kononenkoae* have been used in food related applications especially due to 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 fermented milk.

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 as SP::His_{10}::Picpa-ω3D or His_{10}::Picpa-ω3D. Table 2 shows the enzyme activity of Picpa-ω3D expressed as fusion proteins in *P. pastoris* with or without secretion peptide. Overexpression of Picpa-ω3D fusion protein in *P. pastoris* substantially increased the desaturation activity of 18:2 to 18:3 compared to vector alone (Table 2). In addition, the His_{10}::Picpa-ω3D had higher activity than SP::His_{10}::Picpa-ω3D.

Table 2. 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 fatty acid 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, secretion peptide. n = repeats with individual colonies.

The genome sequence including the T-DNA insertions in DHA canola was analysed. The translated amino acid sequence of Picpa-ω3D in the insert was confirmed to be identical to the original sequence (see Figure 8, Report Nº 2016-006).

Digestibility of Picpa-ω3D

Digestibility / stability of the Picpa-ω3D protein was assessed with an *in vitro* stability assay (Thomas et al. 2004) using a standard protocol. Picpa-ω3D protein was tested with simulated gastric fluid (SGF) comprising the proteolytic enzyme, pepsin, and in combination with a

novel pepsin-trypsin assay employing state-of-the-art mass spectrometric approaches 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 (as a proxy for intact protein). The allergenic potential of a protein is determined by a weight of evidence approach since no single method can predict the allergenicity of a protein. Protein digestibility is one aspect of the overall allergenicity assessment that is conducted for newly introduced proteins into genetically modified crops.

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 (Report Nº 2016-012). 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. The Picpa-ω3D protein was used as the representative of the two yeast acyl-CoA type fatty acid desaturases (Picpa-ω3D and Lackl-Δ12D; see Figure 1) engineered into DHA canola, for stability analysis.

The thermal stability of the trans-membrane enzyme proteins, such as desaturases and elongases, cannot be as easily characterized *in vitro* as it can for non-membrane bound enzymes. Membrane proteins, and especially trans-membrane proteins, are not very thermally stable and are difficult to refold once they are partially or fully denatured (Bowie, 2001). In the processing of canola seeds to produce oil, the seed material reaches temperatures ranging from 80ºC to 115ºC. It is improbable that any trans-membrane proteins will be remain in the native folded state at this temperature and even after cooling, it will be unlikely to refold correctly. Therefore, these desaturases and elongases are not thermally stable.

C. *MICROMONAS PUSILLA* Δ6-DESATURASE

The translated *M. pusilla* Δ6-desaturase (Micpu-Δ6D, EEH58637) contained 463 amino acid residues (Figure 4). The molecular weight of Micpu-Δ6D is predicted as 52.9 kDa, with estimated pI of 9.00. For the Micpu-Δ6D protein, there is no potential glycosylation site within this amino acid sequence derived from the nucleotide sequence of the inserted DNA.

MCPPKTDGRSSPRSPLTRSKSSAEALDAKDASTAPVDLKTLEPHELAATFETRWVRVE
DVEYDVTNFKHPGGSVIFYMLANTGADATEAFKEFHMRSLKAWKMLRALPSRPAEIKR
SESEDAPMLEDFARWRAELERDGFFKPSITHVAYRLLELLATFALGTALMYAGYPIIA
SVVYGAFFGARCGWVQHEGGHNSLTGSVYVDKRLQAMTCGFGLSTSGEMWNQMHNKHH
ATPQKVRHDMDLDTTPAVAFFNTAVEDNRPRGFSRAWARLQAWTFVPVTSGLLVQAFW
IYVLHPRQVLRKKNYEEASWMLVSHVVRTAVIKLATGYSWPVAYWWFTFGNWIAYMYL
FAHFSTSHTHLPVVPSDKHLSWVNYAVDHTVDIDPSRGYVNWLMGYLNCQVIHHLFPD
MPQFRQPEVSRRFVPFAKKWGLNYKVLSYYGAWKATFSNLDKVGQHYYVNGKAEKAH

Figure 4. Amino acid sequence of Micpu-Δ6D.

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

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 ($18{:}3^{\Delta 6,9,12}$). For DHA canola, marine microalga Micpu-Δ6D with ω3-preference was used (Petrie et al. 2010b).

The Micpu-Δ6D protein shares about 17-61% sequence identity to other Δ6-desaturase proteins from fungi, salmon, evening primrose and canola (see Table 1, Report Nº 2016-007). The Micpu-Δ6D protein shares ~20% amino acid sequence identity with plant Δ6-desaturase proteins from *Echium 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.

Functional activity of Micpu-Δ6D

The enzyme functionality of Micpu-Δ6D has 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 (SP), followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal. Overexpression of Micpu-Δ6D fusion protein proteins in *P. pastoris* with or without secretion peptide demonstrated the desaturation of $18{:}3^{\Delta 9,12,15}$ to $18{:}4^{\Delta 6,9,12,15}$ compared to the vector alone where there was not any 18:4 product (Table 3). In addition, the His_{10}::Micpu-Δ6D had higher activity than SP::His_{10}::Micpu-Δ6D in *P. pastoris*.

Table 3. 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 fatty acid in cells. Conversion rate is based on the product 18:4 compared to the total of product 18:4 and remaining substrate 18:3. SP, secretion peptide. 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 analysed. The translated amino acid sequence of Micpu-Δ6D in the insert was confirmed to be identical to the original sequence (see Figure 8, Report Nº 2016-007).

Digestibility of Micpu-Δ6D

Micpu-Δ6D is an algae front-end fatty acid desaturases that introduces a double bond between an existing double bond and the carboxyl end of fatty acids (Zhou et al. 2007), including Micpu-Δ6D, Pavsa-Δ5D and Pavsa-Δ4D (Figure 1, green). One representative of this group, Pavsa-Δ4D, was analysed for protein stability (see section G. below).

The thermal stability of the trans-membrane enzyme proteins, such as desaturases and elongases, cannot be as easily characterized *in vitro* as it can for non-membrane bound enzymes. Membrane proteins, and especially trans-membrane proteins, are not very thermally stable and are difficult to refold once they are partially or fully denatured (Bowie, 2001). In the processing of canola seeds to produce oil, the seed material reaches temperatures ranging

from 80ºC to 115ºC. It is improbable that any trans-membrane proteins will be remain in the native folded state at this temperature and even after cooling, it will be unlikely to refold correctly. Therefore, these desaturases and elongases are not thermally stable.

D. *PYRAMIMONAS CORDATA* Δ6-ELONGASE

The translated *P. cordata* Δ6-elongase (Pyrco-Δ6E, ACR53359) contained 288 amino acid residues (Figure 5). The molecular weight of Pyrco-Δ6E is predicted as 33.1 kDa, with estimated pI of 9.09. For the Pyrco-Δ6E protein, there is no potential glycosylation site within this amino acid sequence derived from the nucleotide sequence of the inserted DNA.

MEFAQPLVAMAQEQYAAIDAVVAPAIFSATDSIGWGLKPISSATKDLPLVESPTPLIL
SLLAYFAIVGSGLVYRKVFPRTVKGQDPFLLKALMLAHNVFLIGLSLYMCLKLVYEAY
VNKYSFWGNAYNPAQTEMAKVIWIFYVSKIYEFMDTFIMLLKGNVNQVSFLHVYHHGS
ISGIWWMITYAAPGGDAYFSAALNSWVHVCMYTYYFMAAVLPKDEKTKRKYLWWGRYL
TQMQMFQFFMNLLQAVYLLYSSSPYPKFIAQLLVVYMVTLLMLFGNFYYMKHHASK

Figure 5. Amino acid sequence of Pyrco-Δ6E.

Similarity to other proteins in consumed foods, used in food production or in animal feeds
Pyrco-Δ6E shares amino acid sequence identities (19-64%) to many other elongases presented in food that is consumed, used in food production or in animal feeds (see Table 1, Report Nº 2016-008). Several human PUFA elongases (Elo) 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 trout bifunctional Δ5/Δ6-elongase (AAV67803) or salmon Elo (AAO13175). Salmon is a well-known salt-water fish consumed as food.

Mortierella 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. alpine* Δ6E (AAF70417).

Finally, Pyrco-Δ5E shares 22% sequence identity to soybean fatty acid elongase (XP_003531583). Soybean is one of the major oil crops produced for food oil.

Functional activity of Micpu-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 *Saccharomyces cerevisiae α*-mating type signal peptide (SP), followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal. Overexpression of Pyrco-Δ6E fusion protein proteins in *P. pastoris* with secretion peptide confirmed the desaturation activity of $18{:}4^{\Delta 6,9,12,15}$ to $20{:}4^{\Delta 8,11,14,17}$ compared to the vector alone where there was not any 20:4 product (Table 4). In addition, the His_{10}::Pyrco-Δ6E had higher activity than SP::His_{10}::Pyrco-Δ6E in *P. pastoris*.

Table 4. 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 fatty acid in cells. Conversion rate is based on the product 20:4 compared to the total of product 20:4 and remaining substrate 18:4. SP, secretion peptide. 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 analysed. The translated amino acid sequence of Pyrco-Δ6E in the insert was confirmed to be identical to the original sequence (see Figure 8, Report Nº 2016-008).

Digestibility of Pyrco-Δ6E

Pyrco-Δ6E protein is an algae fatty acid elongases that adds two carbons to the carboxyl end of fatty acids including Pyrco-Δ6E and Pyrco-Δ5E (Figure 1, purple). One representative of this group, Pyrco-Δ5E, was analysed for protein stability (see section F. below).

The thermal stability of the trans-membrane enzyme proteins, such as desaturases and elongases, cannot be as easily characterized *in vitro* as it can for non-membrane bound enzymes. Membrane proteins, and especially trans-membrane proteins, are not very thermally stable and are difficult to refold once they are partially or fully denatured (Bowie, 2001). In the processing of canola seeds to produce oil, the seed material reaches temperatures ranging from 80ºC to 115ºC. It is improbable that any trans-membrane proteins will be remain in the native folded state at this temperature and even after cooling, it will be unlikely to refold correctly. Therefore, these desaturases and elongases are not thermally stable.

E. *PAVLOVA SALINA* Δ5-DESATURASE

The translated *P. salina* Δ5-desaturase (Pavsa-Δ5D, ABL96295) contained 425 amino acid residues (Figure 6). The molecular weight of Pavsa-Δ5D is predicted as 48.2 kDa, with estimated pI of 8.18. For the Pavsa-Δ5D protein, there are two potential glycosylation sites within this amino acid sequence derived from the nucleotide sequence of the inserted DNA (highlighted in green).

MPPRDSYSYAAPPSAQLHEVDTPQEHDKKELVIGDRAYDVTNFVKRHPGGKIIAYQV
GTDATDAYKQFHVRSAKADKMLKSLPSRPVHKGYSPRRADLIADFQEFTKQLEAEGM
FEPSLPHVAYRLAEVIAMHVAGAALIWHGYTFAGIAMLGVVQGRCGWLMHEGGHYSL
TGNIAFDRAIQVACYGLGCGMSGAWWRNQHNKHHATPQKLQHDVDLDTLPLVAFHER
IAAKVKSPAMKAWLSMQAKLFAPVTTLLVALGWQLYLHPRHMLRTKHYDELAMLGIR
YGLVGYLAANYGAGYVLACYLLYVQLGAMYIFCNFAVSHTHLPVVEPNEHATWVEYA
A**NHT**T**NCS**PSWWCDWWMSYLNYQIEHHLYPSMPQFRHPKIAPRVKQLFEKHGLHYDV
RGYFEAMADTFANLDNVAHAPEKKMQ

Figure 6. Amino acid sequence of Pavsa-Δ5D.

Similarity to other proteins in consumed foods, used in food production or in animal feeds
The Pavsa-Δ5D protein shares similarity (16-87%) to desaturase proteins presented in food that is consumed, used in food production or in animal feeds (Table 1, Report Nº 2016-009). Pavsa-Δ5D was cloned from *P. salina. P. salina* itself, is one of microalgae used in mariculture (Brown 1991). *P. lutheri* is used for oyster larvae and clam larvae feeds (Brown et al. 1997). Pavsa-Δ5D shares 53% sequence identity to *P. lutheri* Δ5-desaturase (ALE15225, partial sequence) in overlapping region.

Pavsa-Δ5D shares amino acid sequence identities to many other fatty acid desaturases from wide range of species. Pavsa-Δ5D 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 *Oenothera biennis* (evening primrose) (ACB47482), *Echium plantagineum* (echium, AAZ08559) and *Borago officinalis* (borage, O04353). These species have been used to produce oils that are relatively high in γ-linolenic acid (C18:$3^{\Delta 6,9,12}$, 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.

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), *Nicotiana 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 (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 5. The result showed the desaturation activity on 20:4$^{\Delta 8,11,14,17}$ (ω3) at Δ5-position producing 20:5$^{\Delta 5,\ 8,11,14,17}$ (ω3), although the conversion efficiency was low, where the vector alone led to no 20:5 product.

Table 5. 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 fatty acid in cells. Conversion rate is based on the product 20:4 $^{\Delta 8,11,14,17}$ (ω3) compared to the total of product 20:5$^{\Delta 5,8,11,14,17}$ (ω3) and remaining substrate 20:4 $^{\Delta 8,11,14,17}$ (ω3). SP, secretion peptide. 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 analysed. The translated amino acid sequence of Pavsa-Δ5D in the insert was confirmed to be identical to the original sequence (see Figure 8, Report Nº 2016-009).

Digestibility of Pavsa-Δ5D

Pavsa-Δ5D is an algae front-end fatty acid desaturases that introduces a double bond between an existing double bond and the carboxyl end of fatty acids (Zhou et al. 2007), including Micpu-Δ6D, Pavsa-Δ5D and Pavsa-Δ4D (Figure 1, green). One representative of this group, Pavsa-Δ4D, was analysed for protein stability (see section G. below).

The thermal stability of the trans-membrane enzyme proteins, such as desaturases and elongases, cannot be as easily characterized *in vitro* as it can for non-membrane bound enzymes. Membrane proteins, and especially trans-membrane proteins, are not very thermally stable and are difficult to refold once they are partially or fully denatured (Bowie, 2001). In the processing of canola seeds to produce oil, the seed material reaches temperatures ranging from 80ºC to 115ºC. It is improbable that any trans-membrane proteins will be remain in the

native folded state at this temperature and even after cooling, it will be unlikely to refold correctly. Therefore, these desaturases and elongases are not thermally stable.

F. *PYRAMIMONAS CORDATA* Δ5-ELONGASE

The translated *P. cordata* Δ5-elongase (Pyrco-Δ5E, ACR53360) contained 267 amino acid residues (Figure 7). The molecular weight of Pyrco-Δ5E is predicted as 31.3 kDa, with estimated pI of 9.33.

```
MASIAIPAALAGTLGYVTYNVANPDIPASEKVPAYFMQVEYWGPTIGTIGYL
LFIYFGKRIMQNRSQPFGLKNAMLVYNFYQTFFNSYCIYLFVTSHRAQGLKV
WGNIPDMTANSWGISQVIWLHYNNKYVELLDTFFMVMRKKFDQLSFLHIYHH
TLLIWSWFVVMKLEPVGDCYFGSSVNTFVHVIMYSYYGLAALGVNCFWKKYI
TQIQMLQFCICASHSIYTAYVQNTAFWLPYLQLWVMVNMFVLFANFYRKRYK
SKGAKKQ
```

Figure 7. Amino acid sequence of Pyrco-Δ5E.

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

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 long-chain 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 (18-37%) to other Δ5-elongase, Δ6-elongase or PUFA Elo proteins.

Pyrco-Δ5E shares amino acid sequence identities to other elongases presented in food that is consumed, used in food production or in animal feeds (Table 1, Report Nº 2016-010). Several human PUFA elongases (Elo) have been isolated (Leonard et al. 2004), including the EPA elongation (Δ5-elongation). They share the 24~28% of sequence identities with Pyrco-Δ5E. Pyrco-Δ5E also shares 27% or 28% sequence identity to trout bifunctional Δ5/Δ6-elongase (AAV67803) or salmon Elo (AAO13175). Salmon is a well-known salt-water fish consumed as food.

Finally, Pyrco-Δ5E shares 23% sequence identity to soybean fatty acid elongase (XP_003531583). Soybean is one of the major oil crops produced for food oil.

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 *Saccharomyces cerevisiae* α-mating type signal peptide (SP), followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal. Overexpression of Pyrco-Δ5E fusion protein proteins in *P. pastoris* with secretion peptide demonstrated the elongation of $20{:}5^{\Delta5,8,11,14,17}$ to $22{:}5^{\Delta7.10.13.16.19}$ compared to the vector alone where there was no 22:5 product (Table 6).

Table 6. 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 fatty acid in cells. Conversion rate is based on the product 18:4 compared to the total of product 18:4 and remaining substrate 18:3. SP, secretion peptide. n = repeats with individual colonies. Yeast cell culture was fed with 0.5 mM $20{:}5^{\Delta5,8,11,14,17}$ substrate.

The genome sequence including the T-DNA insertions in DHA canola was analysed. The translated amino acid sequence of Pavsa-Δ5E in the insert was confirmed to be identical to the original sequence (see Figure 8, Report Nº 2016-010).

Digestibility of Pyrco-Δ5E

Digestibility / stability of the Pyrco-Δ5E protein was assessed with an *in vitro* stability assay (Thomas et al. 2004) using a standard protocol. Pyrco-Δ5E protein was tested with simulated gastric fluid (SGF) comprising the proteolytic enzyme, pepsin, and in combination with a novel pepsin-trypsin assay employing state-of-the-art mass spectrometric approaches 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 (as a proxy for intact protein). The allergenic potential of a protein is determined by a weight of evidence approach since no single method can predict the allergenicity of a protein. Protein digestibility is one aspect of the overall allergenicity assessment that is conducted for newly introduced proteins into genetically modified crops.

The results of the study demonstrated that greater than 75% of the Pyrco-Δ5E protein digested within 5 min and full-length protein was rapidly digested within 60 min of incubation in pepsin producing a suite of pepsin products <3,000 Da that spanned the entire peptide sequence when analysed by LC-MS/MS (Report Nº 2016-013). 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 fatty acid elongases engineered into DHA canola, Pyrco-Δ5E and Pyrco-Δ6E for stability analysis in this report. Rapid digestion of the full-length protein is one of many factors that indicate protein safety.

The thermal stability of the trans-membrane enzyme proteins, such as desaturases and elongases, cannot be as easily characterized *in vitro* as it can for non-membrane bound enzymes. Membrane proteins, and especially trans-membrane proteins, are not very thermally stable and are difficult to refold once they are partially or fully denatured (Bowie, 2001). In the processing of canola seeds to produce oil, the seed material reaches temperatures ranging from 80ºC to 115ºC. It is improbable that any trans-membrane proteins will remain in the native folded state at this temperature and even after cooling, it will be unlikely to refold correctly. Therefore, these desaturases and elongases are not thermally stable.

G. *PAVLOVA SALINA* Δ4-DESATURASE

The translated *P. salina* Δ4-desaturase (Pavsa-Δ4D) contains 447 amino acid residues (Figure 8). The amino acid sequence of Pavsa-Δ4D shares high homology to other Δ4-desaturases. The molecular weight of Pavsa-Δ4D is predicted to be 49.3 kDa, with an estimated pI of 8.66.

MPPSAAKQMGASTGVHAGVTDSSAFTRKDVADRPDLTIVGDSVYDAKAFRSEHPGGAH
FVSLFGGRDATEAFMEYHRRAWPKSRMSRFHVGSLASTEEPVAADEGYLQLCARIAKM
VPSVSSGFAPASYWVKAGLILGSAIALEAYMLYAGKRLLPSIVLGWLFALIGLNIQHD
ANHGALSKSASVNLALGLCQDWIGGSMILWLQEHVVMHHLHTNDVDKDPDQKAHGALR
LKPTDAWSPMHWLQHLYLLPGETMYAFKLLFLDISELVMWRWEGEPISKLAGYLFMPS
LLLKLTFWARFVALPLYLAPSVHTAVCIAATVMTGSFYLAFFFFISHNFEGVASVGPD
GSITSMTRGASFLKRQAETSSNVGGPLLATLNGGLNYQIEHHLFPRVHHGFYPRLAPL
VKAELEARGIEYKHYPTIWSNLASTLRHMYALGRRPRSKAE

Figure 8. Amino acid sequence of Pavsa-Δ4D.

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

The Pavsa-Δ4D protein shares similarity (15-85%) to desaturase proteins present in food that is consumed, used in food production or in animal feeds (see Table 1, Report Nº 2016-011 for details). 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 fatty acids. The front-end desaturases include Δ4-, Δ5-, Δ6- and Δ8-desaturases, which exist in a wide

range of organisms including algae, diatom, fungi, moss and bacteria. Microalgae such as *Spirulina, Chlorella, Dunaliella*, *Haematococcus* and *Schizochytrium* are classified as food sources falling under the Generally Regarded as Safe category by the U.S. Food and Drug Administration (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 and health values. The so-called blue-green algae *Spirulina* are actually a cyanobacterium. Cyanobacteria have been part of the human diet for centuries (Gantar and Svircev, 2008). Evening primrose oil is commonly sold in Australian health food shops. Additionally, the flowers of *Echium* spp. have been consumed as medicinal plants in countries such as Iran (Heidari et al., 2006). 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 7 shows the enzyme activity of Pavsa-Δ4D expressed in yeast S288C cells when exogenously supplied with ω3 and ω6 substrate (Zhou et al., 2007). Pavsa-Δ4D is able to desaturate both ω6 $22{:}4^{\Delta7,10,13,16}$ (ω6 DTA) or ω3 $22{:}5^{\Delta7,10,13,16,19}$ (ω3 DPA) substrates at Δ4 positions, making ω6 $22{:}5^{\Delta4,7,10,13,16}$ (ω6 DPA) or ω3 $22{:}6^{\Delta4,7,10,13,16,19}$ (ω3 DHA). The conversion rates with the yeast cell expressed the Pavsa-Δ4D protein were 3.0% or 2.4% respectively.

Table 7. Fatty acid composition of yeast S288C cells expressing Pavsa-Δ4D showing the activity of Δ4-desaturase activity

Fatty acid	Exogenous fatty acid fed in growth medium	
	$22{:}4^{\Delta7,10,13,16}$ (ω6)	$22{:}5^{\Delta7,10,13,16,19}$ (ω3)
$22{:}4^{\Delta7,10,13,16}$ (ω6, DTA)	0.97	0
$22{:}5^{\Delta4,7,10,13,16}$ (ω6, DPA)	0.03	0
$22{:}5^{\Delta7,10,13,16,19}$ (ω3, DPA)	0	1.66
$22{:}6^{\Delta4,7,10,13,16,19}$ (ω3, DHA)	0	0.04
Conversion	3.0%	2.4%

The genome sequence including the T-DNA insertions in DHA canola was analysed. The translated amino acid sequence of Pavsa-Δ4D in the insert was confirmed to be identical to the original sequence (see Figure 8, Report Nº 2016-011).

Digestibility of Pavsa-Δ4D

Digestibility / stability of the Pavsa-Δ4D protein was assessed with an *in vitro* stability assay (Thomas et al. 2004) using a standard protocol. Pavsa-Δ4D protein was tested with simulated gastric fluid (SGF) comprising the proteolytic enzyme, pepsin, and in combination with a novel pepsin-trypsin assay employing state-of-the-art mass spectrometric approaches 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 (as a proxy for intact protein). The allergenic potential of a protein is determined by a weight of evidence approach since no single method can predict the allergenicity of a protein. Protein digestibility is one aspect of the overall allergenicity assessment that is conducted for newly introduced proteins into genetically modified crops.

The results of the study demonstrated that greater than 80% was digested within 10 min and greater than 93% of the full-length Pavsa-Δ4D protein was digested within 60 min of incubation in pepsin when analysed by LC-MS/MS (Report Nº 2016-014). 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, Pavsa-Δ5D 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.

The thermal stability of the trans-membrane enzyme proteins, such as desaturases and elongases, cannot be as easily characterized *in vitro* as it can for non-membrane bound enzymes. Membrane proteins, and especially trans-membrane proteins, are not very thermally stable and are difficult to refold once they are partially or fully denatured (Bowie, 2001). In the processing of canola seeds to produce oil, the seed material reaches temperatures ranging from 80ºC to 115ºC. It is improbable that any trans-membrane proteins will be remain in the native folded state at this temperature and even after cooling, it will be unlikely to refold correctly. Therefore, these desaturases and elongases are not thermally stable.

V. CHARACTERIZATION AND SAFETY ASSESSMENT OF THE PAT PROTEIN

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 fatty acid 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 (Wehrman 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.

VI. SAFETY ASSESSMENT OF THE DHA PATHWAY ENZYMES

Codex Alimentarius guidelines developed and adopted by many countries describe an integrated approach for the assessment of the safety of proteins expressed in plants produced through modern biotechnology (*Codex* 2003). In general, the safety assessment is conducted in terms of changes introduced into the recipient plant that includes the intended trait of modified canola oil with high DHA, but also to identify any unintended effects from the transformation process (Delaney et al. 2008). The introduced genes and proteins are analyzed using an *in silico* method, also known as bioinformatics analysis to identify any similarities to known protein toxins or allergens. And the stability or digestibility is determined for each individual protein. Finally, the expression levels of each protein within the different plant tissues across the growing season are determined for DHA canola.

Genes, gene sources and donor organisms

A protein safety assessment is informed by the physio-chemical characteristics of the individual proteins, which are summarized within this report. None of the genes or introduced protein raises any concerns for human, animal or environmental safety. The genes, gene sources and the donor microorganisms are innocuous. The proteins and their donor organisms have an established history of safe use and have been consumed as food, used in food production or in animal feeds (Report Nºs 2016-005, 2016-006, 2016-007, 2016-008, 2016-009, 2016-010, 2016-011).

Bioinformatics analysis

Evaluation of potential risks of food allergy and toxicity associated with DHA canola included a literature search for studies that might indicate possible risks of either hazard from the gene source or protein (Report Nº 2016-017). In addition, bioinformatics searches compared the amino acid sequence of each new protein 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. The search criteria were set to identify matches that are likely to represent minimal risks with identity matches of >35% identity over 80 AA for allergenicity, as well as comparisons with sequences in NCBI Protein database for sequence matches of 50% or more with keyword limits (allergen, allergy) for risks of allergy.

None of the results from the bioinformatics searches of the microorganisms or inserted amino acid 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.

VII. SAFETY ASSESSMENT OF DHA CANOLA

A comparative safety assessment of foods/feeds derived from transgenic plants includes a full battery of studies to characterize the introduced genes and proteins, the transformation process, molecular analysis of the resulting GM crop, and to determine if there are any differences in the agronomics or nutritional composition. A summary of the characterization of the introduced genes and proteins is described in Section VI.

A comprehensive molecular characterization of the DHA canola, along with methodology to identify the elite event and a bioinformatics analysis of potential ORFs have been conducted and documented (Report Nºs 2016-002, 2016-004). To summarize, in collaboration with CSIRO, Nuseed Pty Ltd has developed a genetically modified canola line, DHA canola that accumulates high concentrations of DHA in canola seed oil. DHA canola was produced through *Agrobacterium tumefaciens*-mediated transformation of canola cultivar AV Jade with binary vector pJP3416_GA7-ModB. The vector was specifically designed to convert OA to DHA in canola seed, and contained expression cassettes of seven microalgae and yeast genes (Micpu-Δ6D, Pyrco-Δ5E, Pavsa-Δ5D, Picpa-ω3D, Pavsa-Δ4D, Lackl-Δ12D and Pyrco-Δ6E) in the DHA biosynthetic pathway and the herbicide selection marker gene *pat* between *A. tumefaciens* T-DNA left and right borders.

Sequencing indicated that the DHA canola contained no vector backbone, binary vector's bacterial selectable marker gene NPT II or *A. tumefaciens* genome sequence. Sequencing also indicated that DHA canola contained two T-DNA inserts, which are fully documented (Report Nº 2016-002). Both T-DNA inserts were found to be required to accumulate DHA in canola seed oil.

DHA canola is a stable event as measured across 5 generations (T2-T7) by both genetic and phenotypic analysis. The individual junction DNA sequences of DHA canola were evaluated to identify potential ORFs in all six potential reading frames. None of the results from the bioinformatics searches of the potential fusion protein amino acid 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.

The agronomic analysis of DHA canola was tested across 10 different growing locations in Australia and Canada (Report Nº 2016-018). DHA canola was compared to the non-transgenic parental canola (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 non-transgenic commercial varieties and were comparable to the parental canola variety.

The concentrations of each of the enzymes in the pathway were quantified during the life cycle of DHA canola within different tissues (Report Nº 2016-015). Samples were taken from two field trial sites and included DHA canola and wild type (WT) canola planted at the same sites with the DHA canola. The quantification was achieved by highly sensitive LC-MRM-MS. The results of 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 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.

Finally, samples taken from these same field trial sites (n=8) were analyzed for more than 90 composition analytes. Aside from the intended changes to the fatty acid pathway (e.g., high DHA), there were no unusual results that would impact the nutritional value of DHA canola.

VIII. CONCLUSIONS

The results of these studies demonstrated that the genes, gene sources and proteins raise no safety concerns for DHA canola, event NS-B50027-4. The vector used to produce DHA canola was specifically designed to convert oleic acid to DHA in canola seed, and contained expression cassettes of seven microalgae and yeast genes (Micpu-Δ6D, Pyrco-Δ5E, Pavsa-Δ5D, Picpa-ω3D, Pavsa-Δ4D, Lackl-Δ12D and Pyrco-Δ6E) in the DHA biosynthetic pathway and the herbicide selection marker gene PAT. The elite event contains two T-DNA inserts that are required to produce the desired trait. DHA canola is a stable event as measured across five generations by both genetic and phenotypic analysis. The potential ORF analysis did not find any similarities to known toxins or allergens.

The safety of the introduced proteins are 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.

Agronomic and compositional analyses were conducted across 10 and 8 sites, respectively. The DHA canola values fell within the range of non-transgenic commercial varieties and were comparable to the parental canola variety in both agronomy and composition measurements, aside from the intended changes to the fatty acid pathway (e.g., high DHA).

In conclusions, 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.

IX. REFERENCES

Ahmad, M, Hirz, M, Pichler, H, Schwab, H. 2014. Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *App Microbiol Biotechnol* 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, CA. 2014. Pichia pastoris. In: *Encyclopedia of Food Microbiology (Second Edition).* Academic Press, Oxford, pp 42-46.

Bourdichon, F, Casaregola, S, Farrokh, C, Frisvad, JC, Gerds, ML, Hammes, WP, Harnett, J, Huys, G, Laulund, S, Ouwehand, A, Powell, IB, Prajapati, JB, Seto, Y, Ter Schure, E, Van Boven, A, Vankerckhoven, V, Zgoda, A, Tuijtelaars, S, Hansen, EB. 2012. Food fermentations: Microorganisms with technological beneficial use. *Intl J Food Microbiol* 154:87-97.

Bowie, JU. 2001. Stabilizing membrane proteins. *Curr Opin Struct Biol* 11:397-402.

Brown, MR. 1991. The amino-acid and sugar composition of 16 species of microalgae used in mariculture. *J Exp Mar Biol Ecol* 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. *Appl Microbiol Biotechnol* 100: 6981-6990.

Carver, JD. 1999. Dietary nucleotides: effects on the immune and gastrointestinal systems. *Acta Pædiatric* 88:83-88.

Chaćon-Lee, TL and Gonzalez-Marino, GE. 2010. Microalgae for "Healthy" Foods-Possibilities and Challenges. *Comprehensive Rev Food Sci Food Safety* 9:655-675.

Cho, HP, Nakamura, MT, Clarke, SD. 1999. Cloning, expression, and nutritional regulation of the mammalian Δ-6 desaturase. *J Biol Chem* 274: 471-477.

Codex. 2003. Alinorm 03/34. Principles for the risk analysis of foods derived from modern biotechnology. WHO, FAO.

Domergue, F, Lerchl, J, Zahringer, U, Heinz, E. 2002. Cloning and functional characterization of Phaeodactylum tricornutum front-end desaturases involved in eicosapentaenoic acid biosynthesis. *Eur J Biochem* 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 Physiol* 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. *Biochem J* 389: 483-490.

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. *Appl Microbiol Biotechnol* 97: 3485-3497.

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

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

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 J* 15: 39-48.

Gouveia, L, Coutinho, C, Mendonça, E, Batista, AP, Sousa, I, Bandarra, NM, Raymundo, A. 2008. Functional biscuits with PUFA-ω3 from *Isochrysis galbana*. *J Sci Food Agri* 88: 891-896.

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

Hérouet, C, Esdaile, DJ, Mallyon, BA, 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. *Reg Tox Pharm* 41:134-149.

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.

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 Microbiol Let* 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 co*li and determination of some of its properties. *FEMS Microbiol Let* 233: 53-64.

Kurtzman, CP. 2003. Phylogenetic circumscription of Saccharomyces, Kluyveromyces and other members of the Saccharomycetaceae, and the proposal of the new genera Lachancea, Nakaseomyces, Naumovia, Vanderwaltozyma and Zygotorulaspora. *FEMS Yeast Res* 4:233-245.

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. *Biochem J* 350: 765-770.

Leonard, AE, Pereira, SL, Sprecher, H, Huang, YS. 2004. Elongation of long-chain fatty acids. *Prog Lipid Res* 43: 36-54.

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

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

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. *J Lipid Res* 53:1210-1222.

Meesapyodsuk, D, Qiu, X. 2016. Biosynthetic mechanism of very long chain polyunsaturated fatty acids in *Thraustochytrium* sp. 26185. *J Lipid Res* 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. *J Lipid Res* 45: 1899-1909.

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

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. *Biochem J* 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. *Mar Biotechnol* 12:430-438.

Petrie, JR, Shrestha, P, Belide, S, Kennedy, Y, Lester, G, Liu, Q, Divi, UK, Mulder, RJ, Mansour, MP, Nichols, PD, Singh, SP. 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*. *Metab Eng* 12:233-240.

Petrie, JR, Shrestha, P, Zhou, X-R, 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.

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*. *Arch Biochem Biophys* 376:399-408.

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 Mol Biol* 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*. *Eur J Biochem* 261:812-820.

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

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

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 b_5 domain results in the accumulation of high levels of delta-6 desaturated fatty acids in transgenic tobacco. *Proc Natl Acad Sci USA* 94: 4211-4216.

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 USA* 94: 1142-1147.

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. *Compar Biochem Physiol Part B: Biochem Mol Biol* 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, and Zawodny, J. 2004. A multi-laboratory evaluation of a common *in vitro* pepsin digestion assay protocol used in assessing the safety of novel proteins. *Reg Tox Pharm* 39:87–98.

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 den Eede, G, Aarts, H, Buhk, H-J, Corthier, G, Glint, HJ, Hammes, W, Jacobsen, B, Midtvedt, T, van der Vossen, J, von Wright, A, Wackernagel, W, Wilcks, A. 2004. The relevance of gene transfer to the safety of food and feed derived from genetically modified (GM) plants. *Food Chem Tox* 42:1127-1156.

Watanabe, K, Oura, T, Sakai, H, Kajiwara, S. 2004. Yeast Δ12 fatty acid desaturase: Gene cloning, expression, and function. *Biosci Biotechnol Biochem* 68:721-727.

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. *Nat Biotechnol* 14:1274-1278.

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. *J Biol Chem* 286:43644-43650.

Zhou, XR, Horne, I, Damcevski, K, Haritos, V, Green, A, Singh, S. 2008. Isolation and functional characterization of two independently-evolved fatty acid Delta 12-desaturase genes from insects. *Insect Mol Biol* 17:667-676.

Zhou, X-R, Robert, SS, 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. *Phytochem* 68:785-796.

X. UNPUBLISHED REFERENCES

Report Nº 2016-002. [redacted] Nuseed Pty Ltd.

Report Nº 2016-004. [redacted]. Nuseed Pty Ltd

Report Nº 2016-005. [redacted] Nuseed Pty Ltd.

Report Nº 2016-006. [redacted] Nuseed Pty Ltd.

Report Nº 2016-007. [redacted] Nuseed Pty Ltd.

Report Nº 2016-008. [redacted] Nuseed Pty Ltd.

Report Nº 2016-009. [redacted] Nuseed Pty Ltd.

Report Nº 2016-010. [redacted] Nuseed Pty Ltd.

Report Nº 2016-011. [redacted] Nuseed Pty Ltd.

Report Nº 2016-012. [redacted]. Nuseed Pty Ltd.

Report Nº 2016-013. [redacted]. Nuseed Pty Ltd.

Report Nº 2016-014. [redacted] Nuseed Pty Ltd.

Report Nº 2016-015. [redacted]. Nuseed Pty Ltd.

Report Nº 2016-017. [redacted] Nuseed Pty Ltd.

Report Nº 2016-018. [redacted]. Nuseed Pty Ltd.