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TITLE:
CHARACTERIZATION OF *PYRAMIMONAS CORDATA* Δ6-ELONGASE

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ABBREVIATIONS

ALA	α-Linolenic acid, 18:3$^{\Delta 9,12,15}$ (ω3)
BMGY	Buffered glycerol-complex medium
BMMY	Buffered complex medium containing methanol
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)
FAME	Fatty acid methyl ester
GC	Gas chromatography
kDa	Kilo dalton
LA	Linoleic acid, 18:2$^{\Delta 9,12}$ (ω6)
Lackl-Δ12D	*Lachancea kluyveri* Δ12-desaturase
Micpu-Δ6D	*Micromonas pusilla* Δ6-desaturase
MMT	Million metric ton
MQ	MilliQ water
OA	Oleic acid, 18:1$^{\Delta 9}$
ω3 LC-PUFA	Omega-3 long-chain (≥C20) polyunsaturated fatty acids
ORF	Open reading frame
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)
SP	Secretion peptide
X:Y	A fatty acid containing X carbons with Y double bonds
YPD	Yeast extract-Peptone-Dextrose

EXECUTIVE SUMMARY

The purpose of this report was to characterise the yeast *Pyramimonas cordata* Δ6-elongase (Pyrco-Δ6E) protein, its amino acid sequence and homology to other proteins, and its enzymatic activity in different expression systems.

The results of the study demonstrated that Pyrco-Δ6E was a functional enzyme elongating stearidonic acid $18{:}4^{\Delta 6,9,12,15}$ (SDA) to produce eicosatetraenoic acid $20{:}4^{\Delta 8,11,14,17}$ (ETA) in different cells for accumulating more precursor of omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA). Pyrco-Δ6E protein contains 288 amino acid residues and shares high homology to other Δ6-elongases that have been consumed as food, used in food production or in animal feeds. The molecular weight of Pyrco-Δ6E is predicted to be 33.1 kDa, with an estimated isoelectric point (pI) of 9.09.

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 docosahexaenoic acid (DHA, 22:6ω3) 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 oleic acid (OA) to DHA in a single pathway expression vector. The pathway (Figure 1) was consisted of *Lachancea kluyveri* Δ12-desaturase (Lackl-Δ12D, Watanabe et al. 2004), *Pichia*

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

pastoris ω3-/Δ15-desaturase (Picpa-ω3D, Zhang et al. 2008), *Micromonas pusilla* Δ6-desaturase (Micpu-Δ6D, Petrie et al. 2010b), *Pyramimonas cordata* Δ6-elongase (Pyrco-Δ6E, Petrie et al. 2010a), *Pavlova salina* Δ5-desaturase (Pavsa-Δ5D, Zhou et al. 2007), *P. cordata* Δ5-elongase (Pyrco-Δ5E, Petrie et al. 2010a) and *P. salina* Δ4-desaturase (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 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).

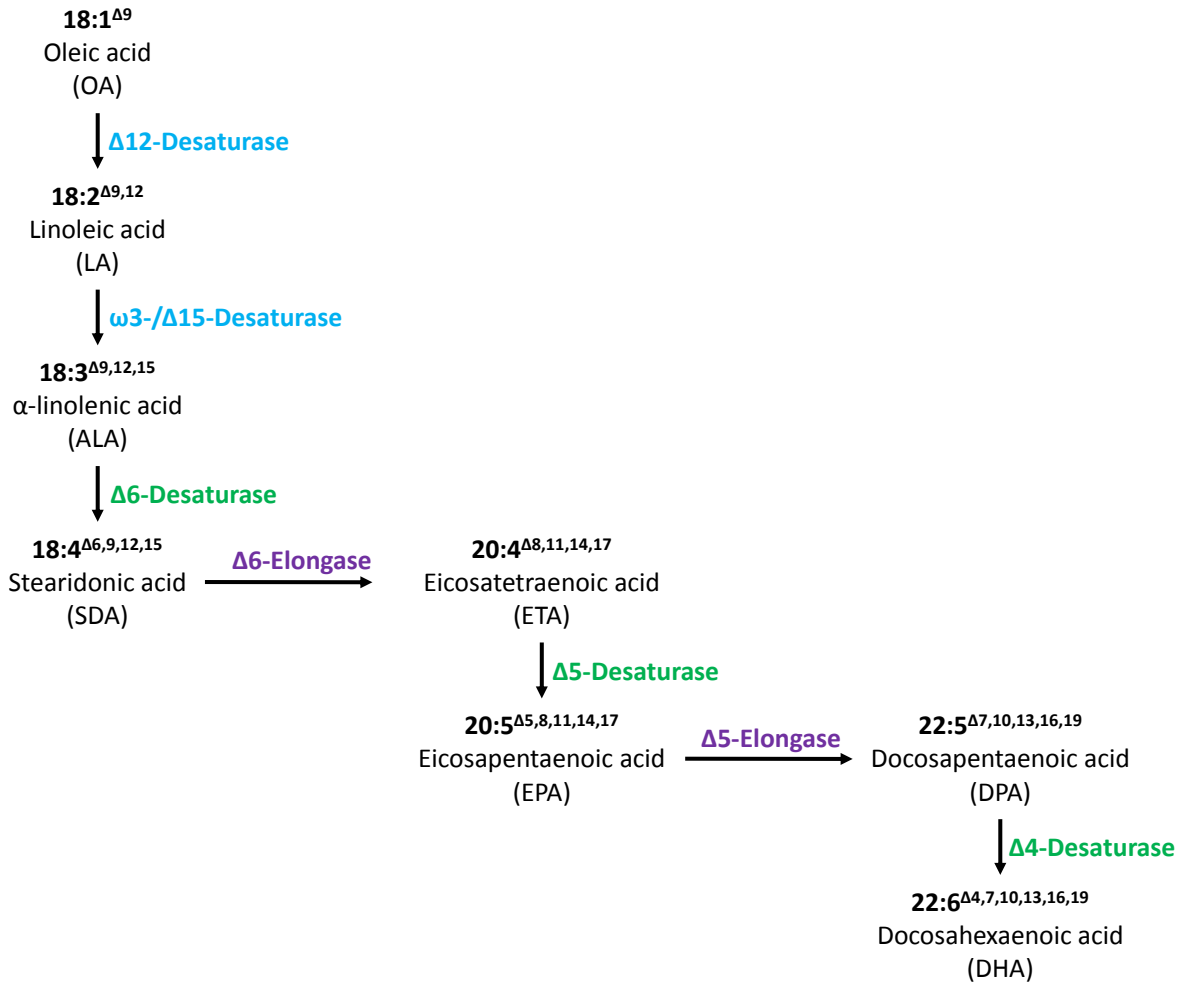

Figure 1. DHA biosynthesis pathway engineered into DHA canola event NS-B50027-4. Seven enzymes introduced in canola to convert oleic acid to final product docosahexaenoic acid were grouped into 3 classes, two fatty acid desaturases from yeast in blue, two elongases from microalgae in purple, and three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

II. PURPOSE

The purpose of this study was to characterise the fatty acid biosynthesis enzymes used in the engineering of DHA canola, including the amino acid sequences, homology to other proteins with similar function or present in consumed food or used in food production, and their enzymatic activities in heterologous expression systems. This particular report is focusing on the *P. cordata* Δ6-elongase (Pyrco-Δ6E) protein to catalyse the elongation of SDA producing ETA ($18{:}4^{\Delta 6,9,12,15} \rightarrow 20{:}4^{\Delta 8,11,14,17}$).

III. MATERIALS

A. 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 (SP). 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 *Pyrco-Δ6E* gene.

B. 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).

IV. METHODS

A. 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).

B. 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 volume of 100% ethanol overnight at -20ºC. The precipitated DNA was resuspended in 10 µL of MilliQ (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.

C. 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).

D. Fatty Acid Analysis

For fatty acid analysis, cells were dried overnight with 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 gas chromatography (GC) vials, dried down to 30 µL with nitrogen. GC analysis was done according to previously described (Zhou et al. 2011).

V. RESULTS AND DISCUSSION

A. GENE SOURCE AND DONOR ORGANISM

The *Pyrco-Δ6E* gene was previously cloned from microalga *P. cordata* (Petrie et al. 2010a). The open reading frame (ORF) of *Pyrco-Δ6E* gene consisted of 867 bp, and is shown in Figure 2.

ATGGAGTTCGCTCAGCCTCTTGTGGCTATGGCACAGGAGCAGTATGCCGCAATTGACGC
GGTGGTAGCCCCTGCAATTTTCTCAGCTACCGACAGCATCGGTTGGGGTCTTAAGCCCA
TTAGCAGCGCGACAAAGGATCTTCCTCTCGTTGAGAGTCCGACGCCGCTCATACTGAGC
CTGTTGGCCTATTTTGCGATCGTCGGCTCTGGGCTGGTGTACCGCAAAGTATTCCCTCG
CACAGTAAAGGGGCAAGACCCCTTCCTGCTGAAGGCGCTCATGCTTGCGCACAACGTGT
TCCTCATTGGCCTCAGTCTATACATGTGCTTGAAGCTTGTCTACGAGGCTTACGTCAAC
AAGTACTCCTTCTGGGGAAACGCCTACAACCCCGCACAGACCGAGATGGCGAAGGTCAT
CTGGATTTTCTACGTCTCCAAGATCTATGAGTTCATGGACACGTTCATCATGCTCTTGA
AGGGCAACGTCAACCAGGTCTCTTTCCTGCATGTGTACCATCATGGCTCCATCTCTGGT
ATCTGGTGGATGATCACCTACGCTGCCCCTGGCGGTGACGCGTACTTCTCGGCGGCGCT
CAACTCGTGGGTGCACGTGTGCATGTACACGTACTACTTCATGGCGGCGGTGCTGCCCA
AGGACGAGAAGACCAAGCGCAAGTACCTCTGGTGGGGCCGCTACCTGACCCAGATGCAG
ATGTTCCAGTTCTTCATGAACCTGCTCCAGGCGGTCTACCTCCTCTACTCCTCTAGCCC
CTACCCCAAGTTCATCGCCCAGCTGCTGGTGGTGTACATGGTCACGCTGCTGATGCTCT
TCGGCAACTTCTACTACATGAAGCACCACGCGAGCAAG**TAG**

Figure 2. Nucleotide sequence of native Pyrco-Δ6E gene.
Start codon (ATG) and stop codon (TAG) are in bold.

B. PROTEIN SEQUENCE

The translated *P. cordata* Δ6-elongase (Pyrco-Δ6E, ACR53359) contained 288 amino acid residues (Figure 3). The molecular weight of Pyrco-Δ6E is predicted as 33.1 kDa, with estimated pI of 9.09.

MEFAQPLVAMAQEQYAAIDAVVAPAIFSATDSIGWGLKPISSATKDLPLVESPTPLILSL
LAYFAIVGSGLVYRKVFPRTVKGQDPFLLKALMLAHNVFLIGLSLYMCLKLVYEAYVNKY
SFWGNAYNPAQTEMAKVIWIFYVSKIYEFMDTFIMLLKGNVNQVSFLHVYHHGSISGIWW
MITYAAPGGDAYFSAALNSWVHVCMYTYYFMAAVLPKDEKTKRKYLWWGRYLTQMQMFQF
FMNLLQAVYLLYSSSPYPKFIAQLLVVYMVTLLMLFGNFYYMKHHASK

Figure 3. Amino acid sequence of Pyrco-Δ6E.

The fatty acid Δ6-elongases have been cloned from nematode (Leonard et al. 2004), plant (Kajikawa et al. 2004), moss (Zank et al. 2002), alga (Meyer et al. 2004) and fungus (Parker-Barnes et al. 2000). 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 4.

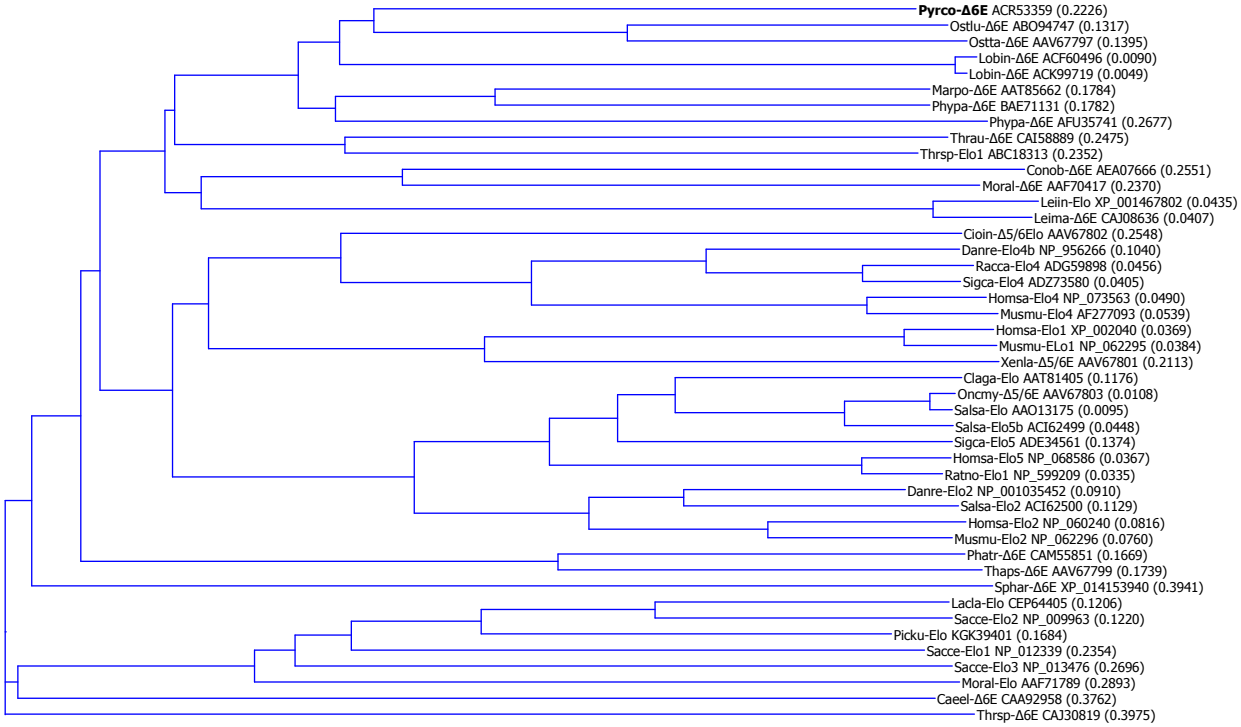

Figure 4. Phylogenetic tree for sequence comparison of Pyrco-Δ6E with representative Δ6-elongases or other PUFA elongases.

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, *Homo 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, *Mortierella alpine* (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, *Phaeodactylum 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, *Salmo salar* (salmon); Sigca, *Siganus canaliculatus* (rabbitfish); Sphar, *Sphaeroforma arctica* (protist); Thaps, Thalassiosira pseudonana (alga); Thrau, *Thraustochytrium aureum* (protist); Thrsp, *T. sp*.; Xenla, *Xenopus laevis* (frog). Δ6E, Δ6-elongase; Δ5/6E, bifunctional Δ5- and Δ6-elongase; Elo, PUFA elongase.

C. Similarity of Pyrco-Δ6E to Other Proteins in Consumed Foods, Used in Food Production or in Animal Feeds

Pyrco-Δ6E shares amino acid sequence identities to many other elongases presented in food that is consumed, used in food production or in animal feeds (Table 1). 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 for 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).

Pyrco-Δ6E shares 27% of sequence identity to *Phaeodactylum tricornutum* Δ6-elongase (CAM55851) or 25% of sequence identity to *Thalassiosira pseudonana* Δ6-elongase (AAV67799). *P. tricornutum* is used to produce pigments or antioxidant for food (Chacón-Lee and González-Marino, 2010). *P. tricornutum* and *T. pseudonana* are foods of oyster (Epifanio et al., 1981).

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

Table 1. Amino acid sequence identity between Pyrco-Δ6E in DHA canola (event NS-B50027-4) and other desaturase proteins present in consumed foods, used in food production or in animal feeds

No.	Protein	Accession	Common Name	Sequence identity						
				1	2	3	4	5	6	7
	NS-B50027-4									
1	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

Δ6E, Δ6-elogase; Elo, PUFA elongase; Glyma, *Glycine max*; Homsa, *Homo sapiens*; Moral, *Mortierella alpine*; Phatr, *Phaeodactylum tricornutum*; Pyrco, *Pyramimonas cordata*; Salsa, *Salmo salar;* Thaps, *Thalassiosira pseudonana*.

D. 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 (SP), followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal (Figure 5). In His_{10}::Pyrco-Δ6E, the Pyrco-Δ6E sequence was fused to His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal (Figure 6). No secretion peptide was used in His_{10}::Pyrco-Δ6E.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTN
NGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMEFAQPLVAMAQEQY
AAIDAVVAPAIFSATDSIGWGLKPISSATKDLPLVESPTPLILSLLAYFAIVGSGLVYRK
VFPRTVKGQDPFLLKALMLAHNVFLIGLSLYMCLKLVYEAYVNKYSFWGNAYNPAQTEMA
KVIWIFYVSKIYEFMDTFIMLLKGNVNQVSFLHVYHHGSISGIWWMITYAAPGGDAYFSA
ALNSWVHVCMYTYYFMAAVLPKDEKTKRKYLWWGRYLTQMQMFQFFMNLLQAVYLLYSSS
PYPKFIAQLLVVYMVTLLMLFGNFYYMKHHASK

Figure 5. 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 (SP, underlined), followed by His-tag (His_{10}, double underlined) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP, dotted underlined) at its N-terminal.

MRPHHHHHHHHHHSLEVLFQGPMEFAQPLVAMAQEQYAAIDAVVAPAIFSATDSIGWGL
KPISSATKDLPLVESPTPLILSLLAYFAIVGSGLVYRKVFPRTVKGQDPFLLKALMLAH
NVFLIGLSLYMCLKLVYEAYVNKYSFWGNAYNPAQTEMAKVIWIFYVSKIYEFMDTFIM
LLKGNVNQVSFLHVYHHGSISGIWWMITYAAPGGDAYFSAALNSWVHVCMYTYYFMAAV
LPKDEKTKRKYLWWGRYLTQMQMFQFFMNLLQAVYLLYSSSPYPKFIAQLLVVYMVTLL
MLFGNFYYMKHHASK

Figure 6. 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$^{\downarrow}$GP, dotted underlined) at its N-terminal.

Overexpression of Pyrco-Δ6E fusion protein proteins in *P. pastoris* with secretion peptide confirmed the desaturation activity of $18{:}4^{\Delta6,9,12,15}$ to $20{:}4^{\Delta8,11,14,17}$ compared to vector alone where there was no any 20:4 product (Table 2). In addition, the His_{10}::Pyrco-Δ6E had higher activity than SP::His_{10}::Pyrco-Δ6E in *P. pastoris*.

Table 2. 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.

E. GLYCOSYLATION ANALYSIS

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. *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. 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 (Figure 7).

MEFAQPLVAMAQEQYAAIDAVVAPAIFSATDSIGWGLKPISSATKDLPLVESPTPLILSL
LAYFAIVGSGLVYRKVFPRTVKGQDPFLLKALMLAHNVFLIGLSLYMCLKLVYEAYVNKY
SFWGNAYNPAQTEMAKVIWIFYVSKIYEFMDTFIMLLKGNVNQVSFLHVYHHGSISGIWW
MITYAAPGGDAYFSAALNSWVHVCMYTYYFMAAVLPKDEKTKRKYLWWGRYLTQMQMFQF
FMNLLQAVYLLYSSSPYPKFIAQLLVVYMVTLLMLFGNFYYMKHHASK

Figure 7. No theoretical glycosylation site (NXT/NXS) in Pyrco-Δ6E.

F. SEQUENCE CONFIRMATION IN TRANSGENIC CANOLA

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 (Figure 8).

```
                        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 8. Alignment of protein sequences of Pyrco-Δ6E.
Δ6E sequence translated from sequenced T-DNA insert in DHA canola NS-B50027-4 event was identical to the original Δ6E sequence from *P. cordata* in binary vector (Pyrco-Δ6E_vec).

VI. CONCLUSIONS

The results of this study demonstrated that the cloned yeast Pyrco-Δ6E protein has activity in heterologous expression systems, including in DHA canola, event NS-B50027-4. The Pyrco-Δ6E protein shares similarity to desaturase proteins present in consumed food, used in food production or in animal feeds. The enzyme functionality of Pyrco-Δ6E has been confirmed in several different heterologous expression systems. Data for Pyrco-Δ6E expressed in Pichia as fusion proteins confirmed this functionality.

Pyrco-Δ6E protein contains 288 amino acid residues. The molecular weight of Pyrco-Δ6E is predicted to be 33.1 kDa, with an 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. The study also demonstrates that canola event NS-B50027-4 contains T-DNA insertions that are translationally identical to the original Pyrco-Δ6E protein sequence.

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