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TITLE:
CHARACTERIZATION OF *MICROMONAS PUSILLA* Δ6-DESATURASE

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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)
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
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
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
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 *Micromonas pusilla* Δ6-desaturase (Micpu-Δ6D) 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 Micpu-Δ6D was a functional enzyme that desaturated α-linolenic acid (ALA) to stearidonic acid (SDA) in different cells for accumulating more precursor of omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA). Micpu-Δ6D protein contains 463 amino acid residues and shares high homology to other Δ6-desaturases that have been consumed as food, used in food production or in animal feeds. The molecular weight of Micpu-Δ6Dis predicted to be 52.9 kDa, with an estimated isoelectric point (pI) of 9.00.

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 the *Lachancea kluyveri* Δ12-desaturase (Lackl-Δ12D, Watanabe et al. 2004), *Pichia pastoris* ω3-/Δ15-desaturase (Picpa-ω3D, Zhang et al. 2008), *Micromonas pusilla* Δ6-

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

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).

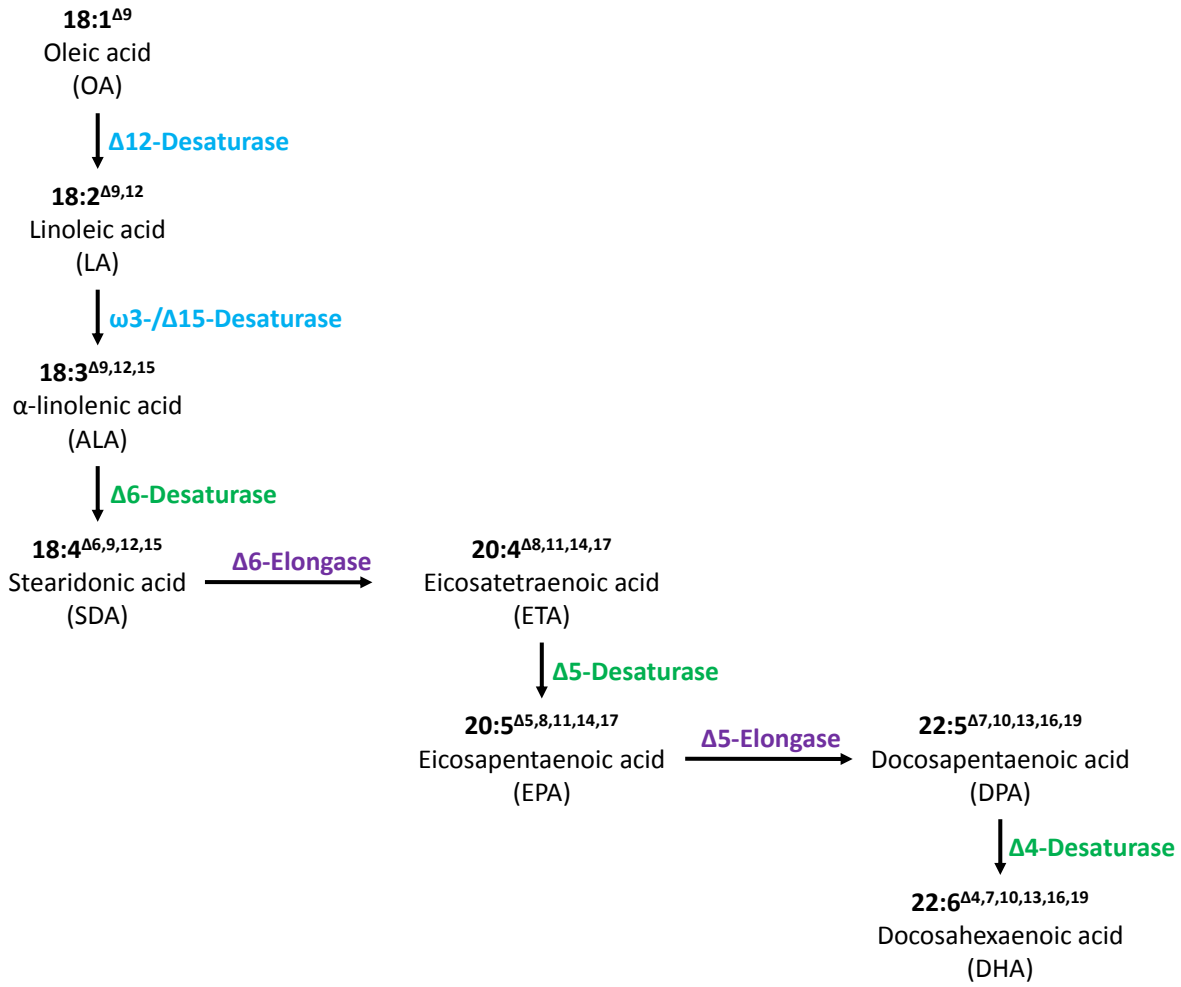

Figure 1. DHA biosynthesis pathway engineered into DHA canola event NS-B50027-4. Seven enzymes introduced in canola to convert oleic acid to final product docosahexaenoic acid were grouped into 3 classes, two fatty acid desaturases from yeast in blue, two elongases from microalgae in purple, and three 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 presented in consumed food or used in food production, and their enzymatic activities in heterologous expression systems. This particular report is focusing on

the *M. pusilla* Δ6-desaturase (Micpu-Δ6D) protein to catalyse the desaturation of ALA at Δ6 position producing SDA ($18:3^{\Delta 9,12,15} \rightarrow 18:4^{\Delta 6,9,12,15}$).

III. MATERIALS

A. TARGET PROTEIN

The Δ6-desaturase gene used in DHA canola event was previously cloned from microalga *M. pusilla* (Petrie et al. 2010b). The Micpu-Δ6D protein was expressed as native sequence in yeast cell and *Nicotiana benthamiana* leaf (Petrie et al. 2010b), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie, 2014), as well as in yeast *P. pastoris* cell as His-tag fusions with or without *Saccharomyces cerevisiae α*-mating type signal peptide as secretion peptide (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 *Micpu-Δ6D* gene.

B. OTHER MATERIALS

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

IV. METHODS

A. SEQUENCE COMPARISON

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

B. TRANSFORMATION OF PICHIA CELL

Pichia transformation was essentially done according to published protocol (Chen et al. 2013). Pichia expression vector DNA containing *Micpu-Δ6D* gene was first linearized by

single restriction enzyme digestion in vector backbone, and precipitated with 1/10 volume of 3 M sodium acetate and 2 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^{TM} 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 *Micpu-Δ6D* gene was previously cloned from microalga *M. pusilla* (Petrie et al. 2010b). The open reading frame (ORF) of *Micpu-Δ6D* gene consisted of 1392 bp, and is shown in Figure 2.

ATGTGCCCGCCGAAGACGGACGGCCGATCGTCCCCGCGATCGCCGCTGACGCGCAGCAA
ATCCTCCGCGGAGGCGCTCGACGCCAAGGACGCGTCGACCGCGCCCGTCGATCTCAAAA
CGCTCGAGCCGCACGAGCTCGCGGCGACGTTCGAGACGCGATGGGTGCGCGTGGAGGAC
GTCGAGTACGACGTCACAAACTTCAAACACCCGGGAGGCAGCGTGATATTCTACATGCT
CGCGAACACGGGCGCGGACGCCACGGAGGCGTTCAAGGAGTTCCACATGCGATCGCTTA
AGGCGTGGAAGATGCTCAGAGCGCTGCCGTCGCGCCCCGCGGAGATCAAACGCAGCGAG
AGCGAGGACGCGCCGATGTTGGAGGATTTCGCGCGGTGGCGCGCGGAGCTCGAACGCGA
CGGGTTCTTTAAGCCCTCGATAACGCACGTCGCGTATCGGTTACTCGAGCTCCTCGCGA
CCTTCGCCCTCGGCACCGCCCTCATGTACGCCGGGTACCCGATCATCGCGTCCGTCGTG
TACGGCGCGTTCTTCGGCGCTCGGTGCGGTTGGGTCCAGCACGAGGGCGGGCACAACTC
GCTCACGGGGTCCGTCTACGTCGACAAGCGCCTCCAAGCGATGACGTGCGGGTTCGGGC
TGTCCACGAGCGGGGAGATGTGGAACCAGATGCACAATAAGCACCACGCGACGCCGCAG
AAAGTGAGGCACGACATGGACCTGGACACGACCCCCGCGGTGGCGTTTTTTAACACCGC
CGTGGAGGACAACCGGCCGAGGGGGTTCTCCCGCGCGTGGGCTCGGCTTCAGGCGTGGA
CGTTCGTCCCGGTGACCTCCGGGCTGCTCGTCCAGGCGTTCTGGATCTACGTCCTGCAC
CCGCGGCAGGTGTTGCGAAAGAAGAACTACGAGGAGGCGTCGTGGATGCTCGTCTCTCA
CGTCGTCAGGACCGCGGTGATTAAACTCGCGACGGGGTACTCGTGGCCCGTCGCGTACT
GGTGGTTCACCTTCGGCAACTGGATCGCGTACATGTACCTCTTCGCGCACTTCTCCACG
AGCCACACGCACCTCCCGGTCGTGCCCTCGGATAAGCACCTGAGCTGGGTGAACTACGC
GGTCGATCACACCGTGGACATCGACCCGTCGCGCGGGTACGTGAACTGGTTGATGGGAT
ATCTGAACTGCCAGGTCATTCATCACCTGTTCCCGGACATGCCGCAGTTTCGCCAGCCG
GAGGTGAGCCGGCGGTTCGTCCCGTTCGCGAAGAAGTGGGGGCTGAACTACAAGGTGCT
GTCCTATTACGGCGCCTGGAAGGCGACGTTCTCGAACTTGGATAAGGTCGGGCAGCACT
ACTACGTCAACGGCAAGGCGGAGAAGGCGCAC**TGA**

Figure 2. ORF nucleotide sequence of native Micpu-Δ6D gene.
Start codon (ATG) and stop codon (TGA) are in bold.

B. PROTEIN SEQUENCE

The translated *M. pusilla* Δ6-desaturase (Micpu-Δ6D, EEH58637) contained 463 amino acid residues (Figure 3). The molecular weight of Micpu-Δ6D is predicted as 52.9 kDa, with estimated pI of 9.00.

```
MCPPKTDGRSSPRSPLTRSKSSAEALDAKDASTAPVDLKTLEPHELAATFETRWVRVED
VEYDVTNFKHPGGSVIFYMLANTGADATEAFKEFHMRSLKAWKMLRALPSRPAEIKRSE
SEDAPMLEDFARWRAELERDGFFKPSITHVAYRLLELLATFALGTALMYAGYPIIASVV
YGAFFGARCGWVQHEGGHNSLTGSVYVDKRLQAMTCGFGLSTSGEMWNQMHNKHHATPQ
KVRHDMDLDTTPAVAFFNTAVEDNRPRGFSRAWARLQAWTFVPVTSGLLVQAFWIYVLH
PRQVLRKKNYEEASWMLVSHVVRTAVIKLATGYSWPVAYWWFTFGNWIAYMYLFAHFST
SHTHLPVVPSDKHLSWVNYAVDHTVDIDPSRGYVNWLMGYLNCQVIHHLFPDMPQFRQP
EVSRRFVPFAKKWGLNYKVLSYYGAWKATFSNLDKVGQHYYVNGKAEKAH
```

Figure 3. Amino acid sequence of Micpu-Δ6D.

The fatty acid Δ6-desaturases have been cloned from bacteria (Reddy et al. 1993), alga (Domergue et al. 2005), diatom (Domergue et al. 2002), fungus (Huang et al. 1999), nematode (Napier et al. 1998), moss (Girke et al. 1998), plant (Sayanova et al. 1997), mouse and human (Cho et al. 1999). The Δ6-desaturases have been widely studied in vertebrates, including many fish species (Vagner and Santigosa 2011, Tanomman et al. 2013). The Δ6-desaturase enzymes can desaturate both ω3 ALA ($18{:}3^{\Delta 9,12,15}$) and ω6 LA ($18{:}3^{\Delta 9,12}$) at Δ6 position producing ω3 SDA ($18{:}4^{\Delta 6,9,12,15}$) and ω6 GLA ($18{:}3^{\Delta 6,9,12}$). In human and mice, Δ6-desaturases also involve in the desaturation of ω3 $24{:}5^{\Delta 9,12,15,18,21}$ to $24{:}6^{\Delta 6,9,12,15,18,21}$ then converted to DHA ($22{:}6^{\Delta 4,7,10,13,16,19}$) by ß-oxidation. For DHA canola, marine microalga Micpu-Δ6D with ω3-preference was used (Petrie et al. 2010b). The Micpu-Δ6D protein shared high homology to other Δ6-desaturase proteins as shown in Figure 4, especially to Δ6-desaturases from other algae, *Mantoniella squamata*, *Ostreococcus lucimarinus* and *O. tauri*.

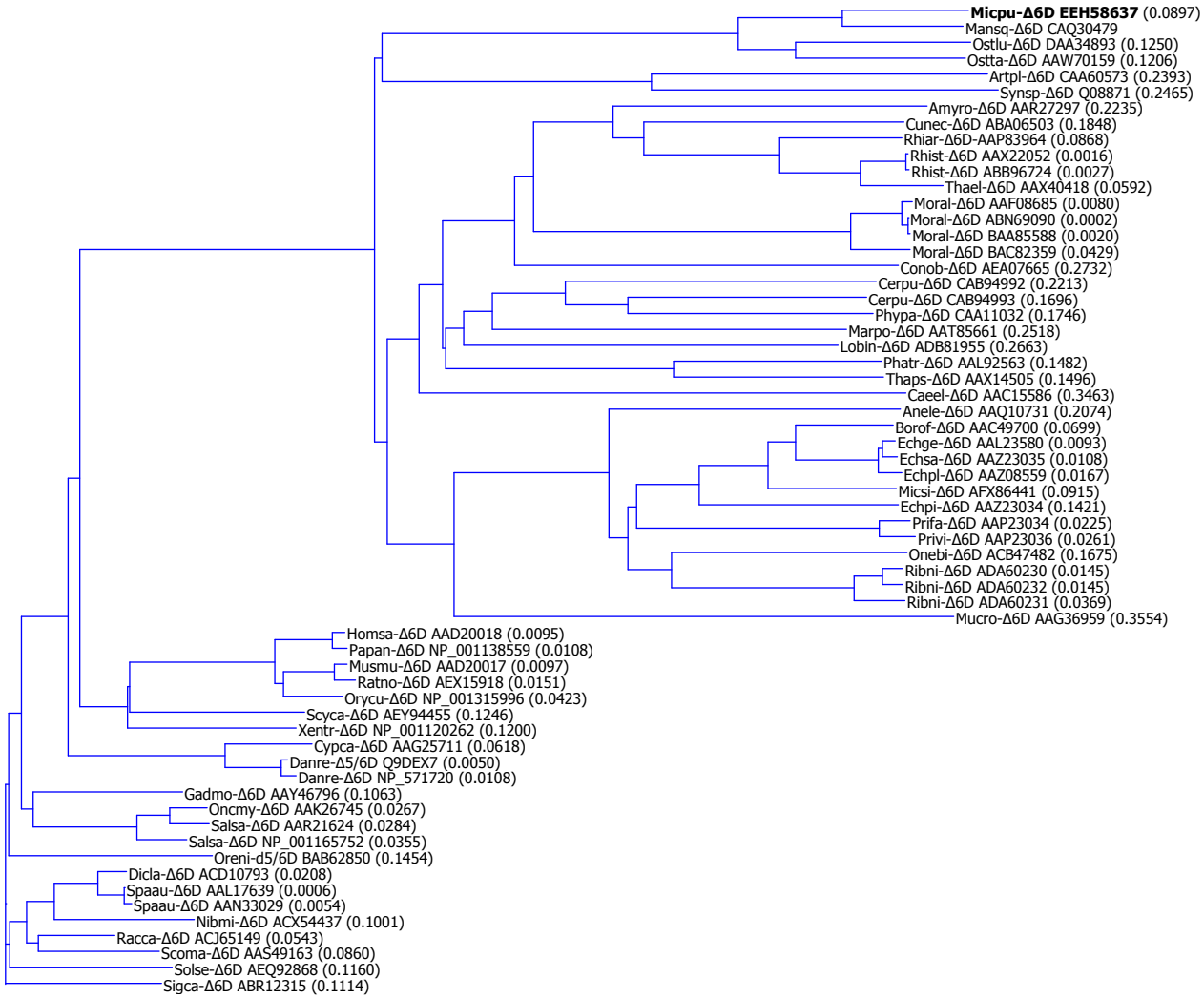

Figure 4. Phylogenetic tree for sequence comparison of Micpu-Δ6D with representative Δ6-desaturases.
The phylogenetic tree was generated with Vector NTI software (Invitrogen, Carlsbad, USA). The protein sequences were named as 5-letter initial of species name, followed by NCBI accession numbers.

Amyro, *Amylomyces rouxii* (fungus); Anele, *Anemone leveillei* (plant); Artpl, *Arthrospira platensis* (bacterium); Borof, *Borago officianalis* (plant); Caeel, *Caenorhabditis elegans* (nematode); Cerpu, *Ceratodon purpureus* (moss); Conob, *Conidiobolus obscurus* (fungus); Cunec, *Cunninghamella echinulate* (fungus); Cypca, *Cyprinus carpio* (carp); Danre, *Danio rerio* (zebrafish); Dicla, *Dicentrarchus labrax* (sea bass); Echge, *Echium gentianoides* (plant); Echpi, *E. pitardii* (plant); Echpl, *E. plantagineum* (plant); Echsa, *E. sabulicola* (plant); Gadmo, *Gadus morhua* (cod); Homsa, *Homo sapiens* (human); Lobin, Lobosphaera incisa (alga); Mansq, *Mantoniella squamata* (alga); Marpo, *Marchantia polymorpha* (liverwort); Micpu, *Micromonas pusilla* CCMP1545 (alga); Micsi, *Microula sikkimensis* (plant); Moral, *Mortierella alpina* (fungus); Mucro, *Mucor rouxii* (fungus);

Musmu, *Mus musculus* (mouse); Nibmi, *Nibea mitsukurii* (croaker); Oncmy, *Oncorhynchus mykiss* (trout); Onebi, *Oenothera biennis* (plant); Oreni, *Oreochromis niloticus* (nile tilapia); Orycu, *Oryctolagus cuniculus* (rabbit); Ostlu, *Ostreococcus lucimarinus* CCE9901 (alga); Ostta, *O. tauri* (alga); Papan, *Papio anubis* (baboon); Phatr, *Phaeodactylum tricornutum* (diatom); Phypa, *Physcomitrella patens* (moss); Prifa, *Primula farinose* (plant); Privi, *P. vialii* (plant); Racca, *Rachycentron canadum* (cobia); Ratno, *Rattus norvegicus* (rat); Rhiar, *Rhizopus arrhizus* (fungus); Rhist, *R. stolonifera* (fungus); Ribni, *Ribes nigrum* (plant); Salsa, *Salmo salar* (salmon); Scoma, *Scophthalmus maximus* (turbot); Scyca, *Scyliorhinus canicula* (catshark); Sigca, *Siganus canaliculatus* (Siganus oramin); Solse, *Solea senegalensis* (sole); Spaau, *Sparus aurata* (bream); Synsp, *Synechocystis sp.* (bacterium); Thael, *Thamnidium elegans* (fungus); Thaps, *Thalassiosira pseudonana* (alga); Xentr, *Xenopus tropicalis* (frog). Δ6D, Δ6-desaturase; Δ5D, Δ5-desaturase.

C. Similarity of Micpu-Δ6D to Other Proteins in Consumed Foods, Used in Food Production or in Animal Feeds

Δ6-desaturases exist in wide range of species including many fungus, plant, fish and mammal species. Micpu-Δ6D shares amino acid sequence identities to other fatty acid desaturases presented in food that is consumed, used in food production or in animal feeds (Table 1). Micpu-Δ6D shares amino acid sequence identities to many those fatty acid desaturases.

Micpu-Δ6D shares 21% sequence identity to *Mortierella alpina* Δ6-desaturase (BAA85588). *M. alpine* 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).

Micpu-Δ6D shares 21% sequence identity to *Thalassiosira pseudonana* Δ6-desaturase (AAX14505). *T. pseudonana* is one of microalgae used in mariculture (Brown 1991), for oyster larvae feed (Brown et al. 1997).

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 oil is commonly sold in Australian health food shops. Additionally, the flowers of *Echium* sp. 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.

The Micpu-Δ6D protein also shares about 19% sequence identity to many plant delta-8 sphingolipid desaturases, including the one from canola (NP_001302507). Micpu-Δ6D shares 18-21% sequence identity to many fish or human Δ6-desaturases.

Table 1. Amino acid sequence identity between Micpu-Δ6D 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
	NS-B50027-4								
1	Micpu-Δ6D			100	20.5	21.1	19.1	17.5	19.1
2	Moral-Δ6D	BAC82359	Fungus		100	37.3	21.9	26.6	26.6
3	Thaps-Δ6D	AAX14505	Diatom			100	23.5	25.5	26.5
4	Salsa-Δ6D	AAR21624	Salmon				100	23.7	23.2
5	Onebi-Δ6D	ACB47482	Evening primrose					100	61.2
6	Brana-Δ8D	NP_001302507	Canola						100

Δ6D, Δ6-desaturase; Δ8D, Δ8-desaturase; Brana, *Brassica napus* (canola); Moral, *Mortierella alpine* (fungus); Onebi, *Oenothera biennis* (evening primrose); Salsa, *Salmo salar* (salmon); Thaps, *Thalassiosira pseudonana* (alga).

D. HETEROLOGOUS EXPRESSION

The enzyme functionality of Micpu-Δ6D have been confirmed in different heterologous expression systems, including yeast cell and *N. benthamiana* leaf (Petrie et al. 2010b), Arabidopsis seed (Petrie et al. 2012) and Camelina seed (Petrie et al. 2014). In this study, Micpu-Δ6D was expressed in *P. pastoris*, as fusion proteins designated as SP::His_{10}::Micpu-Δ6D or His_{10}::Micpu-Δ6D. In SP::His_{10}::Micpu-Δ6D, the Micpu-Δ6D sequence was fused to *Saccharomyces cerevisiae* α-mating type signal peptide (SP), followed by His-tag (His_{10}) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP) at its N-terminal (Figure 5). In His_{10}::Micpu-Δ6D, the Micpu-Δ6D 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}::Micpu-Δ6D.

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNST
NNGLLFINTTIASIAAKEEGVSLEKRPHHHHHHHHHHSLEVLFQGPMCPPKTDGRSSPR
SPLTRSKSSAEALDAKDASTAPVDLKTLEPHELAATFETRWVRVEDVEYDVTNFKHPGG
SVIFYMLANTGADATEAFKEFHMRSLKAWKMLRALPSRPAEIKRSESEDAPMLEDFARW
RAELERDGFFKPSITHVAYRLLELLATFALGTALMYAGYPIIASVVYGAFFGARCGWVQ
HEGGHNSLTGSVYVDKRLQAMTCGFGLSTSGEMWNQMHNKHHATPQKVRHDMDLDTTPA
VAFFNTAVEDNRPRGFSRAWARLQAWTFVPVTSGLLVQAFWIYVLHPRQVLRKKNYEEA
SWMLVSHVVRTAVIKLATGYSWPVAYWWFTFGNWIAYMYLFAHFSTSHTHLPVVPSDKH
LSWVNYAVDHTVDIDPSRGYVNWLMGYLNCQVIHHLFPDMPQFRQPEVSRRFVPFAKKW
GLNYKVLSYYGAWKATFSNLDKVGQHYYVNGKAEKAH

Figure 5. Amino acid sequence of SP::His10::Micpu-Δ6D.
Micpu-Δ6D was expressed in *P. pastoris*, fused to mating type alpha signal peptide as secretion peptide (SP, underlined), followed by His-tag (His_{10}, double underlined) and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP, dotted underlined) at its N-terminal.

MRPHHHHHHHHHHSLEVLFQGPMCPPKTDGRSSPRSPLTRSKSSAEALDAKDASTAPVDL
KTLEPHELAATFETRWVRVEDVEYDVTNFKHPGGSVIFYMLANTGADATEAFKEFHMRSL
KAWKMLRALPSRPAEIKRSESEDAPMLEDFARWRAELERDGFFKPSITHVAYRLLELLAT
FALGTALMYAGYPIIASVVYGAFFGARCGWVQHEGGHNSLTGSVYVDKRLQAMTCGFGLS
TSGEMWNQMHNKHHATPQKVRHDMDLDTTPAVAFFNTAVEDNRPRGFSRAWARLQAWTFV
PVTSGLLVQAFWIYVLHPRQVLRKKNYEEASWMLVSHVVRTAVIKLATGYSWPVAYWWFT
FGNWIAYMYLFAHFSTSHTHLPVVPSDKHLSWVNYAVDHTVDIDPSRGYVNWLMGYLNCQ
VIHHLFPDMPQFRQPEVSRRFVPFAKKWGLNYKVLSYYGAWKATFSNLDKVGQHYYVNGK
AEKAH

Figure 6. Amino acid sequence of His10::Micpu-Δ6D.
Micpu-Δ6D was expressed in *P. pastoris*, fused to His-tag (His_{10}, double underlined), and PreScission protease cleavage site (SLEVLFQ$^{\downarrow}$GP, dotted underlined) 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 vector alone where there was no any 18:4 product (Table 2). In addition, the His_{10}::Micpu-Δ6D had higher activity than SP::His_{10}::Micpu-Δ6D in *P. pastoris*.

Table 2. 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.

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 Micpu-Δ6D protein, there is no potential glycosylation site within this amino acid sequence derived from the nucleotide sequence of the inserted DNA (Figure 7).

```
MCPPKTDGRSSPRSPLTRSKSSAEALDAKDASTAPVDLKTLEPHELAATFETRWVRVEDV
EYDVTNFKHPGGSVIFYMLANTGADATEAFKEFHMRSLKAWKMLRALPSRPAEIKRSESE
DAPMLEDFARWRAELERDGFFKPSITHVAYRLLELLATFALGTALMYAGYPIIASVVYGA
FFGARCGWVQHEGGHNSLTGSVYVDKRLQAMTCGFGLSTSGEMWNQMHNKHHATPQKVRH
DMDLDTTPAVAFFNTAVEDNRPRGFSRAWARLQAWTFVPVTSGLLVQAFWIYVLHPRQVL
RKKNYEEASWMLVSHVVRTAVIKLATGYSWPVAYWWFTFGNWIAYMYLFAHFSTSHTHLP
VVPSDKHLSWVNYAVDHTVDIDPSRGYVNWLMGYLNCQVIHHLFPDMPQFRQPEVSRRFV
PFAKKWGLNYKVLSYYGAWKATFSNLDKVGQHYYVNGKAEKAH
```

Figure 7. No theoretical glycosylation site (NXT/NXS) in Micpu-Δ6D.

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 Micpu-Δ6D in the insert was confirmed to be identical to the original sequence (Figure 8).

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

Figure 8. Alignment of protein sequences of Micpu-Δ6D.
Δ6D sequence translated from sequenced T-DNA insert in DHA canola NS-B50027-4 event was identical to the original Δ6D sequence from *M. pusilla* in binary vector (Micpu-Δ6D_vec).

VI. CONCLUSIONS

The results of this study demonstrated that the cloned yeast Micpu-Δ6D protein has activity in heterologous expression systems, including in DHA canola, event NS-B50027-4. The Micpu-Δ6D protein shares similarity to desaturase proteins present in consumed food, used in food production or in animal feeds. The enzyme functionality of Micpu-Δ6D has been confirmed in several different heterologous expression systems. Data for Micpu-Δ6D expressed in Pichia as fusion proteins confirmed this functionality.

Micpu-Δ6D protein contains 463 amino acid residues. The molecular weight of Micpu-Δ6D is predicted to be 52.9 kDa, with an 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. The study also demonstrates that canola event NS-B50027-4 contains T-DNA insertions that are translationally identical to the original Micpu-Δ6D protein sequence.

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