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Isolation and characterization of genes from the marine microalga *Pavlova salina* encoding three front-end desaturases involved in docosahexaenoic acid biosynthesis

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Abstract

The marine microalga *Pavlova salina* produces lipids containing approximately 50% omega-3 long chain polyunsaturated fatty acids (LC-PUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Three cDNA sequences, designated *PsD4Des*, *PsD5Des*, *PsD8Des*, were isolated from *P. salina* and shown to encode three front-end desaturases with Δ4, Δ5 and Δ8 specificity, respectively. Southern analysis indicated that the *P. salina* genome contained single copies of all three front-end fatty acid desaturase genes. When grown at three different temperatures, analysis of fatty acid profiles indicated *P. salina* desaturation conversions occurred with greater than 95% efficiency. Real-Time PCR revealed that expression of *PsD8Des* was higher than for the other two genes under normal growth conditions, while *PsD5Des* had the lowest expression level. The deduced amino acid sequences from all three genes contained three conserved histidine boxes and a cytochrome b_5 domain. Sequence alignment showed that the three genes were homologous to corresponding desaturases from other microalgae and fungi. The predicted activities of these three front-end desaturases leading to the synthesis of LC-PUFA were also confirmed in yeast and in higher plants.

Keywords: *Pavlova salina*; Microalgae; Gene isolation; Δ4-desaturase; Δ5-desaturase; Δ8-desaturase; Long chain polyunsaturated fatty acids

1. Introduction

Omega-3 long-chain polyunsaturated fatty acids (ω3 LC-PUFA) such as docosahexaenoic acid (DHA, $22{:}6^{\Delta4,7,10,13,16,19}$) and eicosapentaenoic acid (EPA, $20{:}5^{\Delta5,8,11,14,17}$) are widely recognised as having important benefits for human health. Whilst the human body is capable of synthesing DHA from dietary α-linolenic acid (ALA, $18{:}3^{\Delta9,12,15}$), the conversion rate is very inefficient, particularly in the case of modern diets rich in linoleic acid (LA, $18{:}2^{\Delta9,12}$), an ω6 fatty acid that competes with ALA for the enzymes involved in DHA synthesis (Voss et al., 1991). Metabolic engineering of higher plants to produce

Abbreviations: AA, arachidonic acid $20{:}4^{\Delta5,8,11,14}$ (ω6); ALA, α-linolenic acid $18{:}3^{\Delta9,12,15}$ (ω3); CoA, coenzyme A; DGLA, dihomo-γ-linolenic acid $20{:}3^{\Delta8,11,14}$ (ω6); DHA, docosahexaenoic acid $22{:}6^{\Delta4,7,10,13,16,19}$ (ω3); DPA, docosapentaenoic acid $22{:}5^{\Delta7,10,13,16,19}$ (ω3) or $22{:}5^{\Delta4,7,10,13,16}$ (ω6); DTA, docosatetraenoic acid $22{:}4^{\Delta7,10,13,16}$ (ω6); EDA, eicosadienoic acid $20{:}2^{\Delta11,14}$ (ω6); EPA, eicosapentaenoic acid $20{:}5^{\Delta5,8,11,14,17}$ (ω3); ETA, eicosatetraenoic acid $20{:}4^{\Delta8,11,14,17}$ (ω3); ETrA, eicosatrienoic acid $20{:}3^{\Delta11,14,17}$ (ω3); FAME, fatty acid methyl ester; GLA, γ-linolenic acid $18{:}3^{\Delta6,9,12}$ (ω6); LA, linoleic acid $18{:}2^{\Delta9,12}$ (ω6); LC-PUFA, long-chain (⩾C20) polyunsaturated fatty acid; MUFA, mono unsaturated fatty acid; PC, phosphatidylcholine; SDA, stearidonic acid $18{:}4^{\Delta6,9,12,15}$ (ω3); TAG, triacylglycerol; X:Y, a fatty acid containing X carbons with Y double bonds.

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nutritionally important EPA and/or DHA has recently been attempted using genes from a variety of organisms that naturally synthesise and accumulate LC-PUFA by a series of alternating elongation and desaturation reactions. The LC-PUFA desaturases responsible for inserting the fourth, fifth and sixth double bonds required to synthesise DHA from ALA are termed 'front-end desaturases' since they catalyse the introduction of double bonds between the carboxyl end and the pre-existing double bonds (Napier et al., 1999b).

The most common LC-PUFA synthesis pathway appears to be the Δ6-desaturase/Δ6-elongase pathway, in which LA and ALA are first desaturated by a Δ6-desaturase to yield γ-linoleic acid (GLA, $18:3^{\Delta6,9,12}$) and stearidonic acid (SDA, $18:4^{\Delta6,9,12,15}$) respectively, followed by a Δ6-elongation to produce dihomo-γ-linoleic acid (DGLA, $20:3^{\Delta8,11,14}$) and eicosatetraenoic acid (ETA, $20:4^{\Delta8,11,14,17}$), respectively (Girke et al., 1998; Napier et al., 1999a; Sayanova and Napier, 2004; Robert, 2006). A less common Δ9-elongase/Δ8-desaturase pathway has also been discovered, by which LA or ALA is first elongated by a Δ9-elongase to yield eicosadienoic acid (EDA, $20:2^{\Delta11,14}$) or eicosatrienoic acid (ETrA, $20:3^{\Delta11,14,17}$), followed by a Δ8-desaturation to yield DGLA or ETA, respectively (Wallis and Browse, 1999). The products of all of these pathways are then Δ5-desaturated to produce either arachidonic acid (AA, $20:4^{\Delta5,8,11,14}$) or EPA. EPA can then be Δ5-elongated to produce docosapentaenoic acid (DPA, $22:5^{\Delta7,10,13,16,19}$), and finally Δ4-desaturated to produce DHA (Qiu et al., 2001; Sayanova and Napier, 2004; Truksa et al., 2006) (Fig. 1).

The genes involved in these pathways have been isolated from a wide variety of organisms, including nematodes, zebrafish, moss, fungi and algae. Non-mammalian sources of these genes include the Δ8-desaturase of the microalga *Euglena gracilis* (Wallis and Browse, 1999) and soil amoeba *Acanthamoeba castellanii* (Sayanova et al., 2006), the Δ5-desaturases of the nematode *Caenorhabditis elegans* (Michaelson et al., 1998b), the fungus *Mortierella alpina* (Michaelson et al., 1998a; Hong et al., 2002), the thraustochytrid *Thraustochytrium* sp. ATCC 21685 (Qiu et al., 2001), the microalgae *Phaeodactylum tricornutum* (Domergue et al., 2002) and *Thalassiosira pseudonana* (Tonon et al., 2005) and the bryophyte *Marchantia polymorpha* (Kajikawa et al., 2004), as well as the Δ4-desaturases of *Thraustochytrium* sp. ATCC 21685 (Qiu et al., 2001), *Pavlova lutheri* (Tonon et al., 2003), *E. gracilis* (Meyer et al., 2003) and *T. pseudonana* (Tonon et al., 2005). These genes, and others, have been introduced into yeast and higher plants in an attempt to synthesise LC-PUFA.

Engineered plant synthesis of the C20-PUFA EPA and AA was first reported in *Arabidopsis* leaves (Qi et al., 2004) and linseed (Abbadi et al., 2004). Subsequently, the synthesis of all three nutritionally important LC-PUFA (AA, EPA and DHA) was demonstrated in *Arabidopsis* and *Brassica juncea* seed oil by transgenic expression of the Δ6-desaturase/Δ6-elongase pathway (Robert et al., 2005; Wu et al., 2005). Whilst the target of DHA production in higher plants has thus been reached, the level of production is presently relatively low. As part of our efforts to produce DHA and other LC-PUFA in higher plants, we have isolated and characterised three LC-PUFA front-end desaturases from the microalga *P. salina*. Expression of each of these genes in both yeast and higher plants confirmed their functions as Δ8-desaturase, Δ5-desaturase, and Δ4-desaturase. We also report a copy number analysis of the three desaturases and their relative expression in *P. salina* grown at different temperatures.

2. Results and discussion

2.1. Growth and major fatty acid composition of *P. salina*

Cultures of microalga *P. salina* (Class Prymnesiophyceae) strain CS-49 from the CSIRO Collection of Living Microalgae acclimatised at 15 °C, 20 °C and 25 °C, reached

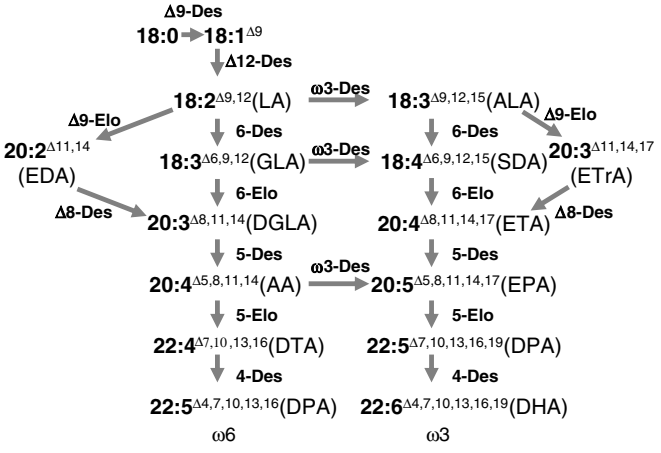

Fig. 1. Biosynthetic pathways for ω3 and ω6 long chain polyunsaturated fatty acids Des, fatty acid desaturase; Elo, fatty acid elongase.

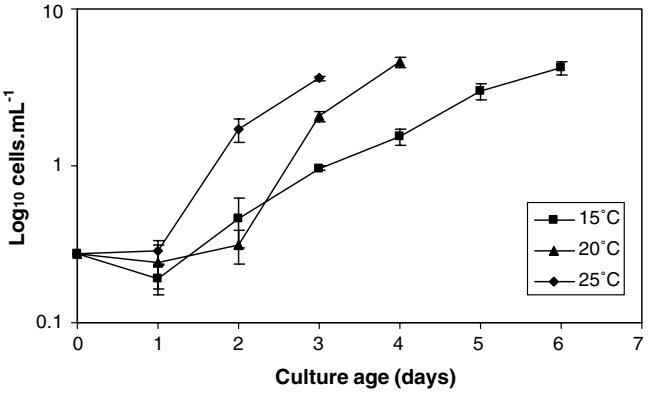

Fig. 2. Growth of *P. salina* at three different temperatures. Error bars are ±1 SD ($n = 3$).

late-logarithmic growth phase after 6, 4 and 3 days, respectively (Fig. 2). The fatty acid composition was not substantially different between the different growth temperatures, although there was a tendency for greater conversion of EPA to DHA at lower temperature (Table 1). The conversion rates of ω3 and ω6 LC-PUFA products and substrates were calculated from the fatty acid profile data at each growth temperature. At each temperature the conversion rate of all three desaturases in *P. salina* were above 95% (Table 1) for ω6 LC-PUFA substrates and above 99% for ω3 LC-PUFA substrates. In contrast, the conversion rate of Δ5-elongase was only 82% for ω6 and 30% for ω3 LC-PUFA at 20 °C (normal growth temperature for *P. salina*). The conversion rate of Δ5-elongase for the ω3 LC-PUFA was greater at the lower temperature (36% at 15 °C) than at the higher temperature (27% at 25 °C). This increased Δ5-elongation efficiency at 15 °C could contribute to the higher accumulation of DHA and is a general reflection of low growth temperatures promoting the accumulation of PUFA. Interestingly, both Δ9-elongated and Δ6-desaturated products of LA and ALA (Fig. 1) were found in the total fatty acids (Table 1), indicating that two alternate pathways (Δ6-desaturase/Δ6-elongation pathway and Δ9-elongation/Δ8-desaturase pathway) could lead to the AA and EPA synthesis in *P. salina*. A similar observation has also been reported in another prymnesiophyte microalga *Isochrysis galbana* (Qi et al., 2002).

Table 1
Fatty acid composition (%) of *Pavlova salina* strain CS-49 grown at different temperatures

Enzyme involved	Fatty acid	15 °C[b]	20 °C	25 °C[b]
	Saturated FA	25.2	27.0	27.3
	Total MUFA	10.5	8.6	8.9
	16:2ω4	0.6	0.6	0.8
	16:3ω4	1.5	1.5	2.2
	18:2ω6	2.7	3.8	1.7
	18:3ω3[c]	1.7	2.4	1.1
	18:3ω6	0.5	2.0	1.3
	18:4ω3	11.8	10.2	13.8
Δ9Elo[a]				
	20:2ω6	0.1	0.0	0.1
	20:3ω3	0.0	0.0	0.0
Δ8Des[a]				
	20:3ω6	0.2	0.3	0.1
	20:4ω3	0.3	0.2	0.2
Δ5Des[a]				
	20:4ω6	0.8	1.0	1.0
	20:5ω3	25.1	26.6	27.2
Δ5Elo[a]				
	22:4ω6	0.0	0.0	0.0
	22:5ω3	0.1	0.3	0.0
Δ4Des[a]				
	22:5ω6	5.1	4.4	4.1
	22:6ω3	13.8	11.2	10.0
	Total FA	100.0	100.0	100.0
Conversion[d]	Δ9Elo ω6	66.0%	49.6%	63.9%
	Δ9Elo ω3	74.4%	75.2%	71.5%
	Δ8Des ω6	98.4%	100.0%	98.1%
	Δ8Des ω3	100.0%	100.0%	100.0%
	Δ5Des ω6	96.7%	94.7%	98.1%
	Δ5Des ω3	99.2%	99.5%	99.5%
	Δ5Elo ω6	86.4%	81.5%	80.4%
	Δ5Elo ω3	35.6%	30.2%	26.9%
	Δ4Des ω6	100.0%	100.0%	100.0%
	Δ4Des ω3	99.3%	97.4%	100.0%

Samples were sampled at late log/early stationary phase.
[a] Enzymes converted two substrates (above the enzyme name) to two products (below the enzyme name).
[b] Mean of triplicates.
[c] Contain trace amount of 18:1ω7.
[d] Conversion rate is calculated as total enzymatic products divided by total available substrates. In case of d9-elongation, all original C18:2ω6 and C18:3ω3 from which portion of them were converted into C18:3ω6 and C18:4ω3 were included as total available substrates.

2.2. Cloning of *P. salina* front-end desaturases

Comparison of known moss and microalgal Δ4-desaturases revealed several conserved motifs including a HPGG motif within a cytochrome b_5-like domain and three histidine box motifs that are presumably required for desaturase activity. Degenerate PCR primers were used to amplify the corresponding region of *P. salina* desaturase genes from a cDNA library template. The amplicons were cloned and sequenced. Amongst the 14 clones sequenced, three clones showed homology to known Δ4-desaturase genes (Qiu et al., 2001). The deduced amino acid sequence of one clone, 1803, showed 65%, 49%, 46%, and 46% identity to that of Δ4-desaturases of *P. lutheri*, *Thraustochytrium* sp. ATCC21685, *T. aureum* and *E. gracilis* (accession nos. AAQ98793, AAM09688, AAN75707, AAQ19605), respectively.

Eighteen plaques were isolated by screening the *P. salina* cDNA library with the insert from clone 1803 as a hybridization probe under high stringency conditions. Plasmids from five of these plaques were excised and sequenced. All five clones had identical coding sequences containing an apparently full-length putative Δ4-desaturase cDNA of approximately 1.7 kb in length. Despite having identical open reading frame and UTR overlapping regions, the five clones differed slightly in the length of the 5′ and 3′ UTRs. Analysis of the 3′ UTR of these five clones showed that there were at least four alternative polyadenylation sites (data not shown).

A synthetic 175 bp fragment was annealed by two 100 bp oligonucleotides designed according to a partial *P. lutheri* desaturase-like sequence deposited in International Patent Application WO03078639-A2. Screening of the *P. salina* cDNA library at low stringency with this fragment as a hybridization probe generated 60 hybridising plaques. Plasmids from 13 of these clones were excised and sequenced. One clone, 1918, contained a partial-length cDNA encoding an amino acid sequence with homology to known Δ5-desaturase genes. The amino acid sequence was 53% identical to amino acid residues 210–430 from

Table 2
Oligonucleotides used

Primer	Sequence	Orientation	Use
PavD4Des-F3	5′-AGCACGACGSSARCCACGGCG	Sense	Gene isolation
PavD4Des-R3	5′-GTGGTGCAYCABCACGTGCT	Antisense	Gene isolation
PsD6Des-F1	5′-TGGTGGAARCAYAARCAYAAY	Sense	Gene isolation
PsD6Des-R1	5′-GCGAGGGATCCAAGGRAANARRTGRTGYTC	Antisense	Gene isolation
PsD5-LF	5′-TGGGTTGAGTACTCGGCCAACCACACGACCAACT-GCGCGCCCTCGTGGTGGTGCGACTGGTGGATGTCTT-ACCTCAACTACCAGATCGAGCATCATCTGT	Sense	Gene isolation
PsD5-LR	5′-ATAGTGCAGCCCGTGCTTCTCGAAGAGCGCCTTGACG-CGCGGCGCGATCGTCGGGTGGCGGAATTGCG-GCATGGACGGGAACAGATGATGCTCGATCTGG	Antisense	Gene isolation
18sRNA-F	5′-CATGGGATAATGGAATAGG	Sense	Gene isolation
18sRNA-R	5′-GATAGTCCCTCTAAGAAGC	Antisense	Gene isolation
PasD4-3F2	5′-AACCTGGCATCCACGCTGAGG	Sense	PsD4Des Real-Time PCR
PasD4-3R2	5′-TCTGCTGGTGCGACCGCAATC	Antisense	PsD4Des Real-Time PCR
PasD5-3F	5′-GTGAAGCAGCTCTTCGAGAA	Sense	PsD5Des Real-Time PCR
PasD5-3R	5′-AAAGCAGAGATGTCAGCTGG	Antisense	PsD5Des Real-Time PCR
PasD8Des-Sense600	5′-GCACTCACCTCGCGCAACAT	Sense	PsD8Des Real-Time PCR
PasD8Des-Anti439	5′-GCAAGCGCACGGATGTTCTG	Antisense	PsD8Des Real-Time PCR
Ps18s-F	5′-GTGGAGCCTGCGGCTTAATT	Sense	Ps18s rRNA Real-Time PCR
Ps18s-R	5′-GGTTAAGGTCTCGTTCGTTA	Antisense	Ps18s rRNA Real-Time PCR

the C-terminal region of a *Thraustochytrium* Δ5-desaturase gene (accession no. AF489588). The partial-length sequence in clone 1918 was then used to design a pair of sequence specific primers which were used to PCR screen the 60 isolated plaques mentioned above. Nineteen out of the 60 were positive, with either the same or similar cDNA sequence, from which, one clone appeared to contain a full-length cDNA sequence.

An alignment of Δ6-desaturase amino acid sequences from Genbank accession nos. AAL73949, AAD01410 and AAC15586 identified the consensus amino acid sequence blocks DHPGGS, WWKDKHN and QIEHHLF corresponding to amino acid positions 49–54, 204–210 and 395–401, respectively, of AAL73949. DHPGGS corresponded to the "cytochrome b_5 domain" block that had been identified previously (Mitchell and Martin, 1995). WWKDKHN was a consensus block that had not previously been identified or used to design degenerate primers for the isolation of desaturase genes. The QIEHHLF block, or variants thereof, corresponded to a required histidine-containing motif is conserved in desaturases. It had been identified and used before as the "third His box" to design degenerate oligonucleotides for desaturase gene isolation (Michaelson et al., 1998a). Based on the second and third conserved amino acid blocks, the degenerate primers PsD6Des-F1 and PsD6Des-R1 (Table 2) were synthesised. The PCR amplified product (515 bp) was cloned, sequenced and subsequently used as a probe to screen the *P. salina* cDNA library. Five hybridising plaques were isolated and the sequences from their excised plasmids were identical where they overlapped. One of these clones appeared to contain a full-length cDNA. Amplification of 3′-UTR of the gene with the sense gene-specific primer and vector Forward primer and sequencing revealed at least 10 alternative polyA sites from 12 clones (Fig. 3). Alternative polyA sites for a single gene are common in many genes (Edwalds-Gilbert et al., 1997) based on the cellular environment, and have impacts on many aspects of mRNA metabolism in the cell, although the average number of alternative polyA sites is 2–3 (Zhang et al., 2005). The reason for existence of up to 10 alternative polyA sites in *P. salina* front-end desaturases remains to be understood.

2.3. Comparison of *P. salina* front-end desaturase sequences

The longest full-length Δ4-, Δ5- and Δ6-desaturase-like cDNAs were 1687, 1627 and 1760 nucleotides long (GenBank accession nos. AY926605, DQ995517 and DQ995518)[1] respectively, and comprised coding region corresponding to 447, 425 and 428 amino acid residues. Predicted amino acid sequences encoded by these three *P. salina* genes showed all the conserved motifs typical of 'front-end desaturases' including the N-terminal cytochrome b_5-like domain and three conserved histidine-rich motifs. Comparison of nucleotide and amino acid sequences with other desaturase genes showed three different desaturases were each homologous to known Δ4-desaturases, Δ5-desaturases and Δ6- or Δ8-desaturases. The greatest homology of *P. salina* putative Δ4-desaturase was with the *P. lutheri* Δ4-desaturase (accession no. AY332747), with 69.4% identity in nucleotide sequence over the coding sequence, and 67.2% identity in amino acid sequence. The amino acid sequence of the *P. salina* putative Δ5-desaturase gene showed 50% identity to a Δ5-desaturase

[1] The nucleotide sequences of *Pavlova salina* Δ4-, Δ5-, and Δ8-desaturases genes are deposited in GenBank at accession nos. AY926606, DQ995517 and DQ9955178.

TGAGCGCTCTCCGCTTCCAAGGGCGGGATCGGCGCATCCGTTGGTG
CTGCGGCACCAACGCTTCCGCTCTCGAGCGCAGATGTTGGTTGCAT
CACGCATCACACCCACCCCCAGCCGGATCACCCAGCTCCCGATGCT
TGGCGTACCTAGCCGCGTCCTCCCAGCATGCACTGCAACTCATTCA
CCACCTGCTACAGTTTCGGCCTAATCCATGGCCCGACTGCTTGCCG
CCTTGCACACCGACGAGTACGCCGACTCGGTCCAATGCCTCGGCGT
AATCTGCTGGCGTGCTGCGGCACTGTGATTCATTCATTGATCGCAC
AGTTCACAGCATGTTCGCTGACACAACGTTTGCTGACCTCATAGAC
AGTAGACGACTGCTTAATGATTAAATCAGACTCGTCAGGTCCTCTG
CAAAAAAAAAAAAAAAAAAA

Fig. 3. 3′-UTR sequence of *P. salina* Δ8-desaturase showing multiple poly(A) sites. Poly(A) sites are double underlined while the stop codon is single underlined.

from *Thraustochytrium* (accession no. AF489588). The full-length amino acid sequence of third *P. salina* front-end desaturase showed homology to both Δ6- and Δ8-desaturase, based on BLAST analysis. These two types of desaturase are very similar at the amino acid level and it was therefore not possible to predict on sequence alone which activity was encoded. The maximum degree of identity between the *P. salina* desaturase and other cloned desaturases was 29% to the *Acanthamoeba castellanii* Δ8-desaturase (Sayanova et al., 2006). Phylogenetic tree analysis (Fig. 4) shows that the *P. salina* desaturase clusters closer to the *A. castellani* Δ8-desaturase, while Δ6-desaturases and sphingolipid Δ8-desaturases from different sources

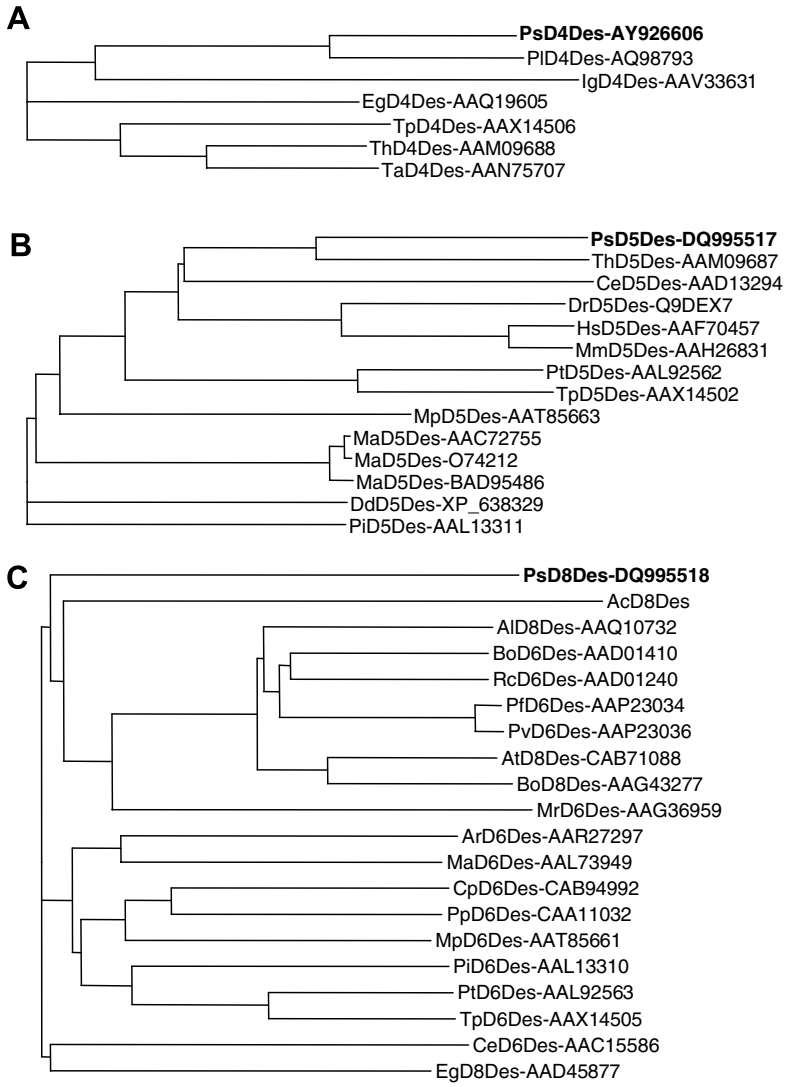

Fig. 4. Phylogenetic tree for sequence comparison of three front-end desaturases. (A–C) Phylogenetic tree of Δ4, Δ5 and Δ8-desaturases, respectively. Amino acid sequences started with initials of species and followed by accession numbers. Ac, *Acanthamoeba castllanii*; Al, *Anemone leveillei*; Ar, *Amylomyces rouxii*; At, *Arabidopsis thaliana*; Bo, *Borago officinalis*; Ce, *Caenorhabditis elegans*; Cp, *Ceratodon purpureus*; Dd, *Dictyostelium discoideum*; Dr, *Danio rerio*; Eg, *Euglena gracilis*; Hs, *Homo sapiens*; Ig, *Isochrysis galbana*; Ma, *Mortierella alpina*; Mm, *Mus musculus*; Mp, *Marchantia polymorpha*; Mr, *Mucor rouxii*; Pf, *Primula farinosa*; Pi, *Pythium irregulare*; Pl, *Pavlova lutheri*; Pp, *Physcomitrella patens*; Ps, *Pavlova salina*; Pt, *Phaeodactylum tricornutum;* Pv, *Primula vialii*; Rc, *Ricinus communis*; Ta, *Thraustochytrium aureum*; Th, *Thraustochytrium* sp. 21685; Tp, *Thalassiosira pseudonana*.

form separate clusters. This gene was confirmed as a Δ8-desaturase gene rather than a Δ6-desaturase gene as described below, and designated *PsD8Des*. The *P. salina* putative Δ4-desaturase and Δ5-desaturase form clusters with known Δ4-desaturases and Δ5-desaturases, respectively. This suggested that the three front-end desaturase genes isolated from *P. salina* encode Δ4, Δ5 and Δ8-desaturases (designated *PsD4Des*, *PsD5Des* and *PsD8Des*) which was confirmed later in an exogenous expression system.

2.4. Genetic analysis and gene expression of *P. salina* front-end desaturases

Southern blot analysis of *P. salina* genomic DNA suggested that *P. salina* genome contains a single copy of each of the Δ4, Δ5 and Δ8-desaturase genes (Fig. 5). This was consistent with the fact that all cDNA clones isolated for each front-end desaturase contained identical coding regions and UTR sequences.

The gene expression pattern of the three desaturases in *P. salina* was analyzed by Real-Time PCR. 18S rRNA was used as a reference for total RNA. The 18S rRNA gene was amplified from the *P. salina* cDNA using primers 18sRNA-F and 18sRNA-R (Table 2) based on the 18S rRNA sequence in GenBank (accession no. AF106059). Primer sets specific to each of the three desaturases, as well as the 18S rRNA gene (Table 2), were used to normalize the PCR condition against identical amounts of cDNA in purified plasmids. The amounts of amplicon generated from all four genes in the same amount of plasmid templates were similar when compared by gel electrophoresis, suggesting the primer binding efficiencies were equivalent.

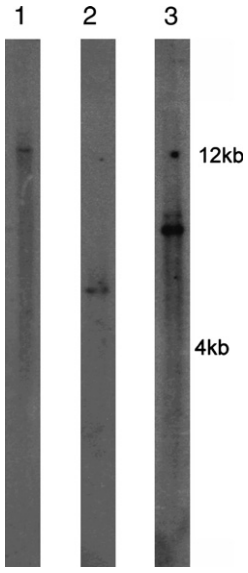

Fig. 5. Southern analysis of *P. salina* genomic DNA. Genomic DNA was digested by *Sma*I (lane 1) or *Sac*I (lanes 2 and 3) and hybridized with probes containing *P. salina* Δ4, Δ5, and Δ8-desaturase gene coding regions (lanes 1–3, respectively). The size at DNA fragments is indicated at the side.

In Real-Time PCR, no 18S rDNA PCR product was detected at the end of a 45-cycle PCR when reverse transcriptase was omitted, indicating that the RNA template was free of genomic DNA after DNase treatment. Real-Time PCR analysis data was normalized using 18S rRNA from the same reaction set as a reference. This analysis showed that among the three desaturase genes tested, the Δ8-desaturase gene was the most highly expressed, taking off at cycle 17.2, followed by the Δ4-desaturase gene, taking off at cycle 23.8. The Δ5-desaturase gene was the least expressed, taking off at cycle 31.2 (Fig. 6A). The relative expression levels of the three genes were also compared (Fig. 6B). The Real-Time PCR demonstrated that the Δ8-desaturase gene had the highest level of expression was consistent with the results of the initial cDNA library screening, where the Δ8-desaturase was far more abundantly represented than the other two desaturases.

Two PUFA elongase genes (*Elo1* and *Elo2*) were also isolated from *P. salina*. Elo1, a Δ5-elongase was used together with the *P. salina* Δ4-desaturase to produce DHA in transgenic *Arabidopsis* seed oil (Robert et al., 2005). Elo2 was confirmed as a Δ9-elongase in yeast expression system (data not shown). The expression levels of these two elongase genes were also tested in the same *P. salina* sample sets by Real-Time PCR. The result of this analysis showed the Δ5-elongase expression was very low (data not shown). This might be related to the apparently low Δ5-elongation rate in *P. salina*. On the other hand, the three desaturases all converted more than 95% of their substrates to products, even though the Δ5-desaturase expressed at a relatively low level compared to the other two desaturases.

The levels of front-end desaturase gene expression (related to the 18S rRNA in the same sample) were also compared for the *P. salina* cultured at 25 °C and 15 °C (5 °C higher or lower than the normal growth temperature of 20 °C) using Real-Time PCR. The results suggested that a higher growth temperature increased the expression of front-end desaturase genes. Comparatively, the expression of all three genes was reduced when *P. salina* was grown at 15 °C. The ratio of expression of desaturases in samples grown at 25 °C and 20 °C, was five times greater for the Δ4-desaturase than for the Δ5-desaturase gene (Fig. 6C).

2.5. Expression of *PsD4Des, PsD5Des and PsD8Des* in yeast

Pavlova salina PsD4Des, *PsD5Des* and *PsD8Des* cDNA coding regions were excised as *Eco*RI–*Sal*I, *Eco*RI–*Sph*I and *Eco*RI–*Xho*I cDNA fragments, respectively and inserted into the yeast expression vector pYES2 at the corresponding sites, generating plasmids pXZP275, pXZP276 and pYES2-psΔ8, respectively. Each plasmid was transformed into yeast cells and the transformants grown in YMM medium. The expression of gene was induced by the addition of galactose in the presence of various exogenously added ω6 and ω3 fatty acids to demonstrate enzyme

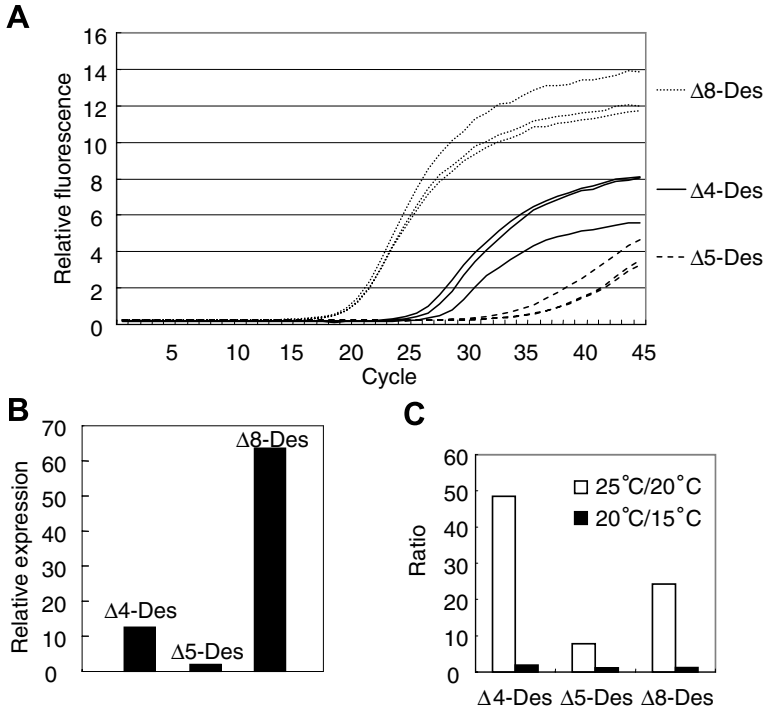

Fig. 6. Expression of *P. salina* front-end desaturase genes based on Real-Time PCR. (A) Relative fluorescence of Δ4, Δ5 and Δ8-desaturase PCR products ($n = 3$) during PCR cycles from *P. salina* grown at 20 °C, using its 18S rRNA template as internal standard. (B) Relative gene expression level (mean; $n = 3$) among Δ4, Δ5, and Δ8-desaturases in *P. salina* grown at 20 °C. (C) Gene expression ratio (mean; $n = 3$) of Δ4, Δ5, and Δ8-desaturases of *P. salina* grown at 25 °C compared to 20 °C (open bar) or at 20 °C compared to 15 °C (solid bar).

activities and the range of substrates that could be acted upon by the expressed genes. Individual fatty acid substrates were each added separately to the medium. In each experiment, yeast cells carrying the pYES2 vector only were also fed with fatty acids as a negative control.

Results from capillary gas chromatography (GC) analysis of yeast fatty acid profiles showed that the *PsD4Des* gene in pXZP275 encoded a Δ4-desaturase which was able to desaturate both $22{:}4^{\Delta7,10,13,16}$ (3.0% conversion to $22{:}5^{\Delta4,7,10,13,16}$) and $22{:}5^{\Delta7,10,13,16,19}$ (2.4% conversion to $22{:}6^{\Delta4,7,10,13,16,19}$) at the Δ4 position (Table 3). The enzyme did not show any Δ5-desaturation activity when the yeast transformants were fed with $20{:}3^{\Delta8,11,14}$ (ω6, DGLA) or $20{:}4^{\Delta8,11,14,17}$ (ω3, ETA) (data not shown). This data confirmed the prediction that *PsD4Des* encoded a Δ4-desaturase and not a Δ5-desaturase. The cloned *PsD5Des* gene in pXZP276 was able to desaturate $20{:}4^{\Delta8,11,14,17}$ (ω3, ETA) to $20{:}5^{\Delta5,8,11,14,17}$ (ω3, EPA) (11.3% conversion) at the Δ5 position, whilst no product was produced from other fatty acids added exogenously (Table 4), confirming that the gene *PsD5Des* encoded a Δ5-desaturase. When yeast cells carrying pYES2-psΔ8 were fed with $18{:}2^{\Delta9,12}$ (ω6, LA) or $18{:}3^{\Delta9,12,15}$ (ω3, ALA), no Δ6-desaturated products ($18{:}3^{\Delta6,9,12}$ (ω6, GLA) or $18{:}4^{\Delta6,9,12,15}$ (ω3, SDA)) were detected. However, when $20{:}3^{\Delta11,14,17}$ (ω3, ETrA) was fed, the presence of the Δ8-desaturated product $20{:}4^{\Delta8,11,14,17}$ (ω3, ETA) was detected (0.02% of total fatty acids) with 4.8% conversion (Table 5). It was concluded that the transgene

Table 3
Fatty acid composition (% total fatty acids) of transgenic yeast showing Δ4-desaturase activity in pXZP275 when fed with alternative PUFA

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	0.0
$22{:}5^{\Delta4,7,10,13,16}$ (ω6, DPA)	0.03	0.0
$22{:}5^{\Delta7,10,13,16,19}$ (ω3, DPA)	0.0	1.66
$22{:}6^{\Delta4,7,10,13,16,19}$ (ω3, DHA)	0.0	0.04
Conversion (%)	3.0	2.4

PsD8Des encoded a polypeptide having Δ8- but not Δ6-desaturase activity in yeast cells.

Extensive efforts, namely cDNA library probing with the Δ8-desaturase coding region and PCR screening of genomic DNA using primers based on regions of the Δ8-desaturase coding region, did not result in the isolation of a Δ6-desaturase gene from *P. salina*, suggesting this alga might not carry this gene. If so, the LC-PUFA biosynthesis pathway in *P. salina* is such that ALA is first elongated by a Δ9-elongase to yield eicosatrienoic acid ($20{:}3^{\Delta11,14,17}$), followed by a Δ8-desaturation to yield eicosatetraenoic acid ($20{:}4^{\Delta8,11,14,17}$). This pathway has been reported in *E. gracilis* (Wallis and Browse, 1999), and may also exist in another microalga *Isochrysis galbana*, as evidenced by the isolation of a Δ9-elongase (Qi et al., 2002), and in a non-photosynthetic free-living soil amoeba *A. castellanii as*

Table 4
Fatty acid composition (% total fatty acids) of transgenic yeast showing *P. salina* Δ5-desaturase activity in pXZP276 when fed with various PUFA

Fatty acid	ALA fed		ETA fed		DPA fed		DTA fed	
	pYES2	pXZP276	pYES2	pXZP276	pYES2	pXZP276	pYES2	pXZP276
$18:3^{\Delta9,12,15}$ (ω3, ALA)	65.9	68.8	0.0	0.0	0.0	0.0	0.0	0.0
$18:4^{\Delta6,9,12,15}$ (ω3, SDA)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$20:4^{\Delta8,11,14,17}$ (ω3, ETA)	0.0	0.0	4.4	4.7	0.0	0.0	0.0	0.0
$20:5^{\Delta5,8,11,14,17}$ (ω3, EPA)	0.0	0.0	0.0	0.0.6	0.0	0.0	0.0	0.0
$22:5^{\Delta7,10,13,16,19}$ (ω3, DPA)	0.0	0.0	0.0	0.0	1.5	1.5	0.0	0.0
$22:6^{\Delta4,7,10,13,16,19}$ (ω3, DHA)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$22:4^{\Delta7,10,13,16}$ (ω6, DTA)	0.0	0.0	0.0	0.0	0.0	0.0	0.6	1.0
$22:5^{\Delta4,7,10,13,16}$ (ω6, DPA)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

evidenced by the isolation of a Δ8-desaturase (Sayanova et al., 2006).

Fatty acid analysis of *P. salina*, however, indicates that typical Δ6-desaturase products (GLA, $18:3^{\Delta6,9,12}$ and SDA, $18:4^{\Delta6,9,12,15}$) are present in the total fatty acids. Considering the fact that there is only a single copy of Δ8-desaturase in the *P. salina* genome (Fig. 5), one possible explanation for the presence of GLA and SDA is that *P. salina* has a divergent Δ6-desaturase, as reported for microalga *Ostreococcus tauri* (Domergue et al., 2005b). This could explain the difficulties encountered when attempts were made to isolate a Δ6-desaturase based on *P. salina* cDNA library screening with a Δ8-desaturase probe.

Table 5
Fatty acid composition (% total fatty acids) of transgenic yeast showing *P. salina* Δ8-desaturase activity in pYES2-psΔ8 when fed with various PUFA

Fatty acid	pYES2	pYES2-psD8		
	ALA fed	ALA fed	LA fed	ETrA fed
$18:2^{\Delta9,12}$ (ω6, LA)	0.0	0.0	42.5	0.0
$18:3^{\Delta6,9,12}$ (ω6, GLA)	0.0	0.0	0.0	0.0
$18:3^{\Delta9,12,15}$ (ω3, ALA)	63.3	60.6	0.0	0.0
$18:4^{\Delta6,9,12,15}$ (ω3, SDA)	0.0	0.0	0.0	0.0
$20:3^{\Delta11,14,17}$ (ω3, ETrA)	0.0	0.0	0.0	0.4
$20:4^{\Delta8,11,14,17}$ (ω3, ETA)	0.0	0.0	0.0	0.02

2.6. Expression of PsD4des, PsD5des, PsD8des in plants

Arabidopsis seeds have endogenous elongated fatty acids $20:1^{\Delta11}$ and $20:2^{\Delta11,14}$ produced from $18:1^{\Delta9}$ and $18:2^{\Delta9,12}$, respectively (Katavic et al., 1995). The $20:2^{\Delta11,14}$ is potentially an ω6 substrate for a Δ8-desaturase, whilst $20:1^{\Delta11}$

Table 6
Fatty acid composition (%) of *Arabidopsis* seed transformed with Δ5- and Δ8-desaturases from *Pavlova salina*

Fatty acid	WT	DR1	DR6	DR7	DR12	DR13	DR14	DR16	DR17	DR18
14:0	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
15:0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16:0	7.8	8.7	7.9	7.9	8.0	8.2	8.5	8.2	8.0	8.1
$16:1^{\Delta9}$	0.5	0.7	0.6	0.5	0.5	0.5	0.6	0.5	0.5	0.5
18:0	3.6	3.4	3.6	3.6	3.5	3.4	3.5	3.5	3.6	3.4
$18:1^{\Delta9}$	17.1	17.4	16.4	16.6	16.7	16.7	18.2	17.4	18.8	17.3
$18:2^{\Delta9,12}$	27.9	31.3	30.1	29.1	30.1	29.8	30.2	28.3	28.5	29.4
$18:3^{\Delta9,12,15}$[a]	16.9	15.7	16.4	17.0	16.6	16.4	15.6	15.7	16.2	16.2
20:0	1.5	1.7	1.8	2.0	1.7	1.7	1.6	1.9	1.8	1.9
22:0	0.8	0.6	0.7	0.6	0.7	0.6	0.7	0.8	0.7	0.6
24:0	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.3
$20:1^{\Delta11}$ (ω9)[b,c]	18.0	14.4	16.7	15.9	15.6	16.6	14.3	16.4	16.5	16.1
$20:2^{\Delta8,11}$ (ω9)[d]	0.0	0.3	0.5	0.8	0.7	0.3	0.9	0.7	0.4	0.6
$20:3^{\Delta5,8,11}$ (ω9)[d]	0.0	0.1	0.1	0.6	0.6	0.1	0.5	0.7	0.2	0.4
$20:2^{\Delta11,14}$ (ω6, EDA)[d]	0.9	0.1	0.0	0.3	0.0	0.6	0.0	0.6	0.5	0.5
$20:3^{\Delta8,11,14}$ (ω6, DGLA)	0.0	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0
$20:4^{\Delta5,8,11,14}$ (ω6, AA)	0.0	0.1	0.2	0.4	0.4	0.2	0.4	0.4	0.2	0.4
Other MUFA	4.6	4.6	4.5	4.2	4.4	4.5	4.4	4.6	4.3	4.2
Sum	100	100	100	100	100	100	100	100	100	100
Δ8Des ω9 conversion		2.8%	3.5%	8.1%	8.2%	2.1%	9.2%	8.0%	3.4%	6.2%
Δ5Des ω9 conversion		20.9%	24.6%	43.1%	46.2%	15.5%	34.6%	47.9%	38.3%	39.5%
Δ8Des ω6 conversion		65.4%	100.0%	57.3%	100.0%	29.0%	100.0%	40.2%	32.9%	43.7%
Δ5Des ω6 conversion		59.9%	65.4%	100.0%	100.0%	100.0%	73.8%	100.0%	100.0%	100.0%

[a] Trace amount of 18:1ω7 co-eluted.
[b] 20:1ω11 which coelutes with 20:1ω9 may be present in trace proportions.
[c] 20:3ω3 coeluted with 20:1ω9 and was detected by GC–MS of dimethyl oxazoline (DMOX) derivatives as a minor component.
[d] Identification confirmed by GC–MS of DMOX derivatives.

is potentially an ω9 substrate for a Δ8-desaturase. We therefore tested the functionality of the cloned *P. salina* Δ5- and Δ8-desaturase genes in *Arabidopsis* seed. Expression of a Δ8-desaturase together with a Δ5-desaturase under the *Brassica* seed specific promoter *Fp1* (Stalberg et al., 1993) resulted in Δ5- and Δ8-desaturated fatty acid products in transgenic lines (Table 6). Firstly, a Δ8-desaturated fatty acid product ($20{:}3^{\Delta 8,11,14}$) produced from the ω6 substrate $20{:}2^{\Delta 11,14}$ was found in trace amounts in three transgenic lines, while the Δ5-desaturated fatty acid product ($20{:}4^{\Delta 5,8,11,14}$) from the ω6 substrate $20{:}3^{\Delta 8,11,14}$ was present in all transgenic lines at levels of up to 0.4%. Neither of these fatty acids were present in the untransformed *Arabidopsis*. This demonstrated that these two enzymes were functional in plant seeds, having the same activities as in yeast cells. Furthermore, the Δ5-desaturated and Δ8-desaturated fatty acid products $20{:}2^{\Delta 8,11}$ and $20{:}3^{\Delta 5,8,11}$ produced from the ω9 substrate $20{:}1^{\Delta 11}$ were found in all transgenic lines. This demonstrated that these enzymes could also desaturate ω9 unsaturated fatty acids. The lines that accumulated a lower amount of $20{:}2^{\Delta 8,11}$ generally also accumulated lower amounts of $20{:}3^{\Delta 5,8,11}$. When compared to the conversion rates of Δ5- and Δ8-desaturases in the transgenic plants, although they were variable from line to line due to the low amount of substrates or products, these enzymes generally showed a preference for ω6 rather than ω9 substrates. In general, Δ5-desaturase was more efficient than Δ8-desaturase, especially when using ω9 fatty acids as substrates. Finally, in addition to desaturating ω3 fatty acid $20{:}3^{\Delta 11,14,17}$ in yeast (Table 5), the plant expression results demonstrate that the *P. salina* Δ8-desaturase can also desaturate ω6 and ω9 fatty acids. This observation is in agreement with other Δ8-desaturases isolated from *A. castellanii* (Sayanova et al., 2006) and *Euglena gracilis* (Wallis and Browse, 1999), where the Δ8 desaturase was able to catalyse the desaturation of both $20{:}2^{\Delta 11,14}$ (ω6) and $20{:}3^{\Delta 11,14,17}$ (ω3) at the Δ8 position.

Aside from providing an alternative route for the production of EPA in cells, the strategy of using a Δ9-elongase in combination with the Δ5- and Δ8-desaturases may provide an advantage in that the elongation, which occurs on fatty acids coupled to Coenzyme A (CoA), precedes the desaturation, which occurs on fatty acids coupled to phosphatidylcholine (PC), for subsequent two desaturation steps by Δ8- and Δ5-desaturases, possibly leading to more efficient EPA synthesis. That is, the order of reactions – an elongation followed by two desaturations – will potentially reduce the amount of substrate switching that needs to occur for EPA synthesis (Qi et al., 2004; Domergue et al., 2005a; Robert et al., 2005; Singh et al., 2005).

We have previously reported that the *P. salina* Δ4-desaturase is functional in *Arabidopsis* seeds, and produced up to 0.5% DHA from 3.0% EPA when co-expressed with the *P. salina* Δ5-elongase, the zebrafish Δ5/Δ6-desaturase and the nematode Δ6-elongase (Robert et al., 2005). The identification and confirmation of activity of the three front-end desaturases in this report and two elongase (Robert et al., unpublished) from *P. salina* will provide an all plant suite of genes for the overall goal to achieve ω3 LC-PUFA in higher plants.

3. Conclusions

We have isolated and characterized three genes from the microalga *P. salina* encoding Δ4-, Δ5, and Δ8-acting members of the front-end class of fatty acid desaturases. Analysis of fatty acid profiles from *P. salina* grown at 15 °C, 20 °C and 25 °C showed that each of these three enzymes desaturated ω3 and ω6 substrates with greater than 95% efficiency at all growth temperatures. All three desaturases contain the expected conserved histidine boxes and cytochrome b_5 domain. The sequences are homologous to the known Δ4-, Δ5- and Δ8-desaturases (Fig. 4) and their catalytic functions were confirmed in both yeast cells and a higher plant. Transgenic expression of these genes in combination with appropriate fatty acid elongases resulted in conversion of endogenous C18-PUFA into ω3 LC-PUFA such as EPA and DHA in seeds (Table 6, and Robert et al., 2005).

4. Experimental

4.1. Pavlova salina culturing and biomass preparation

Pavlova salina strain CS-49 was obtained from the CSIRO Collection of Living Microalgae (http://www.cmar.csiro.au/microalgae) and cultivated under standard culture conditions: f/2 medium – a modification of Guillard and Ryther's (1962) f medium containing half-strength nutrients; 20 ± 1 °C; 100 μmol photons PAR m^{-2} s^{-1}, 12:12 h light:dark photoperiod; bubbling with 1% CO_2 in air at a rate of 200 mL L^{-1} min^{-1}. Under these conditions, biomass for cDNA library construction was obtained by scaling-up a stock culture to 10 L in a polycarbonate carboy and harvested after four days by centrifugation at 3500 rpm for 10 min before directly extracting. For a comparison of the effects of temperature on gene expression and fatty acid composition, three newly inoculated 1 L cultures were acclimatised at 15 °C, 20 °C and 25 °C in temperature-controlled cabinets for a minimum of five generations. Acclimatised cultures then provided standardised inocula for three replicate 1 L cultures in 2 L glass Erlenmeyer flasks and incubated according to their respective temperatures under the conditions outlined above. Cell counts were estimated daily by removing 1 mL aliquots from all replicate cultures and counted using a haemocytometer. At late-logarithmic phase, 800 mL of each culture was taken for quantitative Real-Time PCR analysis and 50 mL aliquots taken for fatty acid analysis. Cultures were harvested by centrifugation at 2000 rpm for 15 min and the cell pellets frozen in liquid nitrogen before storage at −80 °C.

4.2. cDNA library construction

Pavlova salina mRNA was isolated from two grams (wet weight) of cells using a mortar and pestle in liquid nitrogen and sprinkled slowly into a beaker containing 22 ml of extraction buffer that was stirred constantly. To this, 5% insoluble polyvinylpyrrolidone, 90 mM β-mercaptoethanol, and 10 mM dithiothreitol were added and the mixture stirred for a further 10 min prior to being transferred to a Corex™ tube. 18.4 mL of 3 M ammonium acetate was added and mixed well. The sample was then centrifuged at 6000*g* for 20 min at 4 °C. The supernatant was transferred to a new tube and nucleic acid precipitated by the addition of 0.1 vol. of 3 M NaAc (pH 5.2) and 0.5 vol. of cold isopropanol. After an 1 h incubation at −20 °C, the sample was centrifuged at 6000*g* for 30 min in a swing rotor. The pellet was resuspended in 1 ml of water and extracted with phenol/chloroform. The aqueous layer was transferred to a new tube and nucleic acids were precipitated once again by the addition of 0.1 vol. 3 M NaAc (pH 5.2) and 2.5 vol. of ice cold ethanol. The pellet was resuspended in water, the concentration of nucleic acid determined and then mRNA was isolated using the Oligotex mRNA system (Qiagen, Valencia, CA, USA).

First strand cDNA was first synthesised using an oligo-dT linker-primer (Stratagene, La Jolla, CA, USA) and the reverse transcriptase Superscript III (Invitrogen, Carlsbad, CA, USA), and the *P. salina* cDNA library was then constructed using the ZAP-cDNA synthesis kit (Stratagene) as described in the manufacturer's instruction manual.

4.3. Isolation of P. salina front-end desaturase genes

RT-PCR was used for the isolation of a Δ4-desaturase gene fragment. Degenerate PCR primers PavD4Des-F3 and PavD4Des-R3 (Table 2) corresponding to the conserved amino acid sequence of known Δ4-desaturase histidine box I and complementary to a nucleotide sequence encoding the amino acid sequence of histidine box II, respectively, were designed to amplify the corresponding region of *P. salina* desaturase genes. PCR amplification reactions using these primers were carried out using *P. salina* first strand cDNA as template with cycling of 95 °C, 5 min for 1 cycle, 94 °C 30 s, 57 °C 30 s, 72 °C 30 s for 35 cycles, and 72 °C 5 min for 1 cycle. A *P. salina* Δ6-desaturase-like gene fragment was amplified from genomic DNA. Degenerate primers PsD6Des-F1 and PsD6Des-R1 (Table 2) corresponding to Δ6-desaturase motifs WWKDKHN and QIEHHLF were used. A set of oligonucleotides was designed based on a partial desaturase-like sequence of *P. lutheri* found in International Patent Application WO03078639-A2. This sequence showed homology to known Δ5-desaturases by BlastX analysis. Oligo PaD5-LF and PaD5-LR (Table 2) corresponded to nucleotides 115–214 and 195–294 of above partial sequence. These oligonucleotides were annealed and extended in a PCR reaction. All PCR products were cloned in pGEM-T Easy vector (Promega, Madison, WI, USA), and sequenced with reverse primer from the pGEM-Teasy vector.

The full-length cDNAs for three front-end desaturases were isolated by screening the *P. salina* cDNA library at high stringency (for the Δ4- and Δ8-desaturase genes), or low stringency (for the Δ5-desaturase gene), using corresponding PCR fragments as probes. About 500,000 plaque-forming units (pfu) of the *P. salina* cDNA library were screened in each case. The hybridization was performed at 60 °C overnight and washing was done with 2× SSC/0.1% SDS for 30 min at 65 °C, followed by washing with 0.2× SSC/0.1% SDS for 30 min at 65 °C for high stringency; 55 °C overnight and washing the membranes at 60 °C with 2× SSC/0.1% SDS three times for 10 min for low stringency. The plasmids were excised from the positive plaques, and the nucleotide sequences of the inserts were determined.

4.4. Genomic Southern blot analysis

Pavlova salina genomic DNA was isolated from 20 mg of freeze-dried cells using DNeasy Plant Mini Kit (Qiagen), followed by extra purification with DNAzol ES (Molecular Research Center, Inc, Cincinnati, OH, USA) according to manufacturer's instructions. The DNA was then digested overnight in 6 μg aliquots with various restriction enzymes (*Sac*I, *Sma*I and *Xho*I) and electrophoresed (1% agarose TAE gel, 38 V, 16 h). The DNA was transferred to a Biodyne B positively charged 0.45 μm nylon membrane (Pall Corporation, Pensacola, FL, USA), and hybridized with ^{32}P radio-labeled probes of *P. salina* Δ4-, Δ5-, and Δ8-desaturase full-length coding sequences at 65 °C overnight. Following hybridization, the membrane was washed at high stringency (0.1× SSC + 0.1% SDS at 65 °C), and exposed to X-ray film.

4.5. Real-Time PCR

Total RNA was isolated from *P. salina* cultured at 15 °C, 20 °C and 25 °C as above. Total *P. salina* RNA was treated with Turbo DNA-free™ (Ambion, Austin, TX, USA) according to manufacturer's instructions. Then 1 μg of treated RNA was converted to cDNA with oligo(dT)$_{20}$ primer and an 18S rRNA-specific antisense primer (Ps18s-R) by SuperScript III RNaseH$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) by using 50 pmol of oligo(dT)$_{20}$ and 5 pmol of Ps18s-R primer, in the presence of RNaseOut™ (Invitrogen). A fraction (0.1 μg) of total RNA was treated as above, but no reverse transcriptase was added to confirm that there was no genomic DNA contamination of the treated RNA. Each primer set was used to amplify cDNA from 25 ng of total RNA for Real-Time PCR in triplicates, each with 1 μL of diluted SYBR Green (Invitrogen). The PCR conditions were 15 s at 95 °C, 15 s at 67 °C and 20 s at 72 °C for 45 cycles on Rotor-gene 2000 Real-Time Cycler (Cornett Research,

Sydney, Australia). The PCR products were also run on a 1% agarose gel afterwards to confirm the amplification.

4.6. Expression of genes in yeast

Plasmids were introduced into yeast by heat shock and transformants were selected on yeast minimal medium (YMM) plates containing 2% raffinose as the sole carbon source. Clonal inoculum cultures were established in liquid YMM with 2% raffinose as the sole carbon source. Experimental cultures were inoculated from these, in YMM + 1% NP-40, to an initial OD_{600} of ~0.3. Cultures were grown at 30 °C with shaking (~60 rpm) until OD_{600} was approximately 1.0. At this point galactose was added to a final concentration of 2% and precursor fatty acids were added to a final concentration of 0.5 mM. Cultures were incubated at 20 °C with shaking for a further 48 h prior to harvesting by centrifugation. Cell pellets were washed sequentially with 1 vol. of 1% NP-40, 0.5% NP-40 and water to remove any unincorporated fatty acids from the surface of the cells.

4.7. Expression of genes in higher plants

The plant expression vectors containing the microalga *P. salina* desaturases were constructed as follows. The Δ8-desaturase gene was inserted as a *Bam*HI–*Nco*I fragment from its cDNA (Accession no. DQ995518) into pXZP143 (Robert et al., 2005) between the *Brassica* seed specific napin promoter *Fp1* (Stalberg et al., 1993) and the *Nos* terminator to generate plasmid pXZP146. Similarly, the Δ5-desaturase gene was inserted as an *Eco*RI–*Bss*HII fragment from its cDNA (Accession no. DQ995517) into pXZP143 to generate plasmid pXZP147. The *Fp1-Δ5Des-Nos3′* cassette of pXZP147 was excised as a *Hind*III–*Apa*I fragment and cloned into pXZP146 at *Stu*I–*Apa*I sites downstream of the Δ8-desaturase gene expression cassette, generating pXZP149. The pyramided gene expression cassettes from pXZP149 were then excised as a *Hind*III–*Apa*I fragment and cloned into binary vector pWVec8-Fp1 (Singh et al., 2001), generating expression plasmid pXZP354, with a hygromycin resistance gene as a selectable marker for selection in plant cells. The *P. salina* Δ4-desaturase gene was cloned in the binary vector generating 'DHA construct' as described previously (Robert et al., 2005). The plant expression vectors were first transformed into *Agrobacterium* by electroporation, and then transformed into *Arabidopsis* ecotype Columbia as described previously (Zhou et al., 2006).

4.8. Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared by transesterification of the centrifuged yeast pellet or *Arabidopsis* seeds as described (Zhou et al., 2006) and analysed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). For capillary GC, FAME were analysed with a Hewlett Packard (HP) 5890 GC or Agilent 6890 gas chromatograph fitted with HP 7673A or 6980 series automatic injectors respectively as previously described (Zhou et al., 2006). Identification of peaks was based on comparison of relative retention time data with standard FAME with further confirmation using mass-spectrometry. For quantification Empower software (Waters) or Chemstation (Agilent) was used to integrate peak areas. GC–MS was carried out essentially as previously described (Zhou et al., 2006). Mass spectra were acquired and processed with Xcalibur™ software (Thermo Electron Corporation). For PUFA present as minor or trace components (e.g. <0.05%), confirmation of identification was achieved by comparison of GC retention time with standards under two different oven programs, and by mass spectral data obtained for the yeast samples after concentration. Reconstructed ion chromatograms of molecular and other major diagnostic ions were also used to confirm component identifications. Control samples did not contain these minor and trace PUFA.

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