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Identification of a very long chain polyunsaturated fatty acid Δ4-desaturase from the microalga *Pavlova lutheri*[1]

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Abstract ***Pavlova lutheri*, a marine microalga, is rich in the very long chain polyunsaturated fatty acids (VLCPUFAs) eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. Using an expressed sequence tag approach, we isolated a cDNA designated *Pldes1*, and encoding an amino acid sequence showing high similarity with polyunsaturated fatty acid front-end desaturases. Heterologous expression in yeast demonstrated that PlDES1 desaturated 22:5n-3 and 22:4n-6 into 22:6n-3 and 22:5n-6 respectively, and was equally active on both substrates. Thus, PlDES1 is a novel VLCPUFA Δ4-desaturase. *Pldes1* expression is four-fold higher during the mid-exponential phase of growth compared to late exponential and stationary phases.**

Key words: Very long chain polyunsaturated fatty acid; Docosahexaenoic acid; Δ4-Desaturase; *Pavlova lutheri*

1. Introduction

Very long chain polyunsaturated fatty acids (VLCPUFAs) such as docosahexaenoic acid (DHA) 22:6n-3 are important components of infant as well as adult nutrition, serving as structural elements of cell membranes [1]. They can be classified into the ω6 and ω3 families, derived mainly from the diet or from the metabolism of dietary linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3). VLCPUFAs play key roles in various biological functions, such as inflammatory response [2], foetal growth and development [3], retina function [4] and brain development [5]. Although humans can synthesise VLCPUFAs, dietary changes over the last decades have resulted in the consumption of fatty acids in very high ω6 to ω3 ratios, which have negative impacts on both health and development [6].

PUFAs are currently obtained from a number of sources including higher plants and oily fish [7], but it has become evident that PUFA production from current sources is inadequate for supplying the expanding market [8,9]. In order to address this increasing demand and to preserve marine species, alternative sources have been researched, among which are the production of PUFAs in transgenic oilseed crops [10]. In order to meet this target, it is essential that genes responsible for the various steps involved in VLCPUFA synthesis, including substrate specific desaturases and elongases, are isolated and shown to be functional in plant species [7]. Front-end desaturases involved in the production of ω6-arachidonic acid (20:4n-6) and ω3-eicosapentaenoic acid (EPA) (20:5n-3) have already been isolated from prokaryotic and eukaryotic organisms [11]. The conventional view is that DHA is synthesised by further elongation to ω3-docosapentaenoic acid (22:5n-3), followed by Δ4-desaturation. So far, only one gene encoding a VLCPUFA Δ4-desaturase has been isolated, from the marine fungus *Thraustochytrium* sp. [12]. However, in mammals, DHA biosynthesis occurs via a Δ4-independent pathway [13], involving two consecutive elongations of 20:5n-3 to 24:5n3, a Δ6-desaturation in microsomes and a two-carbon shortening via limited β-oxidation in peroxisomes.

Microalga are some of the most important feed sources in aquaculture [14] because of their nutritional value owing to their ability to synthesise and accumulate great amounts of PUFAs. The flagellate *Pavlova lutheri* has been recently shown to be a good candidate for biochemical and molecular analysis in order to understand and manipulate the processes that are responsible for the production and the incorporation of these VLCPUFAs into storage oils [15,16,17]. On the other hand, construction and analysis of expressed sequence tag (EST) libraries have become an effective means of gene discovery in focused metabolic situations. As described in this work, random sequencing of a cDNA library prepared from *P. lutheri* cells resulted in the identification of a cDNA encoding a Δ4-desaturase active on both 22:4n-6 and 22:5n-3 fatty acids.

2. Materials and methods

2.1. *Cultivation of* P. lutheri

P. lutheri (CCAP 931/1) was obtained from the Culture Collection of Algae and Protozoa (Dunstaffnage Marine Lab., Oban, PA34 4AD, Scotland, UK). The growth medium used was enriched artificial seawater medium, made up in 20 l batches as described previously [17]. The cultures were grown in 1 l flasks at 15°C with 50 μE m^{-2} s^{-1} constant illumination, and aeration provided by shaking the flasks at 150 rpm.

Cell density was monitored by counting cells with a haemocytometer, after immobilising the cells by incubation in 20 mM sodium azide. Nitrate concentration was determined periodically during the culture time by measuring the change of the medium absorbance at 220 nm [18].

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[1] The sequence reported in this paper has been submitted to GenBank database under the accession number AY332747.

Abbreviations: DHA, docosahexaenoic acid; EST, expressed sequence tag; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; RACE, rapid amplification of cDNA ends; RT, reverse transcription; VLCPUFA, very long chain polyunsaturated fatty acid

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2.2. cDNA library construction

Total RNA was extracted from cells harvested in the middle of the exponential growth phase by centrifugation and frozen in liquid nitrogen. RNA extracts [19] were treated using RNeasy mini kit (Qiagen) and RNase-free DNase set (Qiagen) following manufacturer's instructions to discard DNA contamination.

Poly(A)$^+$ was isolated from total RNA using a poly(A) Quick® mRNA isolation kit (Stratagene). The cDNA library was subsequently constructed from the isolated mRNA using the ZAP-cDNA® Gigapack® III gold cloning kit (Stratagene) according to the manufacturer's instructions. A primary library of 3×10^6 plaque-forming units was obtained. The library was subsequently amplified and excised using the ExAssist™ Interference-Resistant Helper Phage (Stratagene). The excised phagemids were plated as individual bacterial colonies following the manufacturer's instructions. The presence of insert was checked by polymerase chain reaction (PCR) using universal primers and clones containing cDNA longer than 0.2 kb were selected for sequencing.

2.3. Expression of PlDES1 in yeast

5′-Rapid amplification of cDNA ends (RACE) was carried out to determine the 5′ nucleotide sequence of the desaturase using the SMART™ RACE cDNA amplification kit (BD Biosciences). First-strand cDNA synthesis was performed following manufacturer's instructions, and a first PCR was conducted on 10 μl of 5′-RACE ready cDNA with UPM (universal primer mix) kit specific and Delfor (5′-TGCAGCATGGACGACAAG-3′) primers. Nested PCR was then performed using the NUP (nested universal primer) kit specific primer and Delfor3 (5′-GCACACCGTCGCGCAGATGCACAG-3′). Fragments generated were gel-purified using the gel extraction kit (Qiagen), cloned into the pCR®2.1-TOPO vector (Invitrogen) and sequenced.

The entire *Pldes1* coding region was amplified from *P. lutheri* cDNA with primers Des1NK1 5′-GCGGGTACC**ATG***GCT*CCG-CCTTCGGCCGCGAGC-3′ (open reading frame (ORF) start codon is indicated by bold type; underlined sequence is a *Kpn*I site; italic sequence is an added alanine codon, not present in the original sequence of *Pldes1*) and Des1CS 5′-GCGGAGCTC**TCA**CTCGG-CCTTGCCGCC-3′ (ORF stop codon is indicated in bold type; underlined sequence is a *Sac*I site). The Expand High Fidelity PCR system (Roche) was employed to minimise potential PCR errors. The amplified product was gel purified, restricted and cloned into the corresponding sites behind the galactose-inducible GAL1 promoter of pYES2 (Invitrogen) to yield the plasmid pYDES1. This vector was transformed into *Saccharomyces cerevisiae* strain Invsc1 (Invitrogen) by a lithium acetate method, and transformants were selected on minimal medium plates lacking uracil.

For functional expression, cultures were grown at 22°C in the presence of 2% (w/v) raffinose and 1% (w/v) Tergitol NP-40 (Sigma). Expression of the transgene was induced when OD_{600nm} reached 0.2–0.3 by supplementing galactose to 2% (w/v). At that time, the appropriate fatty acids were added to a final concentration of 50 μM. Incubation was carried out at 22°C for 3 days and then 15°C for another 3 days. For the co-feeding experiment, the same conditions were applied, except that both substrate fatty acids were added to 25 μM final concentration.

2.4. Fatty acids analysis

Microalgal or yeast cells were harvested by centrifugation. Total fatty acids were extracted and transmethylated as previously described [17]. Sample fatty acid methyl esters (FAMEs) containing methyl heptadecanoate as an internal standard were dissolved in hexane and 2 μl aliquots injected for GC-FID analysis using a BPX70 60 m×0.25 mm i.d.×0.25 μm film thickness capillary column (SGE) and a CE instruments GC8000 Top GC (Thermoquest). Injection was made into a hydrogen carrier gas stream at 1.3 ml min^{-1} (average linear velocity 35 cm s^{-1}) at a 30:1 split ratio. Temperature was ramped as follows: 110°C isothermal 1 min; 7.5°C min^{-1} to 260°C; cool down 70°C min^{-1} to 110°C; total analysis time 23 min. Most FAMEs were identified by comparison of retention times to a 37 FAME mix (Supelco). PUFA FAMEs were identified by comparison to a sample of standard menhaden oil (Supelco) transmethylated as per the samples.

The identity of unknown PUFA FAMEs was confirmed by GC-MS. Injections (2 μl) were made at a 30:1 split ratio and an injector temperature of 250°C into a 30 m×0.2 mm i.d.×0.5 μM film thickness ZB-1 capillary column (Phenomenex, UK) with He as the carrier gas at a constant flow of 0.6 ml min^{-1}. The GC oven temperature programme was 120°C for 1 min, and then ramped at 5°C min^{-1} to 340°C, which eluted all FAMEs up to C30. Peaks were detected using a GCQ mass spectrometer (Thermofinnigan, Manchester UK) using electron impact ionisation at 70 kV. Mass spectra of individual peaks were examined using Xcalibur software (Thermofinnigan). Identities were made based on the similarity of spectra between standard and candidate peaks.

2.5. DNA extraction and Southern blot analysis

Genomic DNA was extracted from cells at the late exponential phase using the DNA isolation kit Puregene® (Gentra Systems) and 8 μg was digested, electrophoresed through 1% agarose gel, and then blotted onto a positively charged nylon membrane Hybond N$^+$ (Amersham). The blot was probed with a specific PlDES1 fragment amplified from the plasmid used for yeast expression and corresponding to the sequence between nucleotides 431 and 1240 of the ORF encoding PlDES1. The probe was labelled using the DIG DNA labeling and detection kit (Roche Diagnostics) and hybridisation was performed overnight at 65°C. The membrane filter was washed twice with 2×saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min and then washed twice with 0.1×SSC, 0.1% SDS at 65°C for 15 min. Chemiluminescent detection was performed with CDP-star™ (Roche Diagnostics) according to the manufacturer's instructions.

2.6. Quantitative real time PCR

Total RNA was extracted at different times of cultivation of *Pavlova* cells following the protocol described above. Single-strand cDNA was synthesised from DNase-treated RNA using ProSTAR™ first-strand reverse transcription (RT)-PCR kit (Stratagene) with oligo(dT) primer. The completed first-strand cDNA synthesis reaction mix was then diluted five times before further use. To estimate the relative accumulation of *Pldes1* transcripts, quantitative real time PCR was performed using an ABI Prism 7000 detection system (Applied Biosystems). SYBR Green PCR Master mix (Applied Biosystems) was mixed with 5 μl of diluted cDNA and *Pldes1* specific primers DES1-411F (5′-CGGCTGCACCATTCGTACTAC-3′) and DES1-478R (5′-AAGCCGAGCTTCTCGATGTG-3′) (final concentration 0.8 μM) in 25 μl of reaction mixture. PCR reaction was carried out according to the following thermal profile: 95°C 10 min, (95°C 15 s, 60°C 1 min)×40 cycles. Template concentration was estimated by the number of cycles needed for SYBR Green fluorescence to cross a predetermined threshold. The highest concentrated cDNA was diluted 1–5^4-fold in a 5-fold increase to prepare a standard curve. Relative amounts of *Pldes1* transcript were assessed using the standard curve. In the same way, 18S rRNA was also quantified using the specific primers 18S-469F (5′-TGGTGCATGGCCGTTCTT-3′) and 18S-542R (5′-GCAGGGTAAGGTCTCGTTCGT-3′). Finally, the amount of *Pldes1* transcript was normalised to that of 18S rRNA and compared among samples. The normalised values were then transformed by a factor 1000 for graphical representation.

3. Results

3.1. Identification of a cDNA coding for the front-end desaturase

We have recently reported that *P. lutheri* represented a good candidate to isolate genes involved in VLCPUFA synthesis. In order to improve the production and the storage of DHA in this microalga, growth conditions previously used have been altered. The temperature was decreased from 18°C to 15°C and the light from 240 to 50 μE m^{-2} s^{-1}. As a result, growth rate during the exponential phase was multiplied by 1.5 (Fig. 1A) and the biomass at the end of the culture was increase by a similar factor. Furthermore, the quantities of EPA and DHA produced per cell (Fig. 1B) were significantly increased compared with the previous study [17]. EPA, a precursor of DHA biosynthesis, was more abundant than DHA throughout the culture of *Pavlova* cells

(Table 1). DHA was most abundant in the mid-exponential growth phase. Following this observation, and considering that genes encoding enzymes involved in the DHA biosynthesis should be highly expressed before and during the stage of high production of VLCPUFAs, cells were harvested in the middle of the exponential phase for RNA extraction. From the cDNA library, a total of 4986 ESTs were generated through partial single-pass sequencing, yielding 1742 unigene sequences. Blast similarity search showed that a unigene sequence of 480 bp, constituted by assembling four ESTs, contained an incomplete ORF encoding an amino acid sequence with homology to Δ5-desaturases. A full-length cDNA sequence of 1619 bp for this ORF was obtained by 5′-RACE PCR, and named *Pldes1*. Assuming the coding region of the cDNA to be full-length, *Pldes1* encoded a protein of 445 amino acids, with a molecular mass of 49 kDa. Hydropathy analysis predicted two transmembrane regions and dilysine residues at −3 and −6 relative to the C-terminus (Fig. 2) gave probable location in the endoplasmic reticulum [20].

A homology search indicated that PlDES1 shares amino acid sequence similarity to the front-end desaturases in the public databases. Alignment of PlDES1 amino acid sequence with the *Thraustochytrium* Δ4- and Δ5-desaturase sequences (Fig. 2) indicated an overall identity of 27% and 25% respectively, with the cytochrome *b5*-like domain and the three conserved histidine-rich motif areas showing greatest homology. PlDES1 is 74 amino acids shorter than FAD4, the length difference featuring mainly between the second and third histidine motif. In the second conserved histidine motif of the *Thraustochytrium* Δ4-desaturase and the *Pavlova* desaturase, there were three rather than two amino acids between the first and second histidine.

To establish the copy number of *Pldes1* in the genome, Southern hybridisation was performed using a *Pldes1* specific probe (Fig. 1C, inset). Digestion of genomic DNA with four different restriction enzymes resulted in unique banding patterns with *Hin*fI, *Pvu*II, *Sac*I, and two bands in *Xho*I lane. This observation was expected since a restriction site for this enzyme is present in the middle of the *Pldes1* cDNA. It was therefore determined that the desaturase gene is present as a single-copy gene in the genome of *P. lutheri*.

After establishing that a unique gene encoded PlDES1, real time quantitative PCR analysis was performed to determine the transcript profile of *Pldes1*. In parallel to PCR with *Pldes1* specific primers, the concentration of 18S rRNA was determined to correct for differences in the amount of RNA in each extract. The pattern of transcription (Fig. 1C) showed that the *Pldes1* cDNA was four times more abundant in the middle of the exponential phase than during the rest of the culture period.

Table 1
Composition of the major fatty acids of *P. lutheri* at different culture periods

Fatty acid	Mol % of total fatty acids				
	Time of incubation (h)				
	165	233	334	404	520
14:0	12.20	12.6	11.6	11.2	10.1
16:0	27.0	22.6	16.8	23.3	24.4
16:1n-9	21.0	24.5	20.9	24.8	25.2
18:1n-9	–	1.3	7.3	7.1	5.2
18:4n-3	2.6	6.2	5.8	4.5	3.2
20:5n-3	12.9	18.5	16.7	14.2	11.0
22:6n-3	6.4	9.5	7.4	6.1	4.8

The microalgae were harvested after different times of growth as described in Section 2. Fatty acids were extracted and converted to their methyl esters before analysis by GC. Each peak was identified by the retention time compared to known standards; each value represents the average of two replicates within a representative culture.

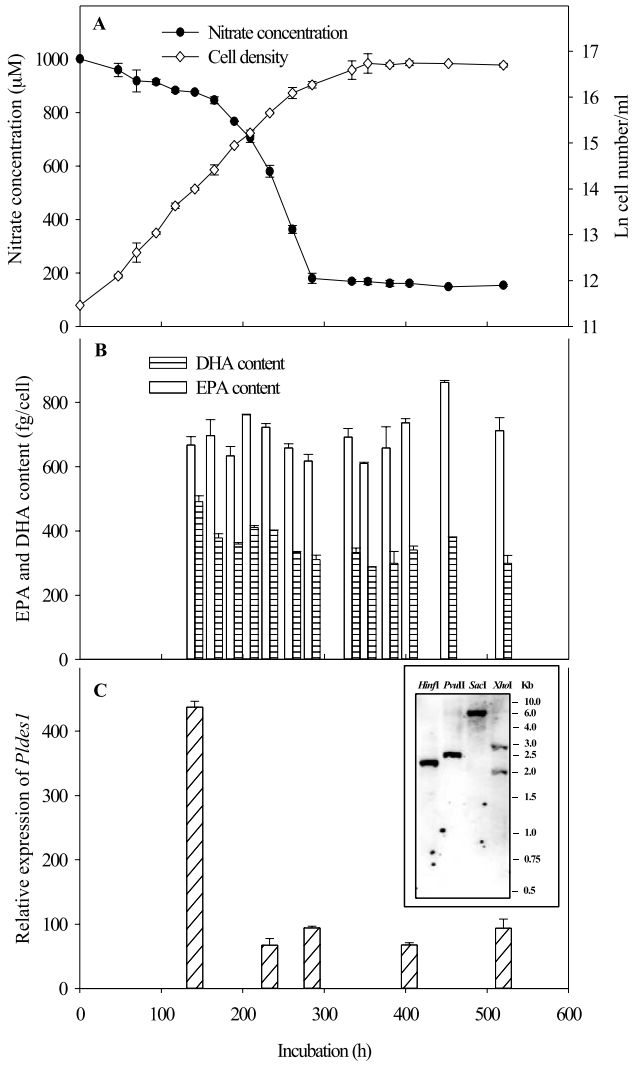

Fig. 1. Growth, VLCPUFAs production and expression of *Pldes1* in *P. lutheri*. Microalga cells were harvested throughout growth to monitor cell number, nitrate consumption (A), quantity of EPA and DHA (B). Relative expression of *Pldes1* was analysed by real time PCR (C). Data was normalised against expression of the 18S rRNA. The inset shows a Southern blot analysis of the *Pldes1* gene. Numbers to the right are sizes of the molecular weight markers. In A–C, error bars represent the range of values between two replicates within an individual experiment.

3.2. Expression of PlDES1 in yeast

To establish the function of PlDES1, the full-length cDNA was expressed in the yeast Invsc1 under the control of an inducible galactose promoter. An alanine codon containing a G as the first letter was added directly downstream from the start codon of *Pldes1* to ensure proper translation in yeast [21]. A number of front-end desaturase substrates, including 18:2n-6, 18:3n-3, 20:3n-6, 20:4n-3, 22:4n-6, and 22:5n-3, were tested. Fig. 3A,B shows that after supplementation of

the medium with 22:5n-3 and 22:4n-6 and 6 days of incubation, yeast cells containing pYDES1 had an extra fatty acid as compared to the empty vector. Extra peaks observed when cells were fed with 22:5n-3 and 22:4n-6 had a retention time respectively identical to the DHA and 22:5n-6 fatty acids contained in the standard menhaden oil. GC-MS analysis of the FAMEs confirmed that the spectrum of the extra peaks was identical to that of the DHA and 22:5n-6 standards (data not shown). These results demonstrate that pYDES1 can transform 22:5n-3 and 22:4n-6 into 22:6n-3 and 22:5n-6 respectively in yeast. No extra peak was observed when other fatty acids were fed, suggesting they were not effective substrates for pYDES1. Analysis of cells harvested after incubation for 3 days at 22°C showed that 10% of the two substrates were transformed into the expected Δ4-desaturated products. After longer incubation at 15°C, this value increased to 30%, both substrates being converted at the same rate.

To better compare the regioselectivity of the *P. lutheri* fatty acid Δ4-desaturase, the yeast culture expressing pYDES1 was simultaneously supplied with equal amounts of both 22:5n-3

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PlDES1  MPPSAASEGGVAELRAAEVASYTRKAVDERPD..LTIVGDAVYDAKAFRD   48
TsFAD4  ...........MTVGYDEEIPFEQVRAHNKPDDAWCAIHGHVYDVTKFAS   39
TsFAD5  ..........MGKGSEGRSAAREMTAEANGDKRKTILIEGVLYDATNFK.   39

PlDES1  EHPGGAHFVSLF...GGRDATEAFMEYHRRAWPKARMSKFFVGSLD....   91
TsFAD4  VHPGG.DIILLA...AGKEATVLYETYHVRGVSDAVLRKYRIGKLPDGQG   85
TsFAD5  .HPGGSIINFLTEGEAGVDATQAYREFHQRSGKADKYLK.SLPKLD....   83
        ******

PlDES1  .ASEKPTQA............DSAYLRLCAE..VNALLPKGSGGFAPPSY  126
TsFAD4  GANEKEKRTLSGLSSASYYTWNSDFYRVMRERVVARLKERGKARRGGYEL  135
TsFAD5  ..ASKVESRFSAKEQARRDAMTRDYAAFREE.....LVAEGYFDPSIPHM  126

PlDES1  WLKAAALVVAAVSIEGYMLLRGKT...LLLSVFLGLVFAWIGLNIQHDAN  173
TsFAD4  WIKAFLLLVGFWSSLYWMCTLDPSFGAILAAMSLGVFAAFVGTCIQHDGN  185
TsFAD5  .IYRVVEIVALFALSFWLMSKASPTSLVLGVVMNGIAQGRCGW.VMHEMG  174

PlDES1  HGALSRHSVIN..YCLGYAQDWIGGNMVLWLQEHVVMHHLHTN.......  214
TsFAD4  HGAFAQSRWVN..KVAGWTLDMIGASGMTWEFQHVLGHHPYTNLIEEENG  233
TsFAD5  HGSFTGVIWLDDRMCEFFYGVGCGMSGHYWKNQHS.KHHAAPNRLEHD..  221

PlDES1  ..............DVDADPDQ..KAHGVLRLKPTDGWMPWHALQ.QLY.  246
TsFAD4  LQKVSGKKMDTKLADQESDPDV.FSTYPMMRLHPWHQKRWYHRFQ.HIYG  281
TsFAD5  ..............VDLNTLPLVAFNERVVRKVKPGSLLALWLRVQAYLFA  258

PlDES1  ILPGEAMYAFKLLLFLDALELLAWR.WEGEKISPLARALFAPAVACKLGFW  295
TsFAD4  PFIFGFMTINKVVTQDVGVVLRKRLFQIDAECRYASPMYVARFWIMKALT  331
TsFAD5  PVSCLLIGLGWTLYLHPRYMLRTK...RHMEFVWIFARYIGWFSLMGALG  305

PlDES1  ARF.VALPLWLQPTVHTALCICATVCTGSFYLAFFFFISHNFDGV.....  339
TsFAD4  VLYMVALPCYMQGPWHGLKLFAIAHFTCGEVLATMFIVNHIIEGVSYASK  381
TsFAD5  YSPGTSVGMYL...........CSFGLGCIYIFLQFAVSHTHLPV.....  339

PlDES1  ....GSVGPK....GSLPRSAT.........................FVQR  357
TsFAD4  DAVKGTMAPPKTMHGVTPMNNTRKEVEAEASKSGAVVKSVPLDDWAVVQC  431
TsFAD5  ..............TNPEDQLH.....................WLEYAA  353

PlDES1  QVETSSNVGGYWLGVLNGGLNFQIEHHLFPRLHHSYYAQIAPVVRTHIEK  407
TsFAD4  QTSVNWSVGSWFWNHFSGGLNHQIEHHLFPGLSHETYYHIQDVFQSTCAE  481
TsFAD5  DHTVNISTKSWLVTWWMSNLNFQIEHHLFPTAPQFRFKEISPRVEALFKR  403

PlDES1  LGFKYRHFPT VGSNLSSMLQ HMGKMGTRPG AEKGGKAE           445
TsFAD4  YGVPYQHEPS LWTAYWKMLE HLRQLGNEET HESWQRAA           519
TsFAD5  HNLPYYDLPY T.SAVSTTFA NLYSVGHSVG ADTKKQD.           439
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Fig. 2. Sequence comparison of the *P. lutheri* Δ4-desaturase. Multiple peptide sequence alignment was performed using the Multialign tool [26]. Protein sequences used in the alignment include: AY332747 from *P. lutheri* (PlDES1), AF489589 (FAD4) and AF489588 (FAD5) from *Thraustochytrium* sp. Conserved amino acids are on a shaded background, the cytochrome *b5* haem-binding domain is underlined by dots, the three histine boxes are underlined, and the two lysine residues are in bold underlined.

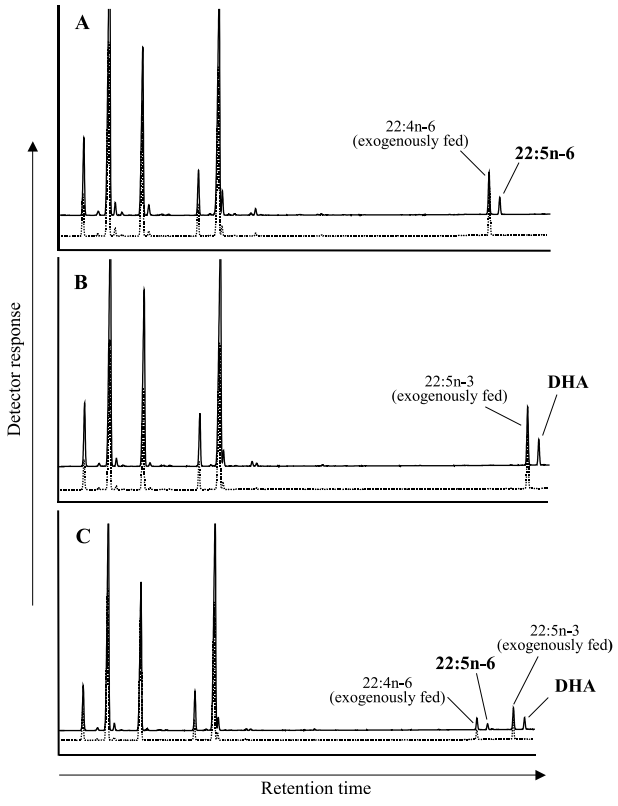

Fig. 3. GC analysis of FAMEs from yeast expressing PlDES1 with exogenous substrates 22:4n-6 (A), 22:5n-3 (B) and 22:4n-6+22:5n-3 (C). Invsc1 yeast strain transformed with either the empty vector (dotted line) or the plasmid pYDES1 (full line) was supplemented with fatty acid 22:4n-6 (A), 22:5n-3 (B) and simultaneously with 22:4n-6 and 22:5n-3 (C). The native yeast FAMEs are unlabelled. Each feeding experiment was repeated twice, and results of a representative experiment are shown.

and 22:4n-6 substrates. Although 22:4n-6 was incorporated less efficiently than 22:5n-3 when fed to yeast, GC analysis of the resulting desaturation products indicated that PlDES1 displayed no selectivity when facing the ω3 or ω6 fatty acids (Fig. 3C). The conversion of the two potential substrates used in this experiment was 35% for both C22 fatty acids, and was slightly higher than those obtained during the single feeding experiment. This result indicated that PlDES1 is a Δ4-desaturase that is able to introduce a double bond at position 4 from the carboxy-terminal end of 22:5n-3 and 22:4n-6, and is equally active on both substrates.

4. Discussion

In a previous study, we have shown that *P. lutheri* represented a good candidate for the discovery of genes involved in DHA production. After improving culture conditions of this microalga to increase DHA content, we report here the cloning and functional characterisation of *Pldes1*, encoding a novel VLCPUFA Δ4-desaturase. To increase the chance of isolating genes involved in the different steps of DHA synthesis, an EST approach rather than a RT-PCR approach using degenerate primers targeted to conserved domains of desaturases was undertaken. PlDES1 contains the typical features of membrane-bound desaturases and a cytochrome *b5* domain fused to its N-terminal extremity, similar to other front-end

desaturases. Heterologous expression in *S. cerevisiae* was necessary to establish the regioselectivity of this desaturase. PlDES1 displayed desaturase activity only in the presence of 22:5n-3 and 22:4n-6, without ω3 or ω6 preference. Similar results have been obtained with the previously isolated Δ4-desaturase, and more generally with Δ5- and Δ6-desaturases [22], implying that both families of fatty acids share a common desaturation system. However, despite the dual action of PlDES1, only ω3 fatty acids such as stearidonic acid (18:4n-3) have been detected in *P. lutheri* cells (Table 1), and no C24 PUFAs involved in the Δ4-independent pathway for DHA biosynthesis [13] have been identified. Moreover, absence in the EST database of sequences with homology to genes encoding enzymes of the polyketide synthase complex catalysing DHA production in the marine microbes *Shewanella* and *Schizochytrium* [23] suggest that the ω3 pathway is the major route of DHA production in this microalga.

In *P. lutheri*, the precursor EPA is more abundant than the final product DHA. The Δ5-elongation and/or the Δ4-desaturation therefore represent potential limiting steps in the biosynthesis of DHA. The high level of *Pldes1* transcript during mid-exponential compared to late exponential and stationary growth phases suggests at least part of this regulation is at the level of expression of this gene. Intense research is underway to develop more sustainable methods for DHA production, considering its beneficial effect on human health. Engineering oilseed crops able to produce DHA represents an attractive way to relieve pressure for unsustainable extraction of these key nutrients from natural marine sources and a more efficient method of production than oil extraction from algae or fungi mass culture. To this aim, the isolation of elongases and desaturases sequentially involved in DHA biosynthesis is being carried out intensively by several research groups. So far, the only characterised Δ5-elongases available in public databases have been isolated from mammals [24], and are not useful for metabolic engineering in plants for ethical reasons. PlDES1 is only the second VLCPUFA Δ4-desaturase reported in the literature, and the activity reported here suggests it could be a useful tool to allow the production of DHA in oilseed crops. The EPA production pathway has already been reconstituted in yeast [25,22], and encouraging levels of EPA have been monitored. Reconstitution of the entire pathway of DHA production in model and crop plants is now a realistic goal.

Note added in proof

During the review process a report of another Δ4-desaturase, from *Euglena gracilis*, was published by Meyer et al. (2003), Biochemistry 42, 9779–9788.

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