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Identification of a fatty acid Δ11-desaturase from the microalga *Thalassiosira pseudonana*[1]

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Abstract **A set of genomic DNA sequences putatively encoding front-end desaturases were identified by in silico analysis of the draft genome of the marine microalga *Thalassiosira pseudonana*. Among these candidate genes, an open reading frame named *TpdesN* was found to be full-length, intronless, and constitutively expressed during cell cultivation. The predicted amino acid sequence of the corresponding protein, TpDESN, exhibited typical features of desaturases involved in the production of polyunsaturated fatty acids (PUFAs) in algae, i.e. a cytochrome *b5*-like domain at the N-terminus and three conserved histidine-rich motifs in the desaturase domain. Expression of TpDESN in *Saccharomyces cerevisiae* revealed that this enzyme was not involved in PUFA synthesis, but specifically desaturated palmitic acid 16:0 to 16:1Δ11. To our knowledge, until this report, Δ11-desaturase activity had only been detected in insect cells.**

Key words: Yeast expression; Δ11-Desaturase; *Thalassiosira pseudonana*

1. Introduction

Plants, in particular seeds, contain a wide variety of fatty acids, many of which are considered unusual and are confined to a few plant species [1,2]. Marine microalgal cells also produce a wide variety of fatty acids, and some species have attracted interest because they contain health beneficial polyunsaturated fatty acids (PUFAs) [3,4]. Production of PUFAs involves a consecutive series of desaturations and elongations of the fatty acyl chain to generate arachidonic acid (20:4Δ5,8,11,14) and docosahexaenoic acid (DHA) (22:6Δ4,7,10,13,16,19). Several desaturases involved in this metabolic process have recently been isolated from marine microalgae, including *Phaeodactylum tricornutum* [5], *Euglena gracilis* [6] and *Pavlova lutheri* [7]. Features of fatty acid desaturases from animals and lower eukaryotes, together with their functional analysis in heterologous hosts, have recently been reviewed [8,9]. These membrane-bound desaturases are specific with respect to both chain length of the substrate and the double bond positions on the fatty acid. They belong to the class known as front-end fatty acid desaturases due to the fact that they introduce double bonds between the carboxy group and preexisting bond(s) of the fatty acid [1]. These desaturases contain a cytochrome *b5* domain at their N-terminus and three histidine motifs that are important for catalytic activity [10].

Besides the common fatty acids 16:0, 16:1Δ9, 18:0 and 18:1Δ9 found in most living organisms, trace amounts of more unusual fatty acids can be found in a wide range of species. For instance, presence of 16:1Δ11 has been reported in several species of *Pavlova*, in the Eustigmatophyte *Nannochloropsis oculata*, and in the diatoms *P. tricornutum* and *Thalassiosira pseudonana* [11–13]. This fatty acid accounted for a very small portion of the total fatty acids in these microalgae, and its specific role in the algal cells is unknown. However, this fatty acid is a very important precursor in the synthesis of sex pheromones in insects. Sex pheromones are species-specific blends of unsaturated fatty acid (UFA) derivatives that differ in terminal functional group and in the number, position and configuration (Z or E) of the double bond(s), which are produced by various acyl-coenzyme A (CoA) desaturases [14,15]. Simple monoene Δ11 UFAs are the most prevalent precursors in the formation of major sex pheromone components in the modern *Lepidoptera* [16,17]. For instance, in the corn earworm *Helicoverpa zea*, which produces a pheromone mixture of Z11-16:Ald and Z9-16:Ald in a 30:1 ratio, the most abundant desaturase-encoding transcript is *HzeaLPAQ* (also called *HzPGDs1*) which encodes a Δ11-desaturase that does not possess a cytochrome *b5* extension, and therefore requires free cytochrome *b5* for activity. Many acyl-CoA Δ11-desaturases with different specificities have been isolated from insects [14,15], but none from other species. Here we present the first characterisation of a cytochrome *b5* desaturase exhibiting Δ11-desaturase activity. This desaturase is closely related to the front-end class of fatty acid desaturases.

2. Materials and methods

2.1. *Identification of putative* T. pseudonana *desaturase-coding sequences and phylogenetic analysis with other functionally characterised desaturases*

The draft genome of the diatom *T. pseudonana* has been sequenced to approximately nine times coverage by the whole genome shotgun method. Sequence data were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) and the

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[1] The sequence reported in this paper has been submitted to GenBank database under the accession number AY493438.

Abbreviations: DHA, docosahexaenoic acid; DMDS, dimethyl disulphide; FAME, fatty acid methyl ester; PUFA, polyunsaturated fatty acid; RT, reverse transcription; UFA, unsaturated fatty acid

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raw sequence data were downloaded and installed on a local server. Batch tblastn searches were carried out using protein sequences of the following 13 known desaturases as query, including PlDES1 (AY332747, *P. lutheri*), TFAD4 (AF489589, *Thraustochytrium* sp. ATCC 21685), TFAD5 (AF489588, *Thraustochytrium* sp. ATCC 21685), PtDEL5 (AY082392, *P. tricornutum*), PtDEL6 (AY082393, *P. tricornutum*), EgDEL8 (AF139720, *E. gracilis*), EgDEL4 (AY278558, *E. gracilis*), ZfDEL (AF309556, *Danio rerio*), BoDEL6 (U79010, *Borago officinalis*), HsDEL5 (AF084558, *Homo sapiens*), HsDEL6 (AF084559, *H. sapiens*), CeDEL6 (AF031477, *Caenorhabditis elegans*) and CeDEL5 (AF078796, *C. elegans*).

All non-redundant sequences with an *E* value less than 0.001 were retrieved and assembled into contigs using the CAP3 sequence assembly programme [18]. The contigs were translated into amino sequences in three frames in the orientation indicated by the tblastn result. Putative desaturase gene models were constructed manually based on sequence homology and in-frame GT-AG intron boundaries were identified.

Deduced amino acid sequences of all 12 putative desaturase sequences of *T. pseudonana* were aligned with the above 13 functionally characterised desaturases from other species, using Clustal X version 1.8 [19]. The alignment was then reconciled and further adjusted. Only nine near full-length *Thalassiosira* sequences were retained for further analyses.

A data set of 250 conserved residue positions was used for construction of the phylogenetic tree. Distance analysis used the programme protdist of the Phylip 3.5c package with a PAM250 substitution matrix and a tree was then built from the matrix using fitch (Fitch–Margoliash method). Bootstrap analyses were carried out with 1000 replicates using the neighbour-joining algorithm.

2.2. Cultivation of T. pseudonana

T. pseudonana (CCAP 1085/12) 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 (EASW), made up in 20 l batches as described previously [4]. 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. Nitrate concentration was determined periodically during the culture time by measuring the change of the medium absorbance at 220 nm [20].

2.3. RNA extraction, cDNA synthesis and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from frozen cells harvested at different stages of growth with an RNeasy plant mini kit (Qiagen). First-strand cDNA was synthesised from 3 μg of DNase-treated RNA using a Prostar First-strand RT-PCR kit (Stratagene). PCR was performed using undiluted and five-fold dilutions of cDNAs as follows: the reactions were heated to 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 50°C or 65°C (for *18S rRNA* and *TpdesN*, respectively) for 30 s and 72°C for 2 min, then a single 72°C for 10 min. As a marker for constitutive expression, the 18S rRNA gene was amplified with the primers TH18S5′ (5′-GGTAACGAATTGTTAG-3′) and TH18S3′ (5′-GTCGGCATAGTTTATG-3′). *TpdesN* cDNA was amplified using primers DESNR2 (5′-GTGAGAGCACTAACCAAGCTT-3′) and DESN2 (5′-CAATCAGTAGGCTTCGTCG-3′). Aliquots of PCR were electrophoresed through a 1% agarose gel. Identity of the diagnostic fragment amplified with *TpdesN*-specific primers was verified by sequencing after cloning in the pGEM-T EasyVector (Promega).

2.4. Functional characterisation of TpDESN in yeast

Genomic DNA from *T. pseudonana* cells was extracted using the DNA isolation kit Puregene (Gentra Systems) and 100 ng was used to amplify the entire *TpdesN* coding region with primers DesNNB 5′-GCGGGATCCACC**ATG***GCT*GACTTTCTCTCCGGC-3′ (open reading frame (ORF) start codon is indicated by bold type; underlined sequence is a *Bam*HI site; italic sequence is an added alanine codon, not present in the original sequence of *TpdesN*) and DesNCE 5′-GCGAATTC**TCA**ATCAGTAGGCTTCGT-3′ (ORF stop codon is indicated in bold type; underlined sequence is an *Eco*RI site). The Expand High Fidelity PCR system (Roche) was employed to minimise potential PCR errors. The amplified product was gel purified, restricted with *Eco*RI and *Bam*HI and cloned into the corresponding sites behind the galactose-inducible GAL1 promoter of pYES2 (Invitrogen) to yield the plasmid pYDESN. The fidelity of the cloned PCR product was checked by sequencing. The vector pYDESN was then transformed into *Saccharomyces cerevisiae* strain Invsc1 (Invitrogen) by a lithium acetate method, and transformants were selected on minimal medium plates lacking uracil.

For the feeding experiment with PUFAs, 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 feeding experiment with saturated fatty acids, a single Invsc1 colony transformed with pYES2 (empty plasmid, control) or pYDESN was inoculated in 10 ml of minimal media minus uracil containing 2% raffinose and grown overnight at 30°C with shaking (300 rpm). After 16–24 h, cells were collected by spinning at 4500 rpm for 10 min. After discarding the supernatant, the cell pellet was resuspended in the same medium mentioned above supplemented with 2% galactose and 1% tergitol, to obtain a cell density of 5×10^7 cells ml^{-1}. 15 ml of this cell suspension were added to a 100 ml flask with or without addition of saturated fatty acids (as mentioned in the text) at 500 μM final concentration. Desaturase induction was then carried out at 20°C with shaking (300 rpm) for 3 days.

2.5. Fatty acid analysis

Microalgae or yeast cells were harvested by centrifugation. Total fatty acids were extracted and transmethylated as previously described [4]. Most fatty acid methyl esters (FAMEs) were identified by comparison of retention times to a 37 FAME mix (Supelco). PUFA FAMEs were also identified by comparison to a sample of standard Menhaden oil (Supelco) transmethylated as per the samples.

Dimethyl disulphide (DMDS) adducts were used to determine the double bond position in identified and unidentified monounsaturated FAMEs. These were made by adding together 50 μl DMDS (Aldrich), 100–1000 ng FAMEs dissolved in 50 μl hexane, and 5 μl 50 mg ml^{-1} iodine in diethyl ether. This solution was heated at 40°C for 15 h and partitioned with 200 μl hexane and 100 μl 5% (w/v) sodium thiosulphate. The hexane phase was removed, dried under vacuum, reconstituted in 50 μl fresh hexane and used for gas chromatography-mass spectrometry (GC-MS) analysis. A Trace GC 2000 (ThermoQuest) fitted with a 30 m×0.25 mm×0.5 μm film thickness ZB-1 column (Phenomenex) was used to chromatograph 2 μl DMDS adducts injected at 250°C and a 50:1 split ratio with He as carrier gas at 0.6 ml min^{-1} in constant flow mode. The oven programme was 120°C for 1 min then to 340°C at 5°C min^{-1}. Mass spectra were obtained using a GCQ (ThermoQuest) mass spectrometer operating in full scan mode over 50–500 *m/z*. Picolinyl esters were also made from FAMEs to confirm their identities. These were obtained by adding 15 μl freshly prepared 2:1 (v/v) 3-(hydroxymethyl)-pyridine (Aldrich):potassium *tert*-butoxide 1 M solution in tetrahydrofuran (Aldrich) to 50 μl FAMEs dissolved in dichloromethane. This solution was heated at 40°C for 30 min and partitioned with 200 μl hexane and 100 μl 2.5% (w/v) sodium hydrogen carbonate. The hexane phase was removed, dried under vacuum and reconstituted in 50 μl fresh hexane. Picolinyl esters were injected and separated by GC-MS using the same conditions as for DMDS adducts.

3. Results

3.1. Identification and phylogenetic analysis of putative T. pseudonana desaturase sequences with other functionally characterised desaturases

Tblastn searches with 13 functionally characterised desaturases revealed 427 non-redundant raw sequences with *E* values less than 0.001. 12 unique contigs were assembled after retrieving these sequences and gene models were constructed manually based on sequence homology. These 12 gene contigs were arbitrarily designated *TpdesA* to *TpdesL*. All 12 showed significant sequence similarity to query sequences with nine containing near full-length ORFs compared to other known

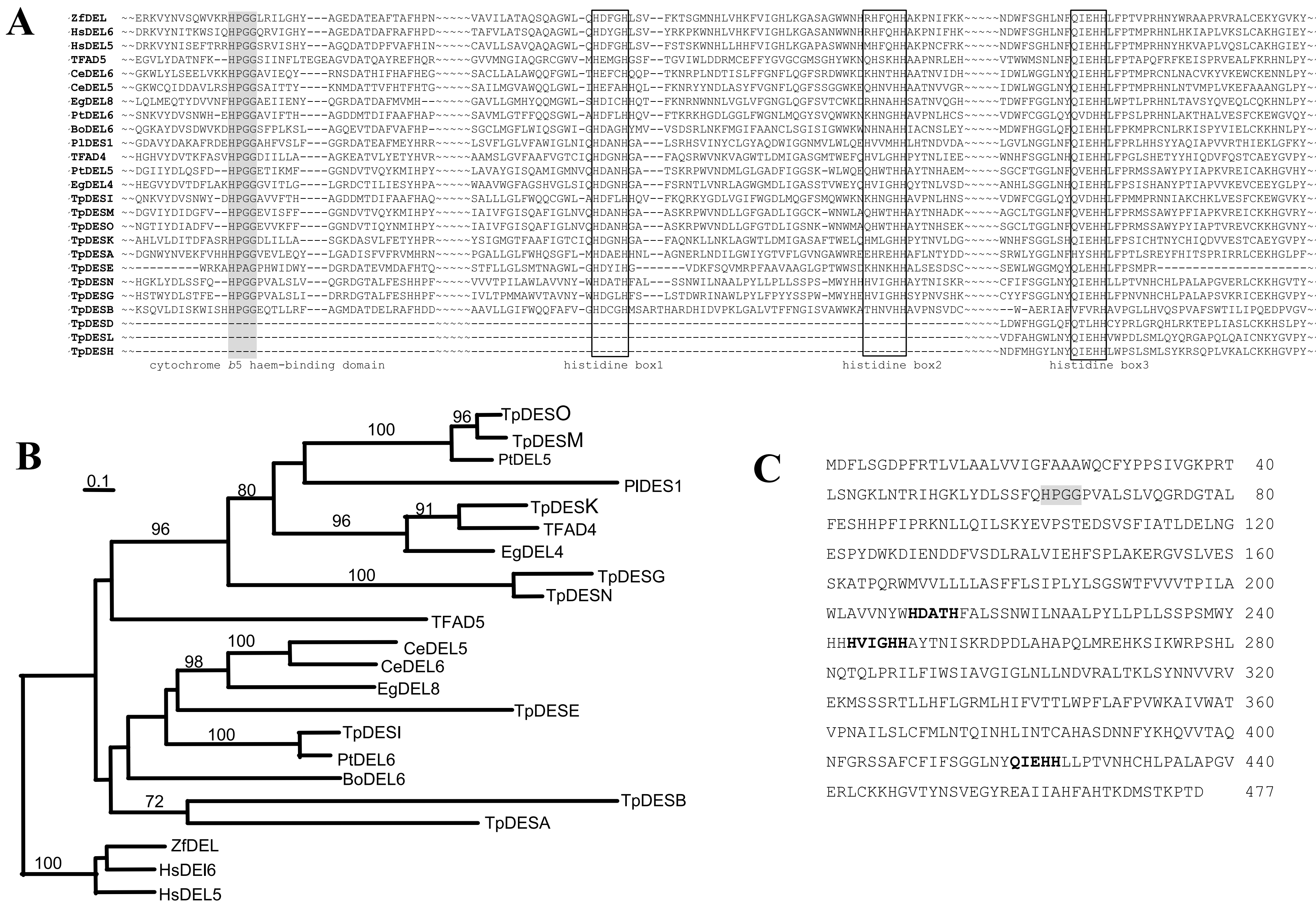

Fig. 1. Predicted protein sequences with homology to front-end desaturases derived from the *T. pseudonana* draft genome. Sequence alignments of 12 putative *T. pseudonana* desaturases with other functionally characterised front-end desaturase enzymes identified three main blocks of homology that represent the functional domains of front-end acyl desaturases (A). The darker shaded box highlights the cytochrome *b5 haem*-binding domain and three histidine-rich motifs are boxed. See Section 2 for GenBank accession numbers and source species of the functionally characterised enzymes. A phylogenetic tree of nine *T. pseudonana* desaturases with other enzymes was constructed (B). By removing the regions containing gaps (ambiguous alignment region), a data set was created from an alignment originally made with Clustal X. The tree was constructed from the data set using Phylip 3.5c software package and bootstrap analyses were carried out with 1000 replicates. Only well-supported nodes (over 70%) are indicated with bootstrap values. All branches are drawn to scale as indicated by the scale bar (=0.1 substitutions per site). TpDESN sequence is 477 amino acids long (C). The cytochrome *b5 haem*-binding domain is on a shaded background and the three histidine boxes **are framed**.

desaturases (Fig. 1A). Interestingly, the predicted amino acid sequences of all nine *T. pseudonana* desaturases have a characteristic fused cytochrome *b5 haem*-binding domain (HP[G/A]G) at their N-terminus and three histidine boxes (H[X]3-4H, H[X]2-3HH and Q[X]2-3HH) with the replacement of the first histidine by glutamine in the third histidine box in all but two of the predicted proteins (TpDESA and TpDESB). These are common characteristics of a large subgroup of front-end acyl group desaturases [21]. These histidine box motifs are critical for desaturase activity, most likely because they serve to coordinate the diiron-oxo component of the active site. Three remaining sequences (TpDESD, TpDESL and TpDESH) appear to be partial, covering only the C-terminal end of desaturases, but nevertheless they do contain a typical third histidine box of the above-mentioned subgroup of desaturases (Fig. 1A).

In order to gain insight into the relationships of these *T. pseudonana* sequences to other functionally characterised desaturases and especially algal desaturases, we constructed an unrooted phylogenetic tree using a Fitch–Margoliash method with statistical confidence measured by bootstrap analysis (Fig. 1B). Relationships of four putative *T. pseudonana* desaturases are in well-supported (>70% bootstrap value) subgroups with at least one functionally characterised desaturase from other species. Both TpDESM and TpDESO grouped with PtDEL5, a Δ5-desaturase from another diatom, *P. tricornutum* [5], suggesting these two enzymes may also have a Δ5-desaturase activity. Similarly TpDESK is grouped with two Δ4-desaturases TFAD4 and EgDEL4 from *Thraustochytrium* sp. ATCC21685 [22] and *E. gracilis*, respectively. TpDESI grouped with PtDEL6, a Δ6-desaturase from *P. tricornutum*. This indicates that TpDESK and TpDESI may have Δ4- and Δ6-desaturase activities, respectively. However, as enzymes with different regioselectivities are also found in a well-supported subgroup (EgDEL8, CeDEL5 and CeDEL6; Δ8-, Δ5- and Δ6-desaturase, respectively) and regioselectivity may even derive independently after a more recent duplication (CeDEL5 and CeDEL6) [23], predictions based on homology can be misleading and it is essential to functionally characterise each enzyme.

The remaining five *T. pseudonana* sequences fall into three separate subgroups (TpDESE; TpDESA and TpDESB;

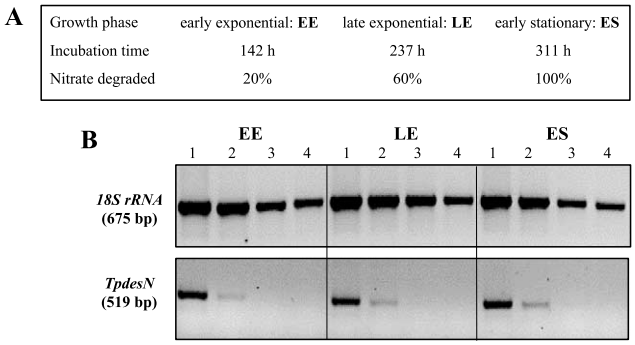

Fig. 2. RT-PCR expression analysis of *TpdesN*. Cells were harvested at different stages of growth for total RNA extraction and cDNA synthesis (A). PCR was performed on cDNA derived from reverse transcribed RNA using *TpdesN*- and *18s rRNA*-specific primer pairs (B). PCR was carried out on undiluted (lane 1) and five-fold serial dilutions (lanes 2–4) of each cDNA. The 18S rRNA gene was used as a control of cDNA synthesis. EE, early exponential phase; LE, late exponential phase; ES, early stationary phase.

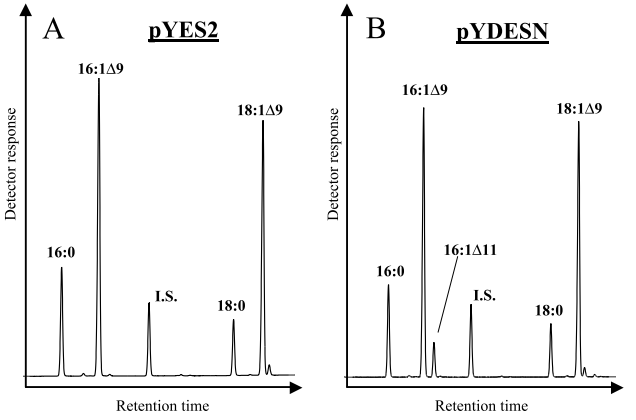

Fig. 3. GC analysis of FAMEs from yeast transformed with the empty plasmid pYES2 or the plasmid containing TpDESN. Invsc1 yeast strains transformed with either pYES2 (A) or pYDESN (B) were induced for 3 days at 20°C without supplementation before sampling for fatty acid analysis. I.S., internal standard (17:0). The experiment was repeated three times and results of a representative experiment are shown.

TpDESG and TpDESN) which do not group with any other known functional desaturases with high confidence. It is therefore possible that these proteins exhibit novel regioselectivity. The current study focussed on the characterisation of one of these proteins, TpDESN.

3.2. Temporal expression of TpDESN gene

RT-PCR analysis of *TpdesN* transcript was conducted at different stages of algal growth in order to establish if and when this gene is expressed. After RNA extraction and cDNA synthesis, *TpdesN*-specific PCR products were amplified. PCR amplification of the 18S rDNA gene was performed as a control for the quantity of cDNA used during PCRs. Fig. 2 shows that the diagnostic 519 bp cDNA amplification product expected for *TpdesN* was present at similar level at the different stages of cultivation of the microalgal cells. Thus, *TpdesN* is transcriptionally active at a constitutive level during *Thalassiosira* growth, suggesting that it may encode a desaturase with a housekeeping function.

3.3. Functional characterisation of TpDESN in yeast

The putative desaturase sequence annotated *TpdesN* was contained on a genomic DNA contig of 2580 bp on which no introns were detected. To establish the function of the protein encoded by this gene, the full-length sequence was amplified from genomic DNA. An alanine codon containing a G as the first letter was added immediately downstream of the start codon of *TpdesN* to ensure optimal translation in yeast [24]. The *TpdesN* ORF is 1434 bp long, and encodes a 477 amino acid protein TpDESN (Fig. 1C), having a molecular weight of 53.8 kDa. Analysis of the secondary structure of TpDESN using SOSUI software (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) [25] predicted four transmembrane regions (not shown). Alignment of TpDESN with functionally characterised desaturase sequences mentioned above indicated an overall identity of 25%, with the cytochrome *b5*-like domain and the three conserved histidine-rich motif areas showing greatest homology.

The primary sequence of TpDESN exhibited typical features of front-end desaturases involved in PUFA synthesis.

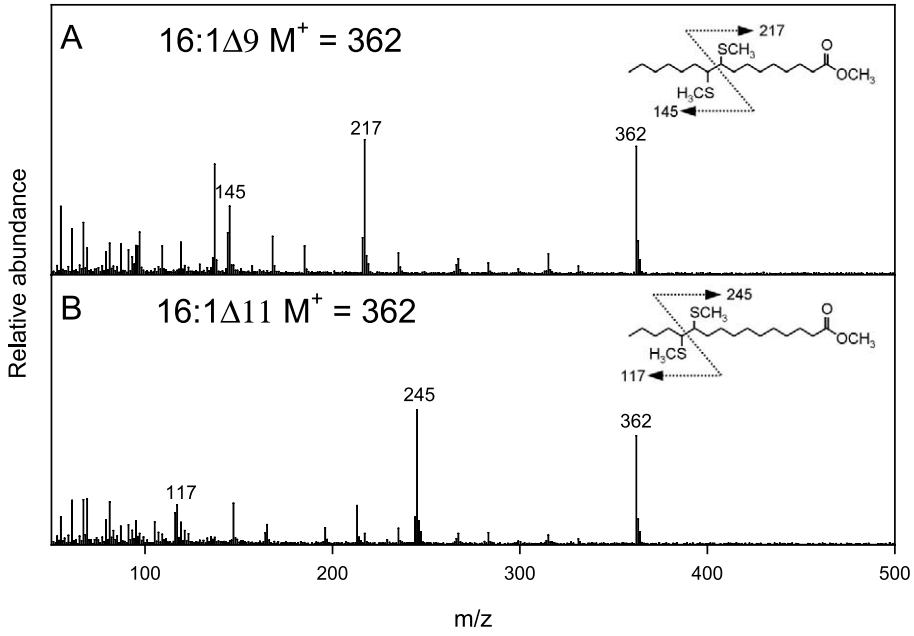

Fig. 4. Mass spectra of DMDS FAME adducts from pYDESN transformed yeast. Mass spectrum of the DMDS adduct of 16:1Δ9 FAME, present in all yeast samples (A). Mass spectrum of the DMDS adduct of 16:1Δ11 FAME, which was only found in yeast transformed with pYDESN (B). Picolinyl esters with spectra characteristic of 16:1Δ11 were also identified in these samples (data not shown).

In order to characterise the specificity of this protein, PUFAs (18:2Δ9,12; 20:2Δ11,14; 20:3Δ8,11,14; 22:4Δ5,8,11,14; 18:3Δ9,12,15; 20:3Δ11,14,17; 20:4Δ8,11,14,17; 22:5Δ7,10,13,16,19) were first fed to the host yeast transformed with pYDESN and the vector alone (pYES2) as a control. Unexpectedly, after 6 days of incubation, TpDESN did not desaturate any of the supplemented PUFA substrates. Furthermore, there did not appear to be any production of 18:2Δ9,12 from endogenous 18:1Δ9. However, a significant increase was observed for a peak eluting in the range of 16 carbon monounsaturated FAMEs in the yeast transformed with pYDESN (Fig. 3). The position of the double bond in this product was determined by GC-MS analysis of FAMEs derived to DMDS adducts [26] and picolinyl esters. The DMDS adduct of 16:1Δ9 FAME yielded two major fragments at *m/z* 145 and 217 (Fig. 4A). Fragmentation of the increased FAME peak found in unfed or fed yeast transformed with pYDESN produced two diagnostic fragments at *m/z* 117 and 245 (Fig. 4B). This fragmentation pattern was indicative of an Δ11 monounsaturated 16 carbon FAME, 16:1Δ11, suggesting that TpDESN encoded a new Δ11-desaturase. Small amounts of this fatty acid have also been measured in *Thalassiosira* cells (Table 1). To further substantiate these results, yeasts transformed with pYDESN and the control empty vector, pYES2, were cultivated in medium supplemented with saturated fatty acid (14:0; 16:0; 18:0) representing potential substrates for the synthesis of the monounsaturated product. Yeast fatty acid profiles were analysed after 3 days of incubation at 20°C. Results in Table 2 showed that a small amount of 16:1Δ11 (0.23% of total fatty acids) was detected in yeast transformed with pYES2, suggesting endogenous synthesis of this fatty acid from 16:0. This fatty acid accumulated at a higher level in both types of transformed yeasts after feeding with 14:0, with values up to 5.84% in pYDESN transformants. A possible explanation for this increase in the pYES2 transformants is that the endogenous yeast Δ9-desaturase was able to use additional 14:0 to produce 14:1Δ9 that was subsequently elongated to 16:1Δ11. Moreover, it has been reported that wild-type yeast cells cultivated in media supplemented with 14:1Δ9 synthesised 16:1Δ11 by Elo1p-dependent carboxy-terminal elongation [27]. After 18:0 supplementation, the percentage of 16:1Δ11, of about 6% total fatty acids, was similar to that observed after feeding with 16:0. Presence of extra 18:0 could lead to an inhibition of the 16:0 chain elongation system, which might allow more 16:0 to be available for Δ11-desaturation. On the other hand, 18:1Δ11 represents 1.2% of

Table 1
Composition of the major fatty acids in *T. pseudonana*

Fatty acid	Mol% of total fatty acids
14:0	11.50
16:0	17.95
16:1Δ9	19.81
16:1Δ11	0.19
16:2Δ9,12	2.47
16:3Δ6,9,12	6.68
18:0	0.47
18:1Δ7	0.26
18:1Δ9	1.50
18:1Δ11	1.52
18:2Δ9,12	2.37
18:3Δ6,9,12	0.98
18:3Δ9,12,15	0.32
18:4Δ6,9,12,15	5.72
20:0	0.44
20:3Δ8,11,14	0.26
20:4Δ5,8,11,14	2.46
20:5Δ5,8,11,14,17	17.51
22:6Δ4,7,10,13,16,19	6.64
24:0	0.49

Microalgal cells were harvested during the exponential phase of growth as described in Section 2. Fatty acid 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 triplicate measurements within a representative culture.

Table 2
Composition of the major fatty acids of pYES2 and pYDESN yeast transformants with and without addition of exogenous saturated fatty acids

Fatty acid	Mol% of total fatty acids							
	−substrate		+14:0		+16:0		+18:0	
	pYES2	pYDESN	pYES2	pYDESN	pYES2	pYDESN	pYES2	pYDESN
14:0	0.78	0.52	1.12	0.96	0.76	0.58	0.76	0.70
14:1Δ9	0.22	0.13	1.20	1.29	0.23	0.14	0.23	0.20
16:0	18.40	15.04	18.37	14.62	23.85	22.09	17.22	15.09
16:1Δ9	39.73	35.55	43.39	36.24	42.24	37.03	36.24	31.67
16:1Δ11	0.23	3.27	2.36	5.84	0.22	5.57	0.25	5.84
18:0	7.37	7.34	6.61	7.23	6.36	6.60	16.72	17.47
18:1Δ9	30.19	34.32	24.44	30.24	23.89	25.19	26.07	26.58
18:1Δ11	1.20	1.21	1.35	1.30	1.19	1.12	1.08	0.96
26:0	1.89	2.63	1.16	2.29	1.26	1.70	1.43	1.50

The values given are expressed as mol% of total FAMEs quantified by GC. All values are the means of duplicates from a typical experiment.

the total fatty acids in transgenic yeast. No variation in its proportion was monitored under the different conditions of incubation, even after supplementation with 18:0 in pYDESN transformants. This suggests that this fatty acid originates from elongation of 16:1Δ9 rather than Δ11-desaturation of 18:0.

4. Discussion

Fatty acid profiling of marine microalgae had shown that *T. pseudonana* represents a good candidate to discover genes involved in the production and storage of PUFAs [4]. Analysis of the recently completed draft genome of this microalga revealed the presence of many candidate genes for elongase and desaturase activities most probably involved in catalysing different steps of the PUFA biosynthetic process. We have identified 12 possible desaturase genes, for nine of which there is sufficient sequence information to demonstrate that they exhibit typical features of front-end desaturases, i.e. a cytochrome *b5* domain in the N-terminus and three histidine clusters located at highly conserved regions. Phylogenetic analysis revealed that several of the genes are closely related to a number of previously characterised front-end desaturases involved in PUFA synthesis. However, the current work highlights the fact that desaturase function, in terms of regioselectivity, cannot solely be based on prediction from primary amino acid sequence homology.

The fatty acid profile of *T. pseudonana* cells is quite diverse (Table 1), with the health beneficial eicosapentaenoic acid (EPA) (20:5Δ5,8,11,14,17) and DHA (22:6Δ4,7,10,13,16,19) accounting for a large proportion. However, the number of desaturase gene sequences found in the genome was higher than we expected based on the number of different desaturation reactions required to produce the diversity of fatty acids in this microalga. This suggested that non-obvious desaturation reactions might also occur in the *Thalassiosira* cells. As a first step to establishing function of the many putative desaturase sequences, we focused on the *TpdesN* contig due to the fact that the sequence was full-length and intronless. A temporal expression study showed that *TpdesN* was constitutively transcribed during algal cultivation. Expression of the *TpdesN* ORF in yeast supplemented with PUFAs as potential substrates for desaturation revealed no new products. There was also no evidence of activity with the endogenous 18:1Δ9 which excludes the possibility that TpDESN acts as a Δ12-desaturase. However, an increase in the peak area of a FAME eluting in the range of the 16 carbon FAMEs was identified and GC-MS-based analysis revealed this to be 16:1Δ11 fatty acid. Small amounts of this fatty acid are also present in wild-type yeast. However, quantitative comparison of fatty acid levels in the empty vector pYES2 and pYDESN transformants showed that proportions of 16:1Δ11 increased in the presence of *TpdesN* in both unfed cells and cells that had been fed different saturated fatty acids. No other changes in either peak area or new peaks were detected in pYDESN transformants, indicating that TpDESN is specifically involved in conversion of 16:0 to 16:1Δ11.

The presence of small amounts of 16:1Δ11 has previously been reported in many microalgae, including *T. pseudonana*. However, a function for this fatty acid in algal cells has not been established. The low quantity observed in many marine microalgae suggests that it may act as an intermediate in an as yet unidentified biosynthetic pathway. In insect cells, 16:1Δ11 represents an important precursor for pheromone synthesis, where it is produced by an acyl-CoA Δ11-desaturase. Interestingly, the insect Δ11-desaturases do not possess a cytochrome *b5* domain in their N-terminal region. This represents a major primary structure difference compared with TpDESN. The cytochrome *b5* domain is not a determinant of the substrate specificity [28]. Alignment of the desaturase domain of TpDESN with the full sequence of insect Δ11-desaturases showed an identity of 20% (data not shown). In insect cells, Δ11-desaturases are more or less specific depending on the origin of the sequence and well-documented reviews exist on this subject [14,15].

In conclusion, therefore, although the TpDESN primary sequence is very similar to front-end desaturases, it should not be considered a member of this family of desaturases because it acts only on 16:0. Identification of such a novel enzyme expands the functional repertoire of the membrane-bound desaturases and it should provide useful comparative information for understanding phylogenetic relationships between these enzymes. One question that remains to be answered regards whether cytochrome *b5* was independently fused to desaturases that had already acquired their different specificities, or whether an ancestral fusion protein for proximal lipid modification duplicated and subsequently evolved into different desaturases. Studies of the primary structure of the different PUFA desaturases support the hypothesis that enzyme conversion (i.e. change of specificity) can be achieved

through a relatively few structural changes [29]. The high degree of homology between the many potential front-end desaturases identified in the genome of *T. pseudonana* supports this notion. Given the fatty acid profile of *T. pseudonana* cells and the complexity of the desaturase gene family it is likely that different genes will encode Δ4, Δ5 and Δ6 desaturases. It will now be very interesting to functionally characterise these remaining putative desaturase genes and study the relationship between regioselectivity, primary amino acid sequence and phylogenetic relationship. A crystal structure for these enzymes is still not available due to technical difficulties in obtaining sufficient quantities of purified membrane-bound protein. Molecular genetic approaches involving site-directed mutagenesis have provided new insight into structure–function relationships, including for example that residues in close proximity to the histidine motifs have been found to be involved in shifting the ratio of desaturation/hydroxylation activities [30]. Detailed comparative analyses and computer modeling of these diverse desaturases from *T. pseudonana* may further guide site-directed mutagenesis studies aimed at defining key residues controlling substrate specificity and regioselectivity of the introduced double bond.

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