

Cloning and Characterization of the $\Delta 6$ Polyunsaturated Fatty Acid Elongase from the Green Microalga *Parietochloris incisa*

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Abstract The very-long-chain polyunsaturated fatty acid (VLC-PUFA), arachidonic acid (ARA, 20:4 ω -6) is a component of neuron tissues such as brain and retina cells and a primary substrate for the biosynthesis of biologically active eicosanoids. The green freshwater microalga *Parietochloris incisa* (Trebouxiophyceae) has been shown to accumulate an extraordinary high content of ARA-rich triacylglycerols. It was thus interesting to characterize the genes involved in lipid biosynthesis in this alga. We report here the identification of a cDNA encoding for a *P. incisa* PUFA elongase (PiELO1) and demonstrate that the expression of *PiELO1* in yeast *Saccharomyces cerevisiae* confers its elongase activity on C18 $\Delta 6$ PUFA. Phylogenetic analysis indicated that PiELO1 is highly similar to functionally characterized $\Delta 6$ PUFA elongase genes from other green algae and lower plants. Quantitative real-time PCR expression studies showed that *PiELO1* is upregulated under nitrogen starvation, the condition triggering and enhancing storage oil and ARA accumulation in *P. incisa*.

Keywords Arachidonic acid · Fatty acid elongation · Nitrogen starvation · *Parietochloris incisa* · Subtractive hybridization · VLC-PUFA

Abbreviations

ARA	Arachidonic acid
DGLA	Dihomo- γ -linolenic acid
DHA	Docosahexaenoic acid
GLA	γ -Linolenic acid
EPA	Eicosapentaenoic acid
STA	Stearidonic acid
VLC-PUFA	Very-long-chain polyunsaturated fatty acid

Introduction

The very-long-chain polyunsaturated fatty acid (VLC-PUFA), arachidonic acid (ARA, 20:4 ω -6), is a component of neuron tissues such as brain and retina cells and an important component of the human diet. ARA is a primary substrate for the biosynthesis of eicosanoids, including the 2-group prostaglandins, 4-group leukotrienes, thromboxanes, and lipoxins that serve as biological effectors involved in inflammatory and immune responses and cell signaling [1, 2]. Being an important and dominant VLC-PUFA in human breast milk, ARA needs to be externally supplied for normal development of preterm babies if they are not breast-fed [1, 3]. Because of its beneficial properties there is a growing interest in the production of ARA for baby formulae. At present, the major commercial source of ARA is the filamentous fungus *Mortierella alpina* [4].

Microalgae are one of the richest sources of VLC-PUFAs [5–8]. Furthermore, algae can be used as sources of genes for implementation of VLC-PUFA biosynthesis in genetically engineered oil crops [9, 10]. Genetic information on enzymes involved in the biosynthesis of VLC-PUFA in some algae led to in-vivo applications of VLC-PUFA production in seed oil [11]. The gene pool

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of the green freshwater microalga *Parietochloris incisa* (Trebouxiophyceae) is of special interest because it is the only microalga known to be able to accumulate extraordinary high amounts of ARA-rich triacylglycerols (TAG) [7, 8]. When *P. incisa* is cultivated under nitrogen starvation, the condition triggering storage oil accumulation, ARA constitutes about 60% of total fatty acids (TFA) and over 95% of cellular ARA is deposited in TAG in cytoplasmic lipid bodies [12].

The biosynthesis of VLC-PUFAs in algae follows various pathways, initiating from oleic acid exported from the chloroplast and employing polar extraplasmic lipids. In the $\omega 6$ and $\omega 3$ pathways, linoleic acid (LA; 18:2 ω -6) and α -linolenic acid (ALA; 18:3 ω -3) are successively converted by $\Delta 6$ desaturase, $\Delta 6$ elongase, and $\Delta 5$ desaturase to ARA and eicosapentaenoic acid (EPA, 20:5 ω -3), respectively [6]. For example, in *P. incisa* and in the red microalga *Porphyridium cruentum*, ARA biosynthesis proceeds via the $\omega 6$ pathway [13, 14]. Unusual elongations and desaturations leading to the biosynthesis of VLC-PUFA have been reported in the marine haptophyte *Isochrysis galbana* [15] and the fresh-water euglenophyte *Euglena gracilis* [16, 17]. In the alternative route, elongation of 18:2 ω -6 and 18:3 ω -3 by a C18- $\Delta 9$ -specific fatty acid elongase to the respective C20 intermediates precedes sequential $\Delta 8$ and $\Delta 5$ desaturations to ARA and EPA, respectively. It is assumed that in *E. gracilis* EPA produced by the $\omega 3$ - $\Delta 8$ pathway is further $\Delta 4$ desaturated and finally elongated to docosahexaenoic acid (DHA, 22:6 ω -3) [17].

Fatty acid elongation is a multi-step process involving four sequential enzymatic reactions: rate limiting condensation (of malonyl-CoA and acyl-CoA), reduction, dehydration and enoyl reduction [18]. Only the expression of the condensing enzyme component is required to reconstitute elongase activity in the heterologous host; there is no requirement for the co-expression of any other component of the elongase complex. Multiple microsomal elongation systems with different specificities to the acyl chain length exist in various organisms. Recent studies have identified and characterized PUFA-specific elongases, responsible for the elongation of PUFA in the nematode *Caenorhabditis elegans*, mammals, fish, algae, lower plants, and fungi [19–25]. The elongation of 18:3 ω -6 to 20:3 ω -6, the immediate precursor of ARA, was shown to be the rate-limiting step in ARA biosynthesis in *M. alpina* [26, 27]. Functional expression of the PUFA elongase condensation component in yeast revealed enzymes of various specificities for C18 and C20 acyl substrates. Thus, two types of PUFA elongases engaged in DHA biosynthesis have been cloned from the green microalga *Ostreococcus tauri* and the diatom *Thalassiosira pseudonana*: OtELO1 and TpELO1 are $\Delta 6$ C18-PUFA-specific and involved in the elongation of 18:3 ω -6 and 18:4 ω -3,

whereas OtELO2 and TpELO2 are $\Delta 5$ C20-PUFA elongases involved in the elongation of 20:5 ω -3 [28]. Bifunctional PUFA elongases able to elongate both $\Delta 6$ and $\Delta 5$ PUFA, and elongases of wide substrate specificity utilizing both C20 and C22 PUFA substrates, have been isolated from aquatic animals [28, 29].

In this paper we report the identification of a cDNA encoding for a *P. incisa* elongase (PiELO1) and that the expression of PiELO1 in yeast cells conferred its $\Delta 6$ PUFA elongase activity. Quantitative real-time PCR expression studies showed that *PiELO1* is up-regulated during nitrogen starvation and that the level of expression is correlated with the production of ARA precursors.

Experimental Procedures

Growth Conditions

Axenic cultures of *P. incisa* were cultivated on BG-11 nutrient medium [30] in 250-ml Erlenmeyer glass flasks in an incubator shaker under an air/CO₂ atmosphere (99:1, v/v) and controlled temperature (25°C) and illumination (115 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) at a speed of 170 rpm [13]. For nitrogen starvation experiments, cells of daily-diluted cultures were collected by centrifugation, washed thrice in sterile double-distilled water, and resuspended in nitrogen-free BG-11 medium. Cultures were further grown under the same conditions for 14 days. To prepare nitrogen-free medium, NaNO₃ was omitted from the BG-11 medium and ferric ammonium citrate was substituted with ferric citrate.

RNA Isolation

Cells for RNA isolation were harvested from 40 ml log-phase culture (Time 0) grown in complete BG11 and cultured on nitrogen-free medium for 3, 7, and 14 days. The cultures were filtered through a glass-fiber paper filter (GF-52, Schleicher & Schuell, Germany), and cells were collected by scraping and immediately flash-frozen in liquid nitrogen and stored at -80°C for further use. Total RNA was isolated either using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) or by the procedure described by Bekesiova et al. [31], with minor modifications. Three independent RNA isolations were conducted for each growth period. For real-time PCR studies, the total RNA samples were further treated with RNAase-free DNAase (Epicentre, Madison, WI, USA).

Construction of Subtracted cDNA Library

The subtracted complementary DNA (cDNA) library was prepared from cDNAs enriched for differentially expressed

sequences obtained from *P. incisa* cultures in the log phase (driver) and after three days of nitrogen starvation (tester) [32], using a PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA, USA). First, a double-stranded cDNA was synthesized from total RNA isolated from cells during log phase and N-starvation and digested with *RsaI*. Subtraction was then done in both directions. To enrich for “log phase” cDNAs, the cDNA sequences of N-starved cells were subtracted from those of log phase cells; conversely, to enrich for cDNAs of N-starved cells, log phase cDNA sequences were subtracted from those of N-starved cells. Two portions of the tester cDNA were ligated to adaptor primers. After two cycles of hybridization with excess of driver over tester, the ends of the enriched tester cDNA population were filled in by DNA polymerase and selectively amplified by PCR. Following a subsequent PCR with nested primers, the two differentially expressed cDNA populations were cloned into a pGEM-T vector (Promega, Madison, WI, USA) to produce clones of the subtracted libraries. Plasmids were sequenced by an ABI automated sequencer. Among the 56 differentially expressed sequence tags (ESTs) clones, one was found to be highly similar to PUFA elongases.

Generation of 5' and 3' End Fragments of the Putative *P. incisa* PUFA Elongase

To generate the full-length cDNA of the putative PUFA elongase, 3' and 5' rapid amplification of the cDNA ends (RACE) was performed using a BD smart RACE cDNA Amplification Kit (BD Biosciences Clontech, Foster City, CA, USA) according to the manufacturer's manual. To amplify the 5' end, the reverse gene-specific primers (GSP) 5'-CCCGGCTGCTGCCATGCTTCTGTG (EL5R1) and the nested 5'-TGGGGTAGGGAGAGTAGGCCCAAGT (EL5RN) were designed using Primer3 online software (<http://frodo.wi.mit.edu>). Based on the nucleotide sequence of the 5'-end fragment obtained, two forward GSPs, 5'-GCCTACATGTCCTCTGCCGCTGCTA (EL3R1) and the nested 5'-GCGGGACATGGGAGGGCTCATCTATA CC (EL3R2) were constructed to amplify the 3' end of the target gene. RACE PCR reactions were conducted using 5' and 3'-RACE-Ready cDNAs made from 1 µg total RNA of N-starved cells with 50× BD Advantage 2 polymerase mix (Clontech Laboratories, Mountain View, CA, USA). The PCR products of the expected size were excised, purified from the gel (NucleoSpin Extract II purification kit; Macherey-Nagel, Duren, Germany) and ligated into a pGEM T-Easy vector (Promega). The full-length cDNA corresponding to the *P. incisa* putative PUFA elongase (designated *PiELO1*) was assembled from the 5' and 3' RACE fragments and its ORF was further subcloned into a pYES2 vector (Invitrogen, Carlsbad, CA, USA).

Expression and Functional Characterization of *PiELO1* cDNA in the Yeast *Saccharomyces cerevisiae*

The ORF encoding for *PiELO1* was amplified using *PfuUltra* II fusion HS DNA polymerase (Stratagene, La Jolla, CA, USA) with the forward primer 5'-AGGAATT CAAAATGGCATTGACGGCGGCCT (PUFAEL5RES1) containing a restriction site (underlined) and a yeast translation consensus followed by ATG (bold) and the reverse primer 5'-CATTCTAGATTACTGCAGCTTTTGCTTGG CTGC (PUFAEL3RES2) containing a restriction site (underlined) and a stop codon (bold). The amplified sequence was then restricted with *EcoRI* and *XbaI* (NEB, Ipswich, MA, USA). The expected bands were gel-purified with NucleoSpin Extract II purification kit (Macherey-Nagel) and ligated into a *EcoRI-XbaI* cut pYES2 vector, yielding pY*PiELO1*. *Saccharomyces cerevisiae* strain W303 was transformed with pY*PiELO1* by the PEG/lithium acetate method [33]. The yeast cells harboring the empty pYES2 vector were used as control. Transformants were selected by uracil prototrophy on yeast synthetic medium (YSM) lacking uracil (Invitrogen). For functional expression, a minimal selection medium containing 2% (w/v) raffinose was inoculated with the pY*PiELO1* transformants and grown at 27°C for 24 h in a water bath shaker. Five milliliters of sterile YSM, containing 1% (w/v) Tergitol-40 and 250 µM of the appropriate fatty acid, was inoculated with raffinose-grown cultures to obtain an OD of 0.2 at 600 nm. Expression was induced by adding galactose to a final concentration of 2% (w/v) and cultures were further grown at 27°C for 48 h. Cells were harvested by centrifugation, washed twice with 0.1% NaHCO₃, freeze-dried, and used for fatty acid analysis.

Fatty Acid Analysis

Fatty acid methyl esters (FAMES) were obtained by transmethylation of the freeze-dried yeast or *P. incisa* cells with dry methanol containing 2% (v/v) H₂SO₄ and heating at 80°C for 1.5 h while stirring under an argon atmosphere. Gas chromatographic analysis of FAMES was performed on a Thermo Ultra gas chromatograph (Thermo Scientific, Italy) equipped with PTV injector, FID, and a fused silica capillary column (30 m × 0.32 mm; ZB WAXplus, Phenomenex). FAMES were identified by co-chromatography with authentic standards (Sigma Chemical, St Louis, MO, USA) and FAME of fish oil (Larodan Fine Chemicals, Sweden). Each sample was analyzed in duplicate.

Real-Time Quantitative RT-PCR

Template cDNA for real-time quantitative PCR (RTQPCR) was synthesized using 1 µg total RNA in a total volume

using the PrimerQuest tool (<http://test.idtdna.com/Scitools/Applications/Primerquest/>). Conditions were set for a primer length of 19–26 bp, primer melting temperature of $60.0 \pm 1.0^\circ\text{C}$, and an amplicon length of 90–150. Primer pairs were validated using seven serial 50-fold dilutions of cDNA samples, and standard curves were plotted to test for linearity of the response. The primer pairs and primer concentrations with reaction efficiencies of $100 \pm 10\%$ were chosen for quantitative RT-PCR analysis of relative gene expression. The nucleotide sequences and characteristics of primers used for quantitative RT-PCR analysis are presented in Table 1.

Gene Expression Profiling

Gene expression profiling was done by real-time quantitative PCR using duplicate reactions for each sample of three independent RNA isolations with a gene-specific primer pair using Absolute Blue QPCR SYBR Green ROX Mix (ABgene) in a real-time PCR 7500 system (Applied Biosystems). The amplification procedure was 50°C for 2 min, 95°C for 15 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. A dissociation curve was obtained for each pair of primers to confirm that a single, specific product was produced in each reaction.

Calculation of Gene Transcript Levels

The mean changes in gene expression, as multiples of the original values, were calculated according to the $2^{-\Delta\Delta\text{Ct}}$ method [34] using the average of threshold cycle (Ct) values from triplicate cDNA-primer samples. The ΔCt followed by the $\Delta\Delta\text{Ct}$ was calculated from the average Ct values of the target and the endogenous genes. The transcript abundance of the *PiELO1* gene was normalized to the endogenous control 18S SSU rRNA gene. The change in gene expression was calculated using $2^{-\Delta\Delta\text{Ct}}$ to find the expression level of the target gene which was normalized to the endogenous gene, relative to the expression of the target gene at time 0.

Results

Identification and Characterization of PiELO1

The BLASTX analysis (<http://www.ncbi.nlm.nih.gov/blast>) of clones obtained through subtractive hybridization revealed a clone of 141 bp whose putative amino acid sequence was highly homologous to the C-terminal region of PUFA elongases. Using GSP primers, the 870-bp 5'-end fragment was amplified and the sequence information was used to obtain the 3' end fragment from the 3' RACE

Ready cDNA. Alignment of the 800 bp 3'-end sequence with that of the 5'-end fragment provided an overlapping nucleotide sequence and included the partial 141 bp sequence, thus confirming the amplification of both ends of the expected gene. The assembled complete 867 bp cDNA sequence, designated *PiELO1*, was preceded and followed by 22 and 150 bp nucleotides at 5' and 3' UTR, respectively. *PiELO1* contained an ORF for 289 predicted amino acid residues consistent with functionally characterized PUFA elongase ORFs from fungi, lower plants, and algae (Fig. 1). The deduced amino acid sequence of the *PiELO1* was 50% identical to functionally characterized $\Delta 6$ PUFA elongases from *O. tauri* and *M. polymorpha*, while sharing 48 and 44% identity with *P. patens* $\Delta 6$ elongase and *M. polymorpha* $\Delta 5$ elongase, respectively. PiELO1 was also similar, yet with a lower score, to $\Delta 6$ elongases of fungal origin. It shared 40 and 36% identity with the $\Delta 6$ PUFA elongases of *Thraustochytrium* sp. and *M. alpina* (not included in the alignment), respectively. PiELO1 was 98% similar to a putative protein of the green microalga *Myrmecea incisa* (accession number ACF60496) which was not functionally characterized.

The predicted amino acid sequence of the PiELO1 contained four conserved motifs that are characteristic of PUFA elongases (Fig. 1, highlighted). The hydropathy plot of the PiELO1-deduced amino acid sequences was obtained using the algorithm available in the DAS transmembrane prediction server (<http://www.sbc.su.se/~miklos/DAS>) [35].

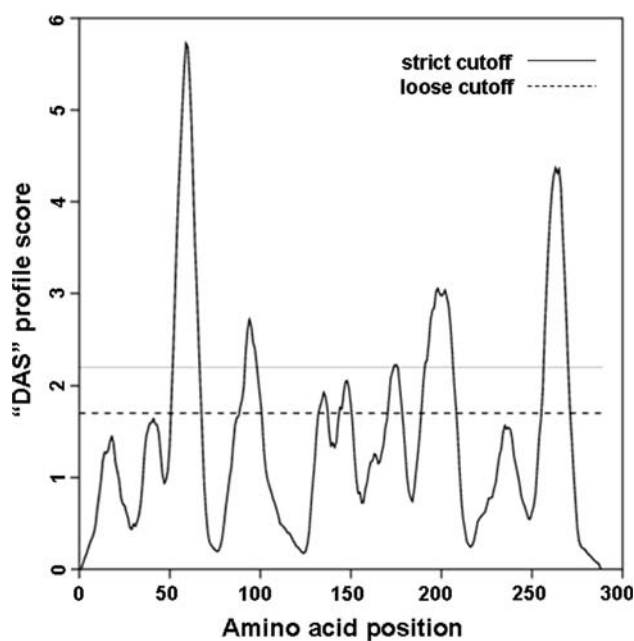


Fig. 2 Hydropathy plot of the amino acid sequence of PiELO1. The lower dashed line and the upper line represent the loose transmembrane region cutoff and the strict transmembrane region cutoff, respectively

The two strictly hydrophobic transmembrane domains were found about 50 amino acids downstream and upstream from the N and C termini, respectively, while the two less hydrophobic domains were located about 100 amino acids downstream and upstream from the N and C termini, respectively (Fig. 2).

Phylogenetic Analysis

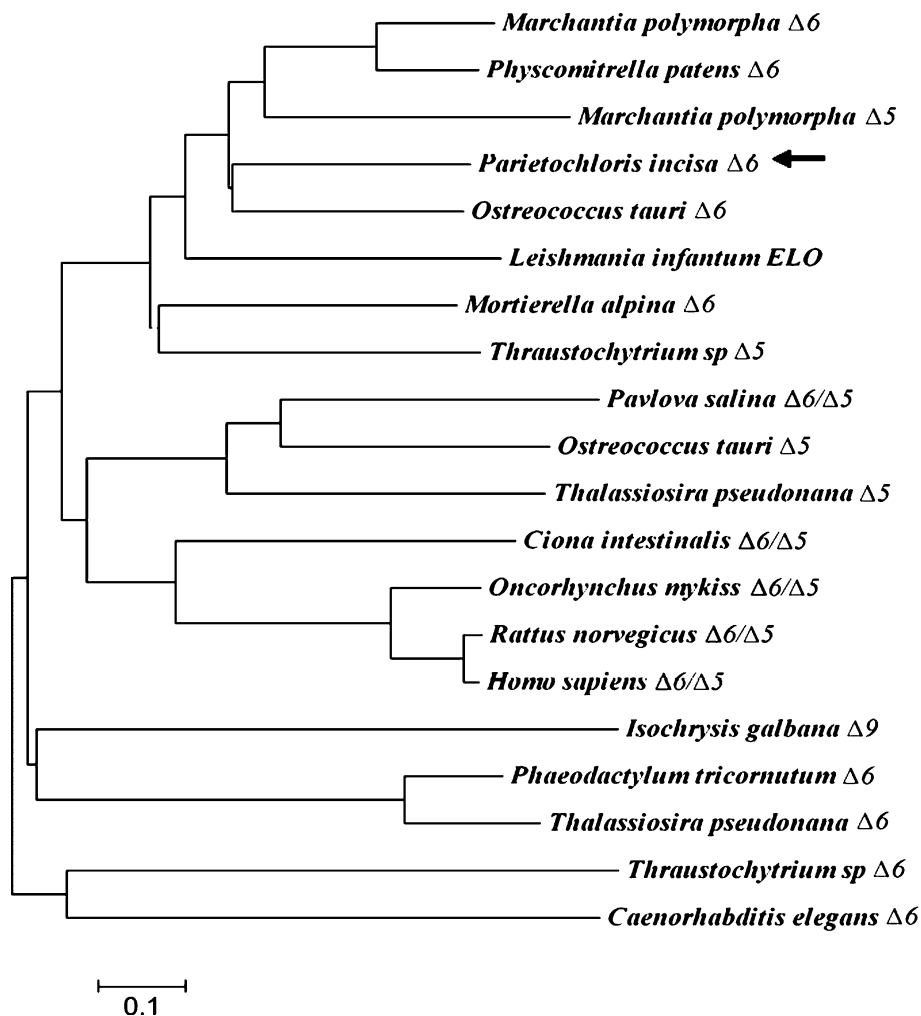
An unrooted phylogenetic tree of PiELO1 and several functionally characterized PUFA elongases was constructed to identify their functional and phylogenetic relationships by the neighbor-joining program in MEGA4 [36]. From Fig. 3 one can see that PiELO1 falls into a group of PUFA elongases of lower eukaryotes. Although the group contains mostly PUFA elongases with $\Delta 6$ activity, some $\Delta 5$ elongases, e.g., those of *M. polymorpha* and *Thraustochytrium* sp., are more related to $\Delta 6$ elongases of lower eukaryotes than to $\Delta 5$ elongases of higher eukaryotes. PiELO1 makes a closely related subgroup with OtELO1, MpELO1, MpELO2, and PpELO1, the OtELO1 being the closest one.

Functional Expression of PiELO1 in *S. cerevisiae*

To characterize the enzymatic activity of PiELO1, the pYES2 plasmid containing the *PiELO1* ORF downstream of the *GALI* promoter was transformed into *S. cerevisiae*. PiELO1 was expressed in the presence of the $\Delta 6$ PUFA elongase substrates, 18:3 ω -6 (γ -linolenic acid, GLA) and 18:4 ω -3 (stearidonic acid, STA). GC analysis of the FAMES of transformed yeast cells showed that PiELO1 elongated both GLA and STA, converting them into di-homo- γ -linoleic acid (DGLA, 20:3 ω -6) and eicosatetraenoic acid (20:4 ω -3), respectively (Fig. 4; Table 2). The yeast cells harboring the empty vector alone did not demonstrate any elongation activity on the added substrates, confirming that the *PiELO1* encoded enzyme has a $\Delta 6$ PUFA elongase activity. Feeding the *PiELO1* transformants with the $\omega 6$ fatty acids LA and ARA did not result in their elongation (not shown).

Real-time quantitative PCR was performed to quantitate the alterations in expression levels of the $\Delta 6$ *PiELO1* in *P. incisa* cells under nitrogen starvation. The change in the

Fig. 3 Unrooted phylogram of PiELO1 and some other functionally characterized PUFA elongases. The alignment was generated by the CLUSTAL W program and the unrooted phylogram was constructed by the neighbor-joining method using MEGA4 software [36]. GeneBank accession numbers for the PUFA elongases are: ACK99719 ($\Delta 6$, *P. incisa*), AAV67797 ($\Delta 6$, *O. tauri*), AAV67798 ($\Delta 5$, *O. tauri*), AAT85662 ($\Delta 6$, *M. polymorpha*), BAE71129 ($\Delta 5$, *M. polymorpha*), AAL84174 ($\Delta 6$, *P. patens*), CAJ30819 ($\Delta 6$, *Thraustochytrium* sp.), CAM55873 ($\Delta 5$, *Thraustochytrium* sp.), AAF70417 ($\Delta 6$, *M. alpina*), XP_001467802 (*L. infantum*), AAV67803 ($\Delta 6/\Delta 5$, *O. mykiss*), NP_001029014 ($\Delta 6/\Delta 5$, *C. intestinalis*), NP_068586 ($\Delta 6/\Delta 5$, *H. sapiens*), AAY15135 ($\Delta 5$, *P. salina*), CAM55851 ($\Delta 6$, *P. tricornutum*), AAL37626 ($\Delta 9$, *I. galbana*), AAV67799 ($\Delta 6$, *T. pseudonana*), AAV67800 ($\Delta 5$, *T. pseudonana*), CAA92958 ($\Delta 6$, *C. elegans*), and NP_599209 ($\Delta 6/\Delta 5$, *R. norvegicus*)



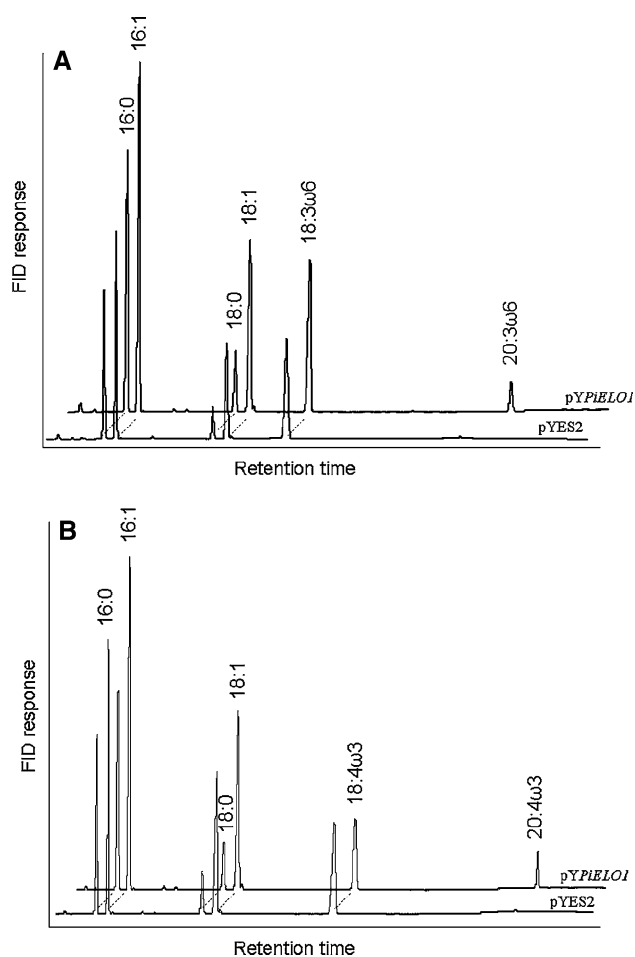


Fig. 4 GC of FAMES of recombinant yeast harboring pYES2 (control) and pYPIELO1 fed with 18:3 ω -6 (a) and 18:4 ω -3 (b)

Table 2 Fatty acid composition of *Saccharomyces cerevisiae* (W303) transformed with the pYES2 vector or the pYPIELO1 construct

Fatty acid (% of total)	Control (pYES2)		pYPIELO1	
	+18:3 ω -6	+18:4 ω -3	+18:3 ω -6	+18:4 ω -3
16:0	20.1	21.6	22.1	20.3
16:1	26.0	28.9	26.8	31.2
18:0	6.0	6.9	7.3	6.5
18:1	18.0	22.7	19.4	25.2
18:3 ω -6	29.9	–	20.4	–
18:4 ω -3	–	19.9	–	12.6
20:3 ω -6	–	–	4.0	–
20:4 ω -3	–	–	–	4.1
Percent elongated			16.4	24.6

The experiments were performed in two biological replicates, each analyzed in duplicate

expression level of the target gene in *P. incisa* cells grown for 3, 7, and 14 days on N-free medium was calculated relative to the expression level of the target gene in the log

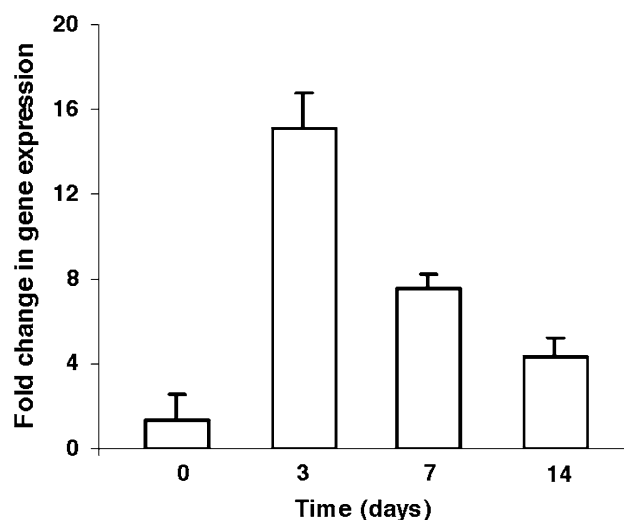


Fig. 5 Quantitative real-time PCR analysis of the *PiELO1* gene expression in the log phase (time 0) and nitrogen starved (3, 7 and 14 days) cells of *P. incisa*. The transcript abundance of the gene was normalized to that of 18S SSU rRNA gene

phase (time 0). The results showed that during nitrogen starvation the mRNA of the *PiELO1* gene was induced to its highest level at day 3 (14-fold increase over time 0), decreasing thereafter to a level still higher than that of day 0 (Fig. 5). After 7 and 14 days, the expression level of the *PiELO1* gene was still 7.5 and 4.3-fold higher, respectively. The level of expression of the *PiELO1* gene correlated with the increase in the share of ARA and the C20/ (C16 + C18) elongation ratio (Table 3). The share of the elongation product, DGLA, increased sharply at day 3 (50% increase over time 0) and decreased thereafter.

Discussion

Recent successes in the production of VLC-PUFA by genetically modified oil plants has elicited further investigations concerning the characterization, role, and regulation of the genes and enzymes involved in VLC-PUFA biosynthesis. We isolated from *P. incisa* a cDNA (*PiELO1*) of an elongase encoding for a deduced protein, structurally similar to Δ 6 PUFA elongase gene products from microalgae, lower plants, and fungi (Fig. 1). The deduced amino acid sequence of the *PiELO1* ORF was about 50% identical with that of Δ 6 elongases from the liverwort *M. polymorpha* (AAT85662), the green marine microalga *O. tauri* (AAV67797), and the moss *P. patens* (AAL84174). In similarity to recently cloned PUFA elongases, the predicted protein is highly hydrophobic and has two strongly hydrophobic transmembrane regions, the first located about 50 amino acids downstream of the N-terminus and the second in the vicinity of the C-terminus. We identified in the *PiELO1* sequence a

Table 3 Major fatty acid composition of *P. incisa* cells grown under N-starvation

Time (days)	Fatty acid composition (% of total fatty acids)												TFA (% DW)
	16:0	16:2	16:3	18:0	18:1	18:2	18:3 ω -6	18:3 ω -3	20:3 ω -6	20:4 ω -6	20:5 ω -3	Elo. ratio ^a	
0	19.1	4.1	2.9	3.1	9.1	20.1	1.2	6.0	0.5	23.0	0.7	0.34	6.4
3	12.7	1.5	1.9	3.8	15.2	13.5	1.6	2.0	0.9	39.7	0.6	0.74	11.0
7	10.7	0.6	1.1	3.5	14.9	10.0	1.1	0.9	1.0	50.0	0.5	1.10	21.2
14	9.0	0.3	0.8	3.1	13.4	8.8	0.9	0.6	0.9	56.9	0.6	1.44	28.9

^a Elongation ratio, C20/(C18 + C16)

C-terminal lysine-rich motif, important for the endoplasmic reticulum targeting [37], and four conserved motifs FYxSKxxEFxDT, QxxxLHVYHHxxI, NSxxHVxMYxYY, and TxxQxxQF, including a conserved histidine-rich box, suggested to be functionally important for PUFA elongation [28] (Fig. 1). These conserved motifs were not found in other classes of plant microsomal elongases, β ketoacyl CoA synthases, and fatty acid elongases (FAE) involved in extraplasmidial elongation of saturated and monounsaturated fatty acids. A variant histidine box, containing three amino acid replacements, in C18- Δ 9-PUFA-specific elongase from *I. galbana* (IgASE1), was shown to be essential for optimal enzymatic activity rather than for substrate specificity [38].

Similarly to GLELO of *M. alpina*, PiELO1 prefers the Δ 6 C18 PUFA substrates, GLA and STA [25]. Only these Δ 6 fatty acids were, when exogenously added, elongated to the respective products by *S. cerevisiae* cells transformed with *PiELO1* (Fig. 4). Transformation of a higher plant so as to render it to produce Δ 6 PUFA requires that the elongase used will have a high selectivity for Δ 6 PUFA, thereby reducing the appearance of side products in the transformed plant [11, 39, 40]. Bifunctional invertebrate PUFA elongases with both Δ 6 and Δ 5 activities (OmELO, XiELO, and CiELO) are less desirable in plant transformations [20, 28].

Phylogenetic analysis showed (Fig. 3) that the PUFA elongases are not strictly divided according to their substrate specificity. The Δ 6 elongases of algal (OtELO1, TpELO1, PiELO1) and moss (PpELO1) origin are functionally restricted to the elongation of Δ 6-C18-PUFAs, however, these elongases are placed in separate groups on the phylogenetic tree (Fig. 3) [28]. PiELO1 is closely related to OtELO1 isolated from a chloropyte and a lower plant rather than to a ELO1 gene isolated from a diatom, although both are specific for the elongation of Δ 6-C18-PUFAs (Fig. 3). PiELO1 is highly similar to, and is placed in the same group with, both Δ 6 and Δ 5 elongases of the liverwort *M. polymorpha*. Kajikawa et al. [41] suggested that MpELO2, a Δ 5 elongase, is likely to have originated through gene duplication of the *MpELO1* gene. In contrast, Δ 5 and Δ 6 PUFA elongases of *Thraustochytrium* sp. are located in distinct branches. The Δ 5 PUFA elongases of a marine chloropyte, a diatom and a haptophyte, OtELO2,

TpELO2 and the *P. salina* ELO1 [42], respectively, are more likely to share a common branch with the mammalian and animal Δ 5 PUFA elongases, OmELO [28] and HELO1 [20], while they are also similar to bifunctional PUFA elongases such as CiELO1/2 [28].

Quantitative real time PCR studies revealed that the expression level of the *PiELO1* gene was up-regulated during the time course of N-starvation (Fig. 5). Nitrogen starvation led to a continuous increase in the share of ARA and the C20/(C16 + C18) elongation ratio (Table 3). However, a major transcriptional activation of *PiELO1* which occurred on day 3 (14-fold increase in transcript level) coincided with the steep rise in ARA accumulation and elongation ratio (Table 3). The increase in *PiELO1* transcription level followed by enhanced biosynthesis of ARA may be interpreted as an increase in the PiELO1 enzyme level and/or enzymatic activity. The importance of the transcriptional activation of PiELO1 is supported by the fact that PUFA elongase gene was the only ARA biosynthesis-related gene that was obtained from the subtractive library. Possibly, the elongation of GLA by PiELO1 could be rate-limiting in ARA biosynthesis as it is in *M. alpina*. However, the significance of the coordinated transcription and action of desaturases, elongases, and enzymes of TAG assembly in biosynthesis of ARA-rich TAG in *P. incisa* is yet to be elucidated. Abbadì et al. [11] suggested that in transgenic plants modified with VLC-PUFA biosynthesis genes, substrate availability rather than enzymatic activity is rate-limiting in the Δ 6 elongation of PUFA. Moreover, the selective acyl channeling to TAG in engineered plant seeds is of major importance in regulating the final TAG acyl quality [11, 39].

In conclusion, PiELO1 is a Δ 6 PUFA elongase, specifically elongating GLA and STA to 20:3 ω -6 and 20:4 ω -3, respectively. The *PiELO1* gene is up-regulated under oleogenic conditions. This gene is a likely candidate for genetic transformations of oil seed plants that will enable the production of VLC-PUFAs in the transgenic plants. The up-regulation of the *PiELO1* gene under nitrogen starvation conditions must have significant physiological importance for adaptation of *P. incisa* cells to nitrogen deficiency.

Accession Numbers GenBank accession numbers for PiELO1 and 18S SSU rRNA of *P. incisa* are ACK99719 and FJ548971, respectively.

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