J. Biochem. 2011;150(4):375–383 doi:10.1093/jb/mvr076

JB THE JOURNAL OF BIOCHEMISTRY

Molecular cloning of a *Pinguiochrysis pyriformis* oleate-specific microsomal Δ12-fatty acid desaturase and functional analysis in yeasts and thraustochytrids†

Received March 31, 2011; accepted April 10, 2011; published online June 23, 2011

Takanori Matsuda[1], Keishi Sakaguchi[1], Takumi Kobayashi[1], Eriko Abe[1], Norihide Kurano[2], Akira Sato[2], Yuji Okita[3], Shinichi Sugimoto[3], Yoichiro Hama[4], Masahiro Hayashi[5], Nozomu Okino[1] and Makoto Ito[1,6,*]

[1]Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581; [2]Marine Biotechnology Institute Co., Ltd. 3-75-1 Heita, Kamaishi, Iwate 026-0001; [3]Nippon Suisan Kaisha, Ltd., 6-2 Otemachi 2-chome, Chiyoda-ku, Tokyo 100-8686; [4]Faculty of Agriculture, Saga University, 1 Honjo, Saga 840-8502; [5]Faculty of Agriculture, Miyazaki University, Miyazaki 889-2192; and [6]Bio-Architecture Center, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

*Makoto Ito, Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan. Tel: +81 92 642 2898, Fax: +81-92-642-2907, email: makotoi@agr.kyushu-u.ac.jp

†The nucleotide sequence reported in this article has been submitted to the GenBank™/EBI Data Bank with accession number AB645862.

We isolated a putative desaturase gene from a marine alga, *Pinguiochrysis pyriformis* MBIC 10872, which is capable of accumulating eicosapentaenoic acid (C20:5$^{\Delta5,8,11,14,17}$). The gene possessed an open reading frame of 1,314 bp encoding a putative 437 amino acid residues showing high sequence identity (37–48%) with fungal and nematode Δ12-fatty acid desaturases. Yeast cells transformed with the gene converted endogenous oleic acid (C18:1$^{\Delta9}$) to linoleic acid (C18:2$^{\Delta9,12}$). However, no double bonds were introduced into other endogenous fatty acids or exogenously added fatty acids. Flag-tagged enzyme was recovered in the microsome fraction when expressed in yeast cells. To express the gene in thraustochytrids, a construct driven by the thraustochytrid-derived ubiquitin promoter was used. Interestingly, exogenously added oleic acid was converted to linoleic acid in the gene transformants but not mock transformants of *Aurantiochytrium limacinum* mh0186. These results clearly indicate that the gene encodes a microsomal Δ12-fatty acid desaturase and was expressed functionally in not only yeasts but also thraustochytrids. This is the first report describing the heterozygous expression of a fatty acid desaturase in thraustochytrids, and could facilitate a genetic approach towards fatty acid synthesis in thraustochytrids which are expected to be an alternative source of polyunsaturated fatty acids.

Keywords: Δ12-fatty acid desaturase/fatty acid/gene expression/PUFA/thraustochytrid.

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester(s); GC, gas chromatography; GC–MS, gas chromatography mass spectrometry; LA, linoleic acid (C18:2$^{\Delta9,12}$); OA, oleic acid (18:1$^{\Delta9}$); PUFA, polyunsaturated fatty acid(s).

A body of accumulating evidence shows the cardiovascular benefits of omega-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, C20:5$^{\Delta5,8,11,14,17}$) and docosahexaenoic acid (DHA, C22:6$^{\Delta4,7,10,13,16,19}$) (*1*). Actually, cardiac societies including the American Heart Association and the European Society for Cardiology recommend the intake of 1 g/day of EPA and DHA for the prevention of cardiovascular disease and sudden cardiac death (*2*). Additionally, DHA, a major fatty acid of phospholipids in the human brain and retina, is thought to be integral to the growth and development of the brain (*3*). The major source of EPA and DHA is fish oils such as sardine oil but recent decreases in fish resources require a substitute (*4*). This has stimulated plant biotechnology aiming to accumulate beneficial PUFA in seed oils of transgenic plants (*5*). An alternative approach to the production of omega-3 fatty acids may target thraustochytrids, unicellular eukaryotic marine protists including the genera *Thraustochytrium*, *Ulkenia* and *Aurantiochytrium* (formerly *Schizochytrium*) (*6*). Thraustochytrids are known to accumulate PUFA, specifically DHA and omega-6 docosapentaenoic acid (DPA, C22:5$^{\Delta4,7,10,13,16}$), mainly in their lipid droplets. Compared with plants such as arabidopsis and tobacco, however, basic genetic information is still lacking for thraustochytrids.

In the present study, a cDNA encoding a putative fatty acid desaturase (PpDes12) was isolated from the marine microalga *Pinguiochrysis pyriformis* MBIC 10872 belonging to a new class of Pinguiophyceae, which was found to accumulate EPA in cells (*7*). The PpDes12 was identified to be a microsomal Δ12-fatty acid desaturase which converts oleic acid (OA, C18:1$^{\Delta9}$) to linoleic acid (LA, C18:2$^{\Delta9,12}$). The Δ12-fatty acid desaturase is a key enzyme in the standard (elongase/desaturase) pathway for production of omega-3 as well as omega-6 fatty acids (Fig. 1A).

To express the PpDes12 in thraustochytrids, a construct driven by the ubiquitin promoter from

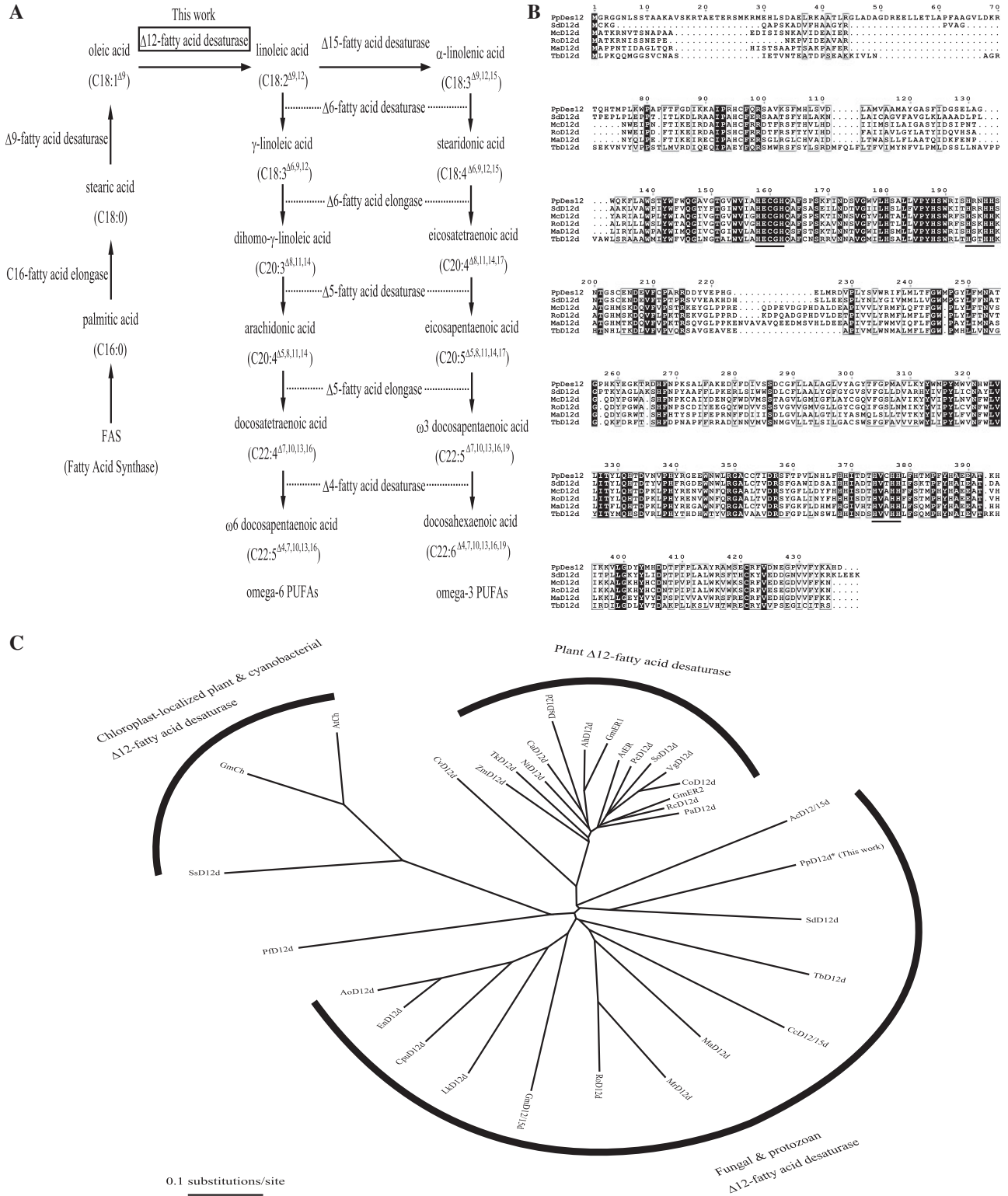

Fig. 1 Putative metabolic role, alignment and phylogenetic tree of PpDes12. (A) PpDes12 in a putative regular (desaturase/elongase) pathway of PUFA synthesis in *P. pyriformis*. (B) alignment of the deduced amino acid sequence of PpDes12 with sequences of fungal and protozoan Δ12-fatty acid desaturases. PpDes12 and fungal and protozoan Δ12-fatty acid desaturases were aligned using ClustalW 1.81 (*29*) and the alignment was shaded in ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). Identical and similar amino acid residues are shown by white letters on a black background and bold face with a black box, respectively. The histidine boxes commonly conserved in fatty acid desaturases are underlined. MaD12d, *M. alpina* Δ12-fatty acid desaturase (*16*); McD12d, *M. circinelloides* Δ12-fatty acid desaturase (*17*); PpD12d, *P. pyriformis* Δ12-fatty acid desaturase (this study); RoD12d, *R. oryzae* Δ12-fatty acid desaturase (*18*); SdD12d, *S. diclina* Δ12-fatty acid desaturase (*19*); TbD12d, *T. brucei* Δ12-fatty acid desaturase (*20*). (C) phylogenetic analysis of Δ12-fatty acid and bi-functional Δ12/Δ15-fatty acid desaturases. Phylogenetic tree was constructed by maximum-likelihood method (*22*) using MOLPHY version 2.3 computer program package. The scale bar represents a distance of 0.1 substitutions per site in the protein sequence. The abbreviations and origins of desaturases used are summarized in Supplementary Table S1.

Thraustochytrium aureum ATCC 34304 was used. *Aurantiochytrium limacinum* mh0186 transformed with the PpDes12 gene, but not with empty construct, converted exogenously added OA to LA, indicating that the gene product functions as a Δ12-fatty acid desaturase in thraustochytrids. This report, the first to describe the heterozygous expression of a fatty acid desaturase in thraustochytrids, could facilitate a genetic approach to the synthesis of fatty acids in thraustochytrids.

Materials and Methods

Strains and culture

Pinguiochrysis pyriformis MBIC 10872 and *T. aureum* ATCC 34304 were obtained from the Marine Biotechnology Institute, Kamaishi (Japan) and American Type Culture Collection (USA), respectively. *Aurantiochytrium limacinum* mh0186 was identified based on the sequence of the 18 S rRNA gene (DDJB, accession number AB362211) (*8*).

Molecular cloning of PpDes 12 from P. pyriformis MBIC 10872

Pinguiochrysis pyriformis was grown at 25°C in ESM medium (*9*). Cells in a late logarithmic phase of growth were harvested by centrifugation (6,000*g*, 4°C, 15 min), and total RNA was extracted by the phenol–SDS method (*10*). Poly(A)$^+$RNA was purified and subjected to the first-strand cDNA synthesis. A pair of degenerate primers targeting the conserved region for fatty acid desaturases, F1 (5′-GGI TGG MGI ATH WSI CAY MGN ACI CAY CA-3′; corresponding to the amino acid sequence GWRISHRTHH) and R1 [5′-CCR TAR TCN CKR TCN AYI GT-3′; corresponding to T(V/I)DRDYG]. Polymerase chain reaction (PCR) was then performed using these primers with first-strand cDNA as a template (PCR cycle: 95°C/30 s, 50°C/30 s, 68°C/2 min, 40 cycles). The amplified PCR products were subcloned into the TOPO TA Cloning vector (Invitrogen, CA, USA) and sequenced. The sequence of an insert showed high identity to known Δ12-fatty acid desaturases, and thus was used as a probe to screen a cDNA library of *P. pyriformis* MBIC 10872. A cDNA library was constructed using Lambda cDNA Library Construction Kits (Stratagene, CA, USA). Phage was packaged and used to infect *Escherichia coli* XL1-Blue MRF. Subsequently, a cDNA library was screened by plaque hybridization with a HRP-labelled probe prepared by ECL Direct Nucleic Labeling. After several rounds of screening, positive clones were excised as a pBluescript SK (−) phargemid by *in vivo* excision. Finally, a full-length cDNA clone encoding Δ12-fatty acid desaturase, named PpDes12, was obtained. The plasmid containing PpDes12 cDNA was designated pBCN8.

Expression of PpDes12 in yeasts

A cDNA of PpDes12 open reading frame (ORF) was amplified by PCR using a 5′ primer containing a HindIII site (P.pyr-F, 5′-TTA AGC TTC AAA ATG TCT CGT GGA GGA AAC CTC TC-3′) and a 3′ primer containing a XbaI site (P.pyr-R, 5′-GTC TAG ATT TAG TCG TGC GCC TTG TAG AAC A-3′) and pBCN8 as a template (94°C/30 s, 61°C/30 s, 72°C/2 min, 30 cycles). The PCR-amplified PpDes12 ORF was digested with HindIII and XbaI, and cloned into the same sites of pYES2/CT (Invitrogen, CA, USA). The resulting PpDes12-expression vector, designated pYpΔ12Des, was introduced into the *Saccharomyces cerevisiae* INVSc1 (Invitrogen, CA, USA) by the lithium–acetate method (*11*). The transformants were selected by plating on synthetic agar plates lacking uracil (SC–ura). *Saccharomyces cerevisiae* harbouring *PpDes12* was cultured in uracil-lacking synthetic complete (SC) medium containing 2% glucose at 25°C for 3 days, and then cultured for an additional 1 day in uracil-lacking SC medium containing 2% galactose. Cells were collected by centrifugation at 3,500*g* for 10 min.

Western blotting of Flag-tagged PpDes12

The Flag-tag sequence was inserted just after the initiation codon of PpDes12 gene by PCR. The PCR was conducted using a forward primer containing the Flag-tag sequences (P.pyr-FLAG-F, 5′-CTA AGC TTC AAA ATG *GAT TAC AAG GAT GAC GAT GAC AAG* TCT CGT GGA-3′) and reverse primer (P.pyr-R, 5′-GTC TAG ATT TAG TCG TGC GCC TTG TAG AAC A-3′). The underline and italics indicate the HindIII site and Flag-tag sequence, respectively. The PCR fragment was directly subcloned into the yeast expression vector pYES2/CT, and subsequencely introduced into *S. cerevisiae* by the method described above. After incubation of the transformant in SC–ura medium, the cells were harvested and suspended in 0.1 M potassium phosphate buffer, pH 7.2, containing 0.33 M sucrose, 0.1% BSA, 1000 U/ml catalase and a protein inhibitor cocktail (Roche Diagnostics K.K., Mannheim, Germany). Glass beads were added and the resultant slurry was sonicated for 20 s and centrifuged (3,000*g* for 10 min). The supernatant (cell lysate) was centrifuged at 100,000*g* for 60 min. The supernatant was used as a cytosolic fraction and the resultant pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.2, containing glycerol (20% by volume) and used as a microsomal fraction. An amount of 10 μg of protein was loaded onto a 10% SDS–PAGE gel and transferred to a PVDF membrane (0.45 μm) using a Bio-Rad Trans-Blot® SD Cell. The membrane was incubated with 5% (w/v) skim milk in TBS buffer containing 0.1% Tween 20 (Tween–TBS) for 1 h at room temperature, washed with Tween–TBS three times and incubated at room temperature for 3 h with an anti-Flag-tag monoclonal antibody (Wako, Osaka, Japan, 1:5000). It was then washed with Tween–TBS three more times and incubated for 3 h at room temperature with an HRP-conjugated anti-mouse IgG [H + L] goat antibody (Nacalai Tesque, Kyoto, Japan; 1:10000). The membrane was again washed with Tween–TBS three times. Protein expression was visualized using a peroxidase staining kit (Nacalai Tesque, 1:20).

Expression of PpDes12 in thraustochytrids

To express the PpDes12 gene in thraustochytrids, we prepared an expression construct (Neo^r/PpDes12 construct, Fig. 4A). For control, PpDes 12 gene with ubiquitin promoter/terminator was omitted from the expression construct (Neo^r construct, Fig. 4B). The EF1-α promoter/terminator and ubiquitin promoter/terminator were obtained from *T. aureum* ATCC 34304. The codons of Neo^r were adjusted according to the codon usage of *T. aureum* ATCC 34304 (*12*). The primers for PCR amplification of these sequences are shown in Supplementary Table S1. The expression construct was introduced into *A. limacinum* mh0186 cells by electrophoration. The cells were then immediately resuspended in 1 ml of GY medium and incubated at 25°C for 1 day, and spread on potato-dextrose agar plates containing G418 at 0.5 mg/ml. After incubation at 25°C for 2–5 days, colonies that appeared on the plates were regarded as putative transformants. *Aurantiochytrium limacinum* mh0186 harbouring *PpDes12* gene was cultured in GY medium at 25°C for 4 days. Cells were collected by centrifugation at 3,500*g* for 10 min.

Genomic PCR and Southern blot hybridization of thraustochytrid transfectants

Genomic PCR was performed using the forward primer 2 F and reverse primer pUC18-R (Supplementary Table S1) (96°C/2 min, 98°C/20 s, 60°C/30 s, 72°C/5 min, 30 cycles). For Southern blot hybridization, 5 μg of genomic DNA was digested at 37°C with XbaI overnight. The digested DNA was separated on 1% agarose gel and transferred onto a Hybond-N$^+$. The membrane was hybridized with a probe prepared using the DIG DNA Labeling Kit (Roche Diagnostics K.K.). PCR primers used were PD12d-probe-F (5′-CTG CCC GGC CCG CCG CGA CGA CTA-3′) and PD12d-probe-R (5′-CGG CGT GAA GCT ACG GTC GAT GGT-3′). Genomic DNA hybridized with probe was detected with an anti-Digoxigenin-AP Fab fragment and an NBT/BCIP stock solution (Roche Diagnostics K.K.).

RT–PCR of Neo^r and PpDes12 in the thraustochytrid transfectants

Total RNA was prepared from transfectants, grown in GY medium containing appropriate amounts of G418, with a Sepasol RNAI Super (Nacalai Tesque, Kyoto, Japan), RNeasy Mini Kit (QIAGEN, Tokyo, Japan) and DNaseI (Takara Bio Inc., Shiga, Japan) and used to produce first-strand cDNA with PrimeScript™ Reverse Transcriptase (Takara Bio Inc., Shiga, Japan). PCR was performed using the forward primer 3F and reverse primer 4 R for amplification of Neo^r cDNA and forward primer ub pro-D12d-F and reverse primer ub term-D12d-R for amplification of PpDes12 cDNA (96°C/2 min, 98°C/20 s, 60°C/30 s, 72°C/90 s, 30 cycles) (Supplementary Table S1).

Fatty acid analysis

The preparation and extraction of fatty acid methyl esters (FAME) were carried out as described previously (*13*). The resulting FAME were analysed by gas–liquid chromatography (GC) by the method described in (*14*). The FAME were also subjected to gas chromatography–mass spectrometry (GC–MS) using a Shimadzu GC-MS QP-5000 (SHIMADZU Co., Kyoto, Japan) equipped with a capillary column (DB-1, 0.25 mm i.d. × 30 m, film thickness 0.25 μm, Agilent). The column temperature was programmed to increase at 4°C/min from 160°C to 260°C. The injection-port temperature was 250°C. The rate of conversion of substrates to products was calculated as follows; conversion rate (%) = GC peak area of product/(GC peak area of product + GC peak area of substrate) × 100. Furthermore, picolinyl esters were prepared from the FAME as described previously (*15*) and subjected to GC–MS using the equipment described above. The column temperature was programmed to increase at 2.5°C/min from 240°C to 260°C and maintained for 15 min, then increased at 2.5°C/min to 280°C.

Results

Molecular cloning of PpDes12 from P. pyriformis MBIC 10872

Pinguiochrysis pyriformis MBIC 10872 has been reported to accumulate omega-3 PUFA, especially EPA, in cells (*7*). In this study, we isolated the cDNA fragment (516 bp) of a putative desaturase (PpDes12) from this organism by degenerate PCR as described in Materials and Methods section. The DNA fragment was used as a probe to isolate a full-length cDNA clone through plaque hybridization with a *P. pyriformis* MBIC 10872 cDNA library. After the screening of 5.5×10^5 recombinants, a cDNA clone including the putative PpDes12 ORF was isolated and designated pBCN8.

DNA and deduced amino acid sequences of PpDes12

We sequenced 1,494 nucleotides of pBCN8, and found a 1,314-bp ORF of PpDes12 encoding a putative 437 amino acid residues. As shown in Fig. 1B, the deduced amino acid sequence of PpDes12 exhibited a high degree of identity with fungal and protozoan Δ12-fatty acid desaturases, such as those from *Mortierella alpina* (43.4%) (*16*), *Mucor circinelloides* (45.3%) (*17*), *Rhizopus oryzae* (44.6%) (*18*), *Saprolegnia diclina* (48.0%) (*19*) and *Trichoderma brucei* (37.2%) (*20*) (the number in parentheses shows the identity relative to PpDes12). Three histidine boxes, conserved in almost all fatty acid desaturases, were found in the deduced amino acid sequence of PpDes12 (Fig. 1B, underlined), whereas the cytochrome b_5 motif, characteristic of front-end desaturases, was not.

Phylogenetic analysis

Δ12- and Δ12/Δ15-fatty acid desaturases have been classified into the following groups based on sequence similarity: a fungal and protozoan group, a plant group, a cyanobacterial group and a chloroplast-localized plant group. The evolutionary relationship between PpDes12 and other Δ12- and Δ12/Δ15-fatty acid desaturases was examined in a phylogenetic analysis. PpDes12 was found to be clustered with the fungal and nematode group in which it was most closely related to the *S. diclina* Δ12-fatty acid desaturase (Fig. 1C).

Expression of PpDes12 in S. cerevisiae

To clarify the function of PpDes12, a PpDes12-expression construct (pYpΔ12Des) and an empty-control construct (pYES2/CT) were separately introduced into the INVSc1 strain of *S. cerevisiae* and the fatty acid composition of pYpΔ12Des and mock transfectants was analysed by GC using fatty acid methyl esters. The peak corresponding to standard LA ($18{:}2^{\Delta 9,12}$) methyl ester was found in pYpΔ12Des transfectants but not in mock transfectants, although OA ($18{:}1^{\Delta 9}$), the precursor of LA, was found in both transfectants (Fig. 2A and B). On the other hand, amounts of endogenous palmitic acid (C16:0), stearic acid (C18:0) and palmitoleic acid ($C16{:}1^{\Delta 9}$) were unchanged in pYpΔ12Des and mock transfectants. GC–MS of the new peak in pYpΔ12Des transfectants revealed its molecular ion (m/z 294) and fragmentation ions to be identical to those of the standard LA methyl ester (Fig. 2C and D). The rate of conversion of OA to LA was calculated to be 14.3 ± 2.71 % under the conditions used (average from three different transfectants, $n = 2$). These results indicate that endogenous OA was converted to LA in pYpΔ12Des transfectants. However, no double bonds were introduced into myristoleic acid ($14{:}1^{\Delta 9}$), palmitoleic acid ($16{:}1^{\Delta 9}$), elaidic acid ($18{:}1^{\Delta 9\ trans}$), LA, γ-linolenic acid ($C18{:}3^{\Delta 6,9,12}$), dihomo-γ-linolenic acid ($C20{:}3^{\Delta 8,11,14}$), arachidonic acid ($C20{:}4^{\Delta 5,8,11,14}$) and docosatetraenoic acid ($C22{:}4^{\Delta 7,10,13,16}$) when they were added to the culture of pYpΔ12Des or mock transfectants at 40 μM (data not shown). Taken together, the PpDes12 gene of *P. pyriformis* MBIC 10872 encodes a Δ12-fatty acid desaturase that catalyses the conversion of OA to LA by introducing a double bond at the Δ12 position of OA.

Western blotting of Flag-tagged PpDes12 expressed in the yeasts

We examined the expression of PpDes12 at the protein level. Yeast cells expressing Flag-tagged PpDes12 were lysed and fractionated into a microsomal fraction and cytosolic fraction, which were subjected to western blotting using anti-Flag-tag antibody. A 51.1-kDa protein band was detected in the cell lysate and microsomal fraction, but not cytosolic fraction (Fig. 3). The molecular weight (51.1 kDa) was well consistent with that estimated from the deduced amino acid sequence of the desaturase with a Flag tag. This result indicates that PpDes12 can be classified as a microsomal fatty acid desaturase.

Expression of PpDes12 in A. limacinum

Thraustochytrids are potentially an alternative to fish for the production of omega-3 PUFAs (*6*). However, the genetic approach to the synthesis of fatty acids in thraustochytrids has not been fully established due to a lack of molecular tools for gene manipulation. In this study, we designed a thraustochytrid-specific expression construct to express the heterozygous gene in thraustochytrids using a promoter and a terminator of house-keeping genes derived from *T. aureum* ATCC 34304. To select the transfectants, we used neomycin (G418) and a neomycin-resistance gene (Neo^r) after

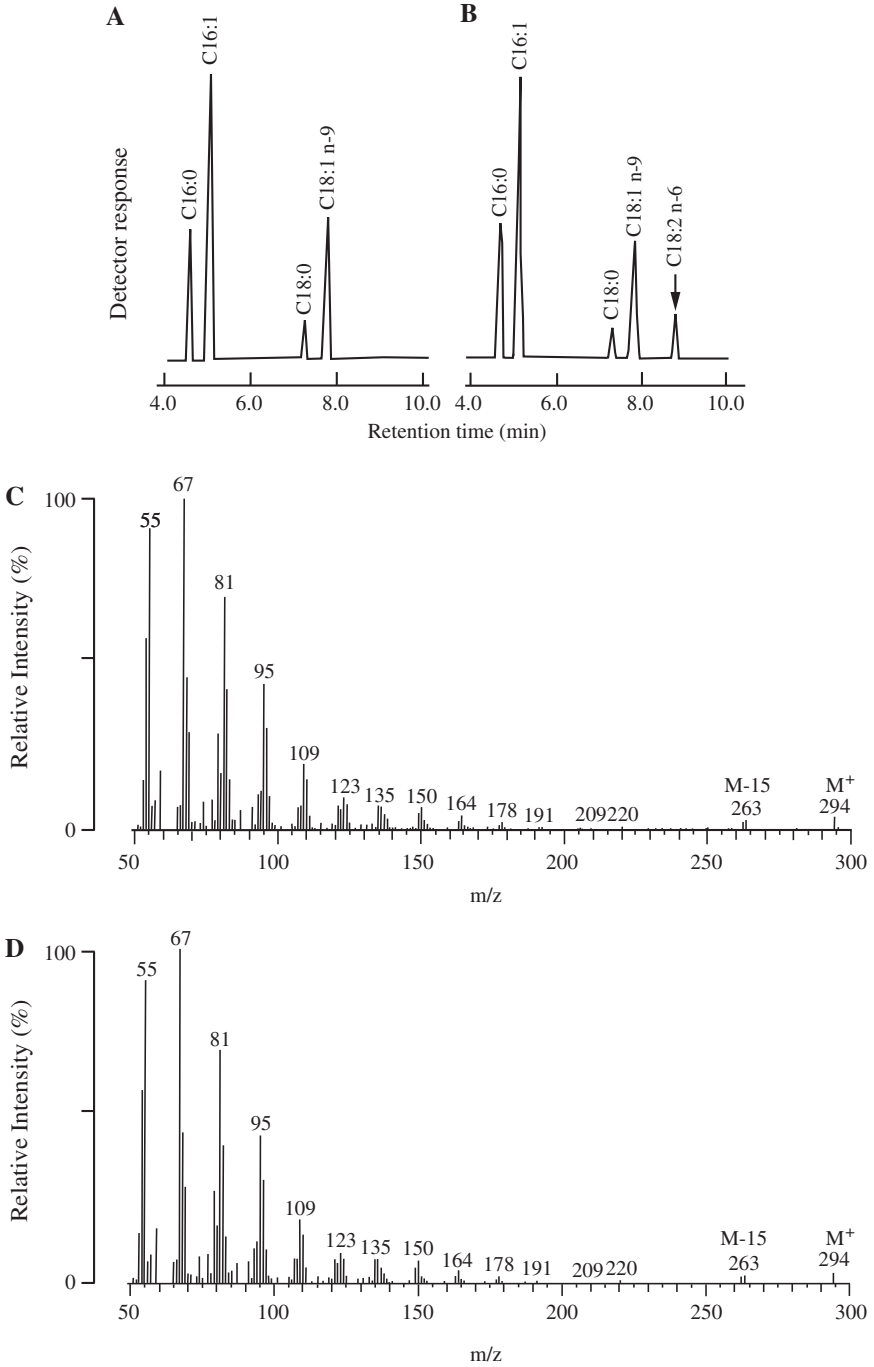

Fig. 2 GC and GC–MS of FAME from *S. cerevisiae* transfectants. GC showing the FAME from *S. cerevisiae* transformed with (A) empty vector, pYES2/CT (mock transfectants) and (B) PpDes12-containing vector, pYpΔ12Des (PpDes12 transfectants). The arrow indicates the new peak in PpDes12 transfectants (B). (C) Mass spectrum of the standard LA methyl ester. (D) Mass spectrum of FAME generated in PpDes12 transfectants. The cells were cultured in uracil-lacking SC medium containing 2% glucose at 25°C for 3 days, and then cultured for an additional 1 day in uracil-lacking SC medium containing 2% galactose with or without exogenous fatty acids. When fatty acids were added to the culture, 0.1% tergitol was also added. Fatty acids were extracted from freeze-dried cells and subjected to GC and GC–MS as described in Materials and Methods section.

adjusting the codons according to the codon usage of *T. aureum* ATCC 34304.

To confirm whether the PpDes12 is able to function in thraustochytrids, a Neo^r/PpDes12-expression construct (Fig. 4A) and a Neo^r control construct (Fig. 4B) were separately injected into *A. limacinum* mh0186 by electroporation. Transfectants grown on a G418-containing GY agar medium were subjected to genomic PCR to examine whether a full-length Neo^r/PpDes12 DNA was integrated into the genome of the mh0186 strain. As shown in Fig. 4C, a 5,394-bp PCR product (corresponding to Neo^r/PpDes12 construct, Fig. 4A) was detected in the Neo^r/PpDes12 transfectants, whereas a 2,657-bp PCR product (corresponding to Neo^r construct, Fig. 4B) was amplified for control Neo^r transfectants. Southern blot

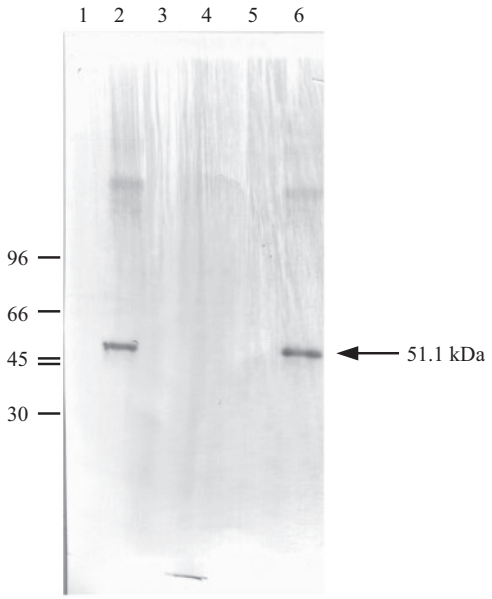

Fig. 3 Western blot analysis of Flag-tagged PpDes12 expressed in the yeast. 1, cell lysate from mock transfectant; 2, cell lysate from transfectant expressing the Flag-tagged PpDes12; 3, cytosol fraction from mock transfectant; 4, cytosol fraction from transfectant expressing the Flag-tagged PpDes12; 5, microsome fraction from mock transfectant; 6, microsome fraction from transfectant expressing the Flag-tagged PpDes12. The *S. cerevisiae* cells harbouring a vector containing the Flag-tagged PpDes12 gene or an empty vector (mock control) were cultured in SC−ura medium and the cell lysates were subjected to the procedure for preparation of micosomes. Western blotting was carried out using 10% SDS–PAGE and anti-Flag-tag mouse monoclonal antibody and HRP-conjugated anti-mouse IgG goat antibody. Details are described in Materials and Methods section.

hybridization using a PpDes12 DNA probe confirmed that the PpDes12 gene was integrated into the mh0186 genome (Fig. 4D). Furthermore, RT–PCR revealed that transcripts of both Neo^r gene (835 bp) and PpDes12 gene (1,354 bp) were present in Neo^r/PpDes12 transfectants while the transcript of Neo^r gene, but not PpDes12, was detected in control Neo^r transfectants (Fig. 4E and F). These results clearly indicate that the PpDes12 and Neo^r genes were integrated into the genome of *A. limacinum* mh0186 and then translated to the respective mRNA.

Finally, the fatty acid composition of Neo^r/PpDes12 transfectants and control Neo^r transfectants was analysed by GC using methyl ester derivatives. The peak corresponding to standard LA methyl ester appeared in Neo^r/PpDes12 transfectants (Fig. 5B) but not in control Neo^r transfectants (Fig. 5A) after adding OA to the culture of both transfectants. GC–MS of this new peak revealed its molecular ion (m/z) and fragmentation ions to be identical to those of the LA picolinyl ester (Fig. 5C). The rate of conversion of OA to LA was calculated to be $7.28 \pm 1.33\%$ (average from five different transfectants, $n = 2$). No significant change in fatty acid composition except OA and LA was observed in Neo^r/PpDes12 transfectants, compared with control Neo^r transfectants (data not shown). Additionally, ^{14}C-LA acid was detected in Neo^r/PpDes12 transfectants but not in control Neo^r transfectants when ^{14}C-OA-CoA was added to the culture of transfectants (Supplementary Fig. S1).

Collectively, the *Pinguiochrysis* gene encoding PpDes12 was integrated into the genome of *A. limacinum* mh0186 (Fig. 4C and D), translated into PpDes12 mRNA (Fig. 4F) and functioned as a Δ12-fatty acid desaturase in thraustochytrid cells (Fig. 5).

Discussion

In this study, we cloned a putative fatty acid desaturase (PpDes12) gene from *P. pyriformis* MBIC 10872 that accumulates omega-3 PUFAs, especially EPA (*7*). The gene was found to encode an enzyme capable of catalysing the introduction of a double bond at the Δ12 position of OA but not other fatty acids tested. Western blotting of Flag-tagged PpDes12 expressed in the yeast revealed that the enzyme was recovered in the microsomal fraction. Furthermore, analysis using TMHMM suggested that the enzyme has two transmembrane domains. These results indicate that PpDes12 is an oleate-specific microsomal Δ12-fatty acid desaturase. The deduced amino acid sequence of PpDes12 contains three histidine boxes (Fig. 1B, underlined), commonly conserved in fatty acid desaturases. This region may act as di-iron coordinating centres for catalytic activity (*21*). Meanwhile, PpDes12 possesses no cytochrome b_5-like domain which is usually present in front-end desaturases and functions as an electron donor. It has been reported that a *T. brucei* oleate desaturase did not carry a cytochrome b_5-like domain but might use a microsomal cytochrome or the cytochrome b_5-like domain of other desaturases as an electron donor (*20*). PpDes12 could accept electrons in a similar manner to the *T. brucei* oleate desaturase.

The phylogenetic analysis of Δ12-fatty acid and bifunctional Δ12/Δ15-fatty acid desaturases by the maximum-likelihood method (*22*) revealed that PpDes12 is part of a fungal and nematode Δ12-fatty acid desaturase group. Among the organisms belonging to this group, only *P. pyriformis* is a 'photosynthetic' stramenopile. Although PpDes12 was recovered in the microsomal fraction when expressed in the yeast (Fig. 3), its intracellular distribution remains to be clarified. However, PpDes12 could be present in chloroplasts like other Δ12-fatty acid desaturases of higher plants, because the strain MBIC 10872 cells used in this study have one or two typical chloroplasts and accumulate PUFA in the chloroplasts (*7*). It is worth noting that the activity of Δ12-fatty acid desaturase could not be detected *in vitro* using the cell lysate or microsomal fraction as an enzyme source possibly because of difficulty with the solubilization of the protein. Thus, reconstitution of the enzyme reaction *in vitro* remains to be achieved.

Although thraustochytrids accumulate PUFA mainly in lipid droplets, their pathway for production of PUFA has not been well documented. Accumulating evidence, however, suggests that two distinct pathways of fatty acid synthesis are present in thraustochytrids, i.e. polyketide-like fatty acid synthesis (PUFA synthase) and a regular (desaturase/elongase) pathway. The former pathway has been well documented in

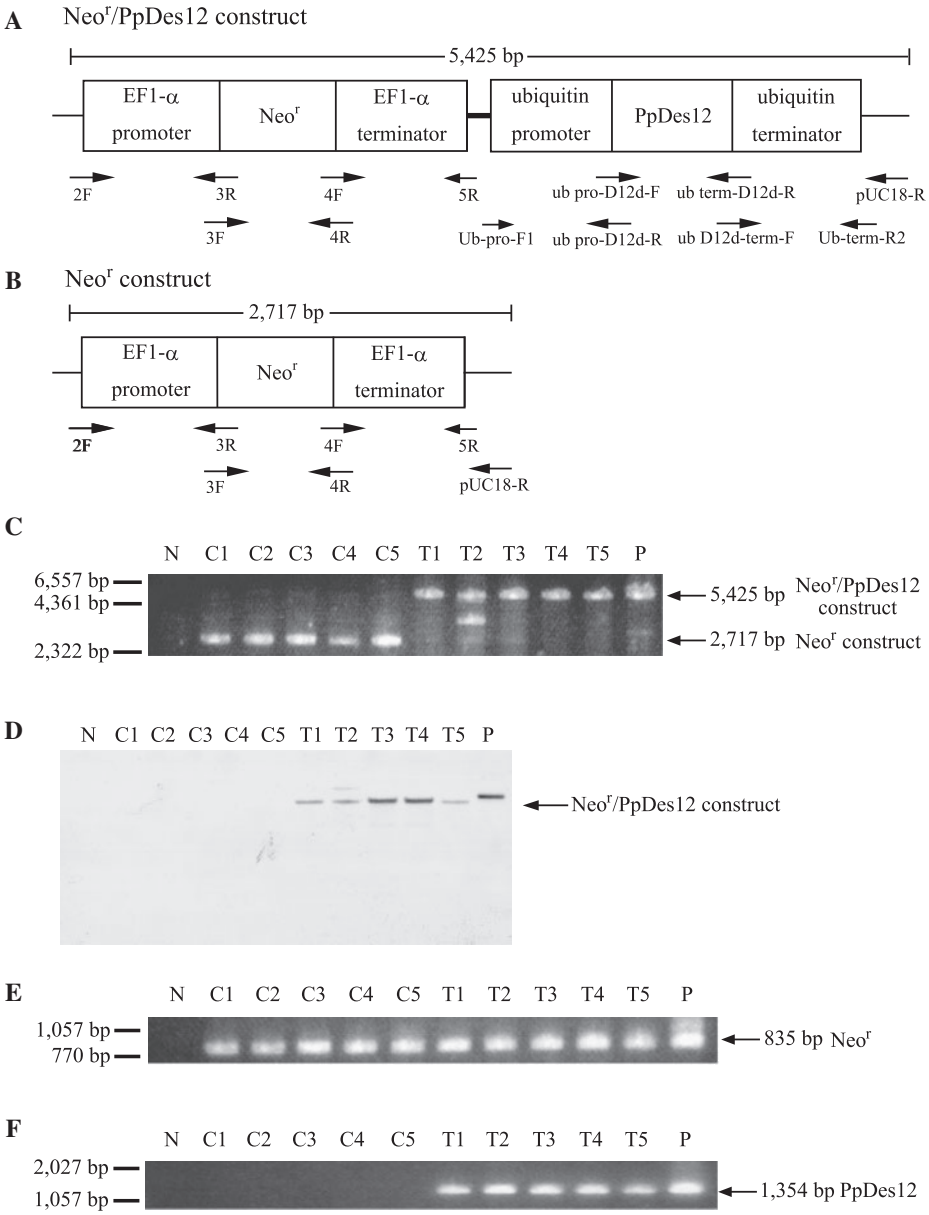

Fig. 4 Molecular characterization of *A. limacinum* mh0186 transfectants. (A) Thraustochytrid-specific expression construct containing Neo^r and PpDes12 genes (Neo^r/PpDes12 construct) with sites for primers used. (B) Thraustochytrid-specific expression construct containing Neo^r gene (Neo^r construct, control vector) with sites for primers used. Neo^r and PpDes12 genes were derived with thraustochytorid-derived EF1-α promoter/terminator and ubiquitin promoter/terminator, respectively. (C) Genomic PCR showing Neo^r construct and Neo^r/PpDes12 construct. (D) Southern blot hybridization using PpDes12-specific probe. RT–PCR amplifying Neo^r mRNA (E) and PpDes12 mRNA (F). N, negative control (wild-type mh0186); C1–C5, Neo^r transfectants (mock transfectants); T1–T5, Neo^r/PpDes12 transfectants; P, positive control (pNeoDes12). The details are shown in Materials and Methods section.

marine bacteria (*23*) and thraustochytrids (*24*) and the latter, in animals from nematodes to mammals (*25*, *26*). It is worth noting that targeted mutagenesis of a PUFA synthase gene of *Schizochytrium* sp. resulted in auxotrophic mutants that required supplementation with PUFA (*27*). This result suggests that the regular pathway in *Schizochytrium* sp. was not capable of synthesizing adequate amount of PUFA under the conditions used, probably due to the absence of a Δ12-fatty acid desaturase (*27*). We also found in the present study that *A. limacinum* mh0186 does not have OA and LA, the former being the substrate of Δ12-fatty acid desaturase and the latter, the product of Δ12-fatty acid desaturase. Furthermore, exogenously added OA was not converted to LA in mh0186 cells until a *Pinguiochrysis* Δ12-fatty acid desaturase was expressed in the strain. Our observations may indicate that *A. limacinum* (formerly *Schizochytrium* sp.) has no Δ12-fatty acid desaturase activity. It is note worthy that the expression of Δ12-fatty acid desaturase did not alter the PUFA composition of *A. limacinum* mh0186 except OA and LA, suggesting the standard pathway of fatty acid synthesis is not sufficiently working in mh0186 cells.

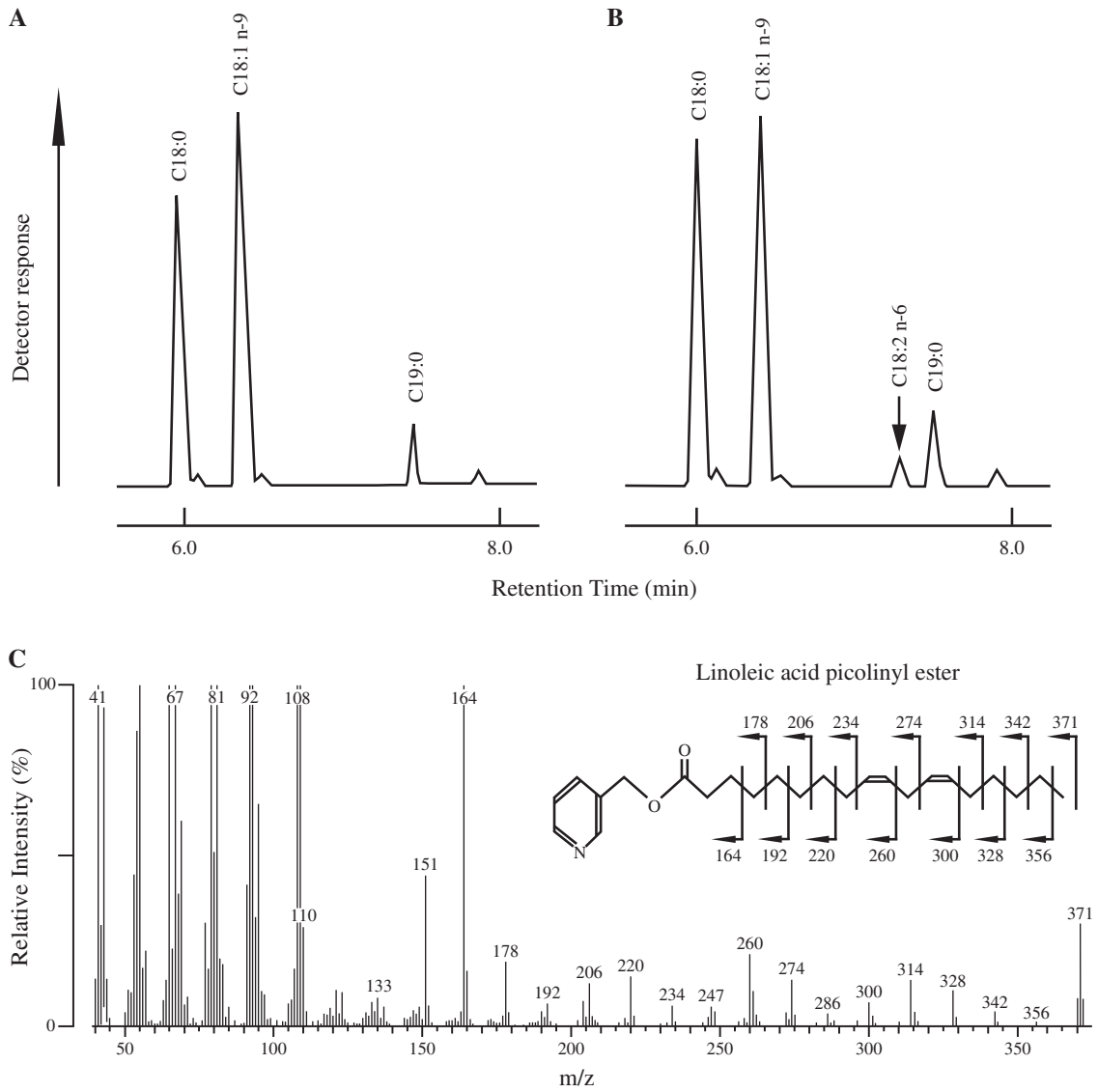

Fig. 5 GC and GC–MS of fatty acid derivatives from *A. limacinum* transfectants. GC showing the FAME from *A. limacinum* mh0186 transformed with (A) control Neo^r construct (mock transfectants) and (B) Neo^r/PpDes12 construct (PpDes12 transfectants). The arrow indicates the new peak in PpDes12 transfectants. (C) Mass spectrum of the picolinyl ester derivatives of the fatty acids generated in mh0186 cells transformed with a Neo^r/PpDes12 construct. Cells were cultured in a GY medium containing G418 at a concentration of 0.5 mg/ml at 25°C for 3 days, and then cultured for an additional 1 day with 100 μM OA. Fatty acids were extracted from freeze-dried cells and subjected to GC and GC–MS as described in Materials and Methods section.

On the other hand, several fatty acid desaturases (*13*) and elongases (*28*) possibly associated with the regular pathway of fatty acid synthesis have been identified in thraustochytrids. Thus, the relationship between the PUFA synthase pathway and desaturase/elongase pathway, and/or the mutual relationships of each enzyme in the desaturase/elongase pathway should be carefully examined using several different species of thraustochytrids. The thraustochytrid-specific gene expression system developed in this study could help us to understand the mechanics of fatty acid synthesis in thraustochytrids and facilitate the biotechnology of thraustochytrids.

In conclusion, we isolated a gene encoding an oleate-specific microsomal Δ12-fatty acid desaturase from *P. pyriformis* MBIC 10872 and expressed it in yeasts as well as thraustochytrids.

Supplementary Data

Supplementary Data are available at *JB* Online.

Conflict of interest

None declared.

References

1. Vrabilk, M., Prusikova, M., Snejdrlova, M., and Zlattohlavek, L. (2009) Omega-3 fatty acids and cardiovascular disease risk: Do we understand the relationship? *Physiol. Res.* **58 (Suppl 1)**, S19–S26
2. von Schacky, C. and Harris, W.S. (2007) Cardiovascular benefits of omega-3 fatty acids. *Cardiovasc. Res.* **73**, 310–315

3. Innis, S.M. (2008) Dietary omega 3 fatty acids and the developing brain. *Brain Res.* **1237**, 35–43
4. Lee, J.H., O'Keefe, J.H., Lavie, C.J., and Harris, W.S. (2009) Omega-3 fatty acids: cardiovascular benefits, sources and sustainability. *Nat. Rev. Cardiol.* **6**, 753–758
5. Sayanova, O.V. and Napier, J.A. (2004) Eicosapentaenoic acid: biosynthetic routes and the potential for synthesis in transgenic plants. *Phytochemistry*. **65**, 147–158
6. Raghukumar, S. (2008) Thraustochytrid marine protists: production of PUFAs and other emerging technologies. *Mar. Biotechnol.* **10**, 631–640
7. Kawachi, M., Inouye, I., Honda, D., O'Kelly, C.J., Bailey, J.C., Bidigare, R.R., and Andersen, R.A. (2002) The pinguiophyceae classis nova, a new class of photosynthetic stramenopiles whose members produce large amounts of omega-3 fatty acids. *Phycological Res.* **50**, 31–47
8. Taoka, Y., Nagano, N., Okita, Y., Izumida, H., Sugimoto, S., and Hayashi, M. (2009) Influences of culture temperature on the growth, lipid content and fatty acid composition of *A. limacinum* strain mh0186. *Mar. Biotechnol.* **11**, 368–374
9. Watanabe, M.M., Kawachi, M., Hiroki, M., and Kasai, F. (2000) *NIES-collection List of Strains, Microalgae and Protozoa.* 6th edn, NIES, Japan
10. Bijli, K.M., Singh, B.P., Sridhara, S., and Arora, N. (2001) Isolation of total RNA from pollens. *Prep. Biochem. Biotechnol.* **31**, 155–162
11. Chen, D., Yang, B., and Kuo, T. (1992) One-step transformation of yeast in stationary phase. *Curr. Genet.* **21**, 83–84
12. Nakamura, Y., Gojobori, T., and Ikemura, T. (2000) Codon usage tabulated from international DNA sequence databases: Status for the year 2000. *Nucleic Acids Res.* **28**, 292
13. Qiu, X., Hong, H., and Mackenzie, S.L. (2001) Identification of a delta 4 fatty acid desaturase from *Thraustochytrium.* sp. involved in the biosynthesis of docosahexanoic acid by heterologous expression in Saccharomyces cerevisiae and Brassica juncea. *J. Biol. Chem.* **276**, 31561–31566
14. Abe, E., Hayashi, Y., Hama, Y., Hayashi, M., Inagaki, M., and Ito, M. (2006) A novel phosphatidylcholine which contains pentadecanoic acid at *sn*-1 and docosahexaenoic acid at *sn*-2 in Schizochytrium sp. F26-b. *J. Biochem.* **140**, 247–253
15. Dubois, N., Barthomeuf, C., and Berge, J.P. (2006) Convenient preparation of picolinyl derivatives from fatty acid esters. *Eur. J. Lipid Sci. Technol.* **108**, 28–32
16. Sakuradani, E., Kobayashi, M., Ashikari, T., and Shimizu, S. (1999) Identification of delta12-fatty acid desaturase from arachidonic acid-producing mortierella fungus by heterologous expression in the yeast *Saccharomyces cerevisiae* and the fungus *Aspergillus oryzae*. *Eur. J. Biochem.* **261**, 812–820
17. Passorn, S., Laoteng, K., Rachadawong, S., Tanticharoen, M., and Cheevadhanarak, S. (1999) Heterologous expression of Mucor rouxii delta(12)-desaturase gene in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **263**, 47–51
18. Wei, D., Li, M., Zhang, X., Ren, Y., and Xing, L. (2004) Identification and characterization of a novel delta12-fatty acid desaturase gene from *Rhizopus arrhizus*. *FEBS Lett.* **573**, 45–50
19. Pereira, S.L., Huang, Y.S., Bobic, E.G., Kinney, A.J., Stecca, K.L., Packer, J.C., and Mukerji, P. (2004) A novel omega 3-fatty acid desaturase involved in the biosynthesis of eicosapentaenoic acid. *Biochem. J.* **378**, 665–671
20. Petrini, G.A., Altabe, S.G., and Uttaro, A.D. (2004) *Trypanosoma brucei* oleate desaturase may use a cytochrome b_5-like domain in another desaturase as an electron donor. *Eur. J. Biochem.* **271**, 1079–1086
21. Shanklin, J., Whittle, E., and Fox, B.G. (1994) Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. *Biochemistry*. **33**, 12787–12794
22. Felsenstein, J. (1996) Inferring phylogenies from protein sequences by parsimony, distance, and likelihood methods. *Methods Enzymol.* **266**, 418–427
23. Orikasa, Y., Yamada, A., Yu, R., Itoh, Y., Nishida, T., Yumoto, I., Watanabe, K., and Okuyama, H. (2004) Characterization of the eicosapentaenoic acid biosynthesis gene cluster from *Shewanella* sp. strain SCRC-2738. *Cell Mol. Biol.* **50**, 625–630
24. Metz, J.G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R., Lardizabal, K., Domergue, F., Yamada, A., Yazawa, K., Knauf, V., and Browse, J. (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* **293**, 290–293
25. Napier, A.J. and Michaelson, L.V. (2001) Genomic and functional characterization of polyunsaturated fatty acid biosynthesis in *Caenorhabditis elegans*. *Lipids* **36**, 761–766
26. Nakamura, M.T. and Nara, T.Y. (2003) Essential fatty acid synthesis and its regulation in mammals. *Prostaglandins Leukot. Essent. Fatty Acids* **68**, 145–150
27. Lippmeier, J.C., Crawford, K.C., Owen, C.B., Rivas, A.A., Metz, J.G., and Apt, K.E. (2009) Characterization of both polyunsaturated fatty acid biosynthetic pathways in *Schizochytrium* sp. *Lipids* **44**, 621–630
28. Jae-Cheol, L., Periasamy, A., Won-Ho, K., Myung-ju, N., Su-Jin, L., Jeong-Woo, S., and Byung-Ki, H. (2008) Identification of Δ9-elongation activity from *Thraustochytrium aureum* by heterologous expression in *Pichia pastoris*. *Biotechnol. Bioprocess Engineer* **13**, 524–532
29. Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.J., and Thompson, J.D. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* **31**, 3497–3500