

# Identification and Functional Characterization of the Moss *Physcomitrella patens* $\Delta^5$ -Desaturase Gene Involved in Arachidonic and Eicosapentaenoic Acid Biosynthesis\*

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The moss *Physcomitrella patens* contains high levels of arachidonic acid and lesser amounts of eicosapentaenoic acid. Here we report the identification and characterization of a  $\Delta^5$ -desaturase from *P. patens* that is associated with the synthesis of these fatty acids. A full-length cDNA for this desaturase was identified by data base searches based on homology to sequences of known  $\Delta^5$ -desaturase cDNAs from fungal and algal species. The resulting *P. patens* cDNA encodes a 480-amino acid polypeptide that contains a predicted N-terminal cytochrome *b<sub>5</sub>*-like domain as well as three histidine-rich domains. Expression of the enzyme in *Saccharomyces cerevisiae* resulted in the production of the  $\Delta^5$ -containing fatty acid arachidonic acid in cells that were provided di-homo- $\gamma$ -linolenic acid. In addition, the expressed enzyme generated  $\Delta^5$ -desaturation products with the C20 substrates  $\omega$ -6 eicosadienoic and  $\omega$ -3 eicosatrienoic acids, but no products were detected with the C18 fatty acid linoleic and  $\alpha$ -linolenic acids or with the C22 fatty acid adrenic and docosapentaenoic acids. When the corresponding *P. patens* genomic sequence was disrupted by replacement through homologous recombination, a dramatic alteration in the fatty acid composition was observed, *i.e.* an increase in di-homo- $\gamma$ -linolenic and eicosatetraenoic acids accompanied by a concomitant disappearance of the  $\Delta^5$ -fatty acid arachidonic and eicosapentaenoic acids. In addition, overexpression of the *P. patens* cDNA in protoplasts isolated from a disrupted line resulted in the restoration of arachidonic acid synthesis.

Polyunsaturated fatty acids (PUFAs),<sup>4</sup> fatty acids containing 18 or more carbon atoms with two or more methylene-interrupted double bonds in the *cis*-position, have received increasing attention in recent years because they are considered to have beneficial effects on human health and development when included in the diet (1, 2). Long-chain PUFAs ( $\geq$ C20) are not commonly found in angiosperms; however, they are present in some gymnosperms (3). High proportions of PUFA accumulation are found in many algae, mosses, and ferns (4–6). The function of these long-chain PUFAs in the membranes of lower plants is still unclear, whereas in humans they play a role in eicosanoid metabolism (7).

The moss *Physcomitrella patens* (Hedw.) B. S. G. (Funariales, Bryophyta) contains high proportions of arachidonic acid (ARA) (20:4  $\Delta^{5,8,11,14}$ ) and some eicosapentaenoic acid (EPA) (20:5  $\Delta^{5,8,11,14,17}$ ) (8). In mammals, ARA (20:4  $\Delta^{5,8,11,14}$ ) and EPA (20:5  $\Delta^{5,8,11,14,17}$ ) are precursors of the short-lived regulatory molecules, the eicosanoids, that comprise the prostaglandins, the leukotrienes, and the thromboxanes (9–11). These compounds act locally through autocrine or paracrine process on G-protein-linked cell surface receptors. This leads to the activation of various signaling mechanisms that have effects on numerous cellular functions including chemotaxis, vascular permeability, inflammation, vasoconstriction, regulation of the immune system, blood clotting, neurotransmission, and cholesterol metabolism (12–14).

ARA (20:4  $\Delta^{5,8,11,14}$ ) and EPA (20:5  $\Delta^{5,8,11,14,17}$ ) are converted from linoleic acid (18:2  $\Delta^{9,12}$ ) and  $\alpha$ -linolenic acid (18:3  $\Delta^{9,12,15}$ ), respectively, by the activities of  $\Delta^6$ -desaturase,  $\Delta^6$ -elongase, and  $\Delta^5$ -desaturase. This reaction series is designated the  $\omega$ -6 pathway for ARA (20:4  $\Delta^{5,8,11,14}$ ) synthesis and the  $\omega$ -3 pathway for EPA (20:5  $\Delta^{5,8,11,14,17}$ ) synthesis (see Fig. 1). The desaturases are thought to function like other microsomal desaturases from higher plants and yeast, catalyzing aerobic reactions requiring cytochrome *b<sub>5</sub>* as a cofactor. Electrons are transferred from NADH-dependent cytochrome *b<sub>5</sub>* reductase via the heme-containing cytochrome *b<sub>5</sub>* molecule to the fatty

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The amino acid sequence of this protein can be accessed through NCBI Protein Database under NCBI accession number CA158861.

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<sup>4</sup> The abbreviations used are: PUFA, polyunsaturated fatty acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; GC, gas chromatography; FAME, fatty acid methyl ester; DHGLA, di-homo- $\gamma$ -linolenic acid; ETA, eicosatetraenoic acid or eicosatrienoic acid.

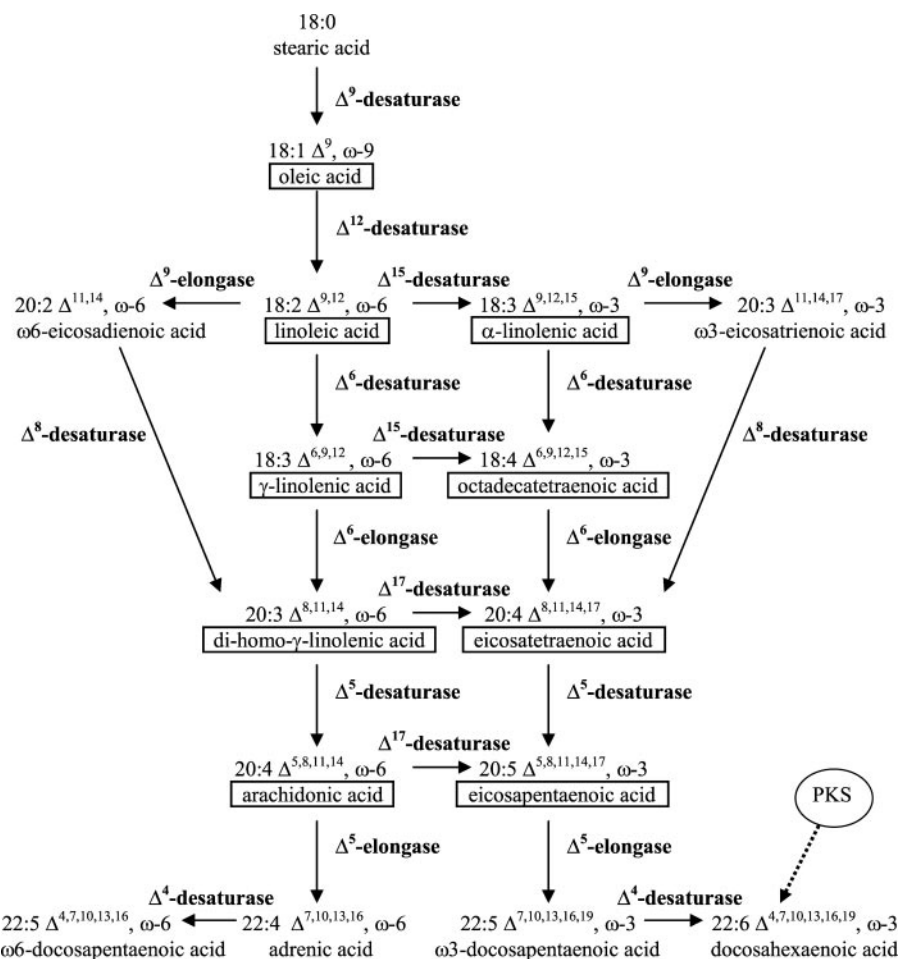


FIGURE 1. Biosynthesis pathway of long-chain PUFAs in lower eukaryotes (17). The common pathway for synthesis of  $\omega$ -6 and  $\omega$ -3 long-chain PUFAs is shown in bold arrows, and the alternative prokaryotic polyketide synthase (PKS) pathway that exists in some organisms is indicated with a dashed arrow. The boxes indicate the fatty acids detected in the moss *P. patens* (8).

acid desaturase (15–18). However, biochemical characterization of membrane-bound desaturases has been limited because they are difficult to purify due to their membrane association. Analysis of the predicted protein sequences for the higher plant desaturases together with those from cyanobacteria, yeast, and mammals revealed the presence of eight highly conserved histidine residues (19).

Because the moss *P. patens* can produce high proportions of ARA (20:4  $\Delta^{5,8,11,14}$ ) and some EPA (20:5  $\Delta^{5,8,11,14,17}$ ) (8), it can serve as a model for studying the mechanism underlying the biosynthesis of PUFAs. Only the genes encoding the  $\Delta^6$ -desaturase and  $\Delta^6$ -elongase have so far been cloned from this organism (20, 21). However, the *P. patens* genes encoding  $\Delta^5$ -desaturase that catalyze the following step in the PUFAs biosynthesis have not yet been functionally identified. This moss can be vegetatively propagated in the haploid state (22, 23), which simplifies the phenotypic analysis after mutation or transformation (24). Genes can be specifically inactivated by targeted gene replacement, which occurs at high frequency in *P. patens* (23, 25). In the present study, a  $\Delta^5$ -desaturase associated with the biosynthesis of ARA (20:4  $\Delta^{5,8,11,14}$ ) and EPA (20:5  $\Delta^{5,8,11,14,17}$ ) in *P. patens* was functionally identified by heterologous expression in *Saccharomyces cerevisiae*, by targeted dis-

ruption of the corresponding gene, and by transient expression in the gene disrupted line.

## EXPERIMENTAL PROCEDURES

**Materials**—Restriction enzymes, polymerases, and DNA-modifying enzymes were obtained from New England Biolabs unless indicated otherwise. All other chemicals used were reagent grade from Sigma. Fatty acids were purchased from Nu-Check-Prep (Elysian, MN).

**Plant Material and Growth Conditions**—The Gransden strain of *P. patens* (22) was used throughout the studies. 7-day-old protonemata were grown on solid BCD medium to which di-ammonium tartrate was added to 5 mM and cultured at 25 °C under continuous light provided by fluorescent tubes (26).

**RNA Isolation and Manipulation**—Total RNA was isolated from 50 mg of fresh weight of protonemal tissue using the RNeasy Plant mini kit (Qiagen), and 5  $\mu$ g was reversed transcribed with the ThermoScript™ reverse transcription PCR system (Invitrogen) according to the manufacturer's instructions. The cDNA was used as a template for PCR amplification with primers.

**Genomic DNA Isolation**—Approximately 1 g of fresh weight of protonemal tissue was ground to a fine powder under liquid nitrogen using a pre-cooled mortar and pestle. Genomic DNA was extracted from the ground tissue using the Nucleon™ PhytoPure™ genomic DNA extraction kit (Amersham Biosciences) according to the manufacturer's instructions. DNA was recovered by ethanol precipitation and dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

**PCR-based Cloning**—Primers were synthesized based on NCBI sequence data (27). The forward primer was PPFOR1, 5'-ATGGCGCCCCACTCTG-3', and the reverse primer was PPREV1, 5'-TCAGCCATCGAGCCGAAACT-3'. The PCR was carried out in a total volume of 50  $\mu$ l. 10  $\mu$ M each of the primers were used for PCR amplification of cDNA reversed transcribed from total RNA. After initial denaturation at 94 °C for 4 min, amplification was performed in 35 cycles of 1 min at 94 °C, 0.5 min at 54 °C, and 2.5 min at 72 °C, followed by a final extension at 72 °C for another 10 min. Amplification products were fractionated on 1.0% agarose gels and directly ligated into pCR®2.1-TOPO® (Invitrogen) and the plasmids used were transformed into One Shot® Chemically competent *Escherichia coli* cells (Invitrogen). Plasmid DNA was purified and

## *Physcomitrella patens*, $\Delta^5$ -Desaturase Gene

sequenced in both directions with an ABI BigDye V3.1 and an automated sequencer, yielding the plasmid PPDES5.

**Functional Analysis: Yeast Transformation**—The open reading frame of the PPDES5 cDNA was cloned next to the *GAL1* galactose-inducible promoter of the yeast expression vector pYES2 (Invitrogen). For this purpose, PCR with the PPFOR1 and PPREV1 primers that add HindIII and XhoI restriction sites at the 5'- and 3'-ends, respectively, was used to amplify the plasmid PPDES5. The amplified PCR product was ligated into pCR<sup>®</sup>2.1-TOPO<sup>®</sup> (Invitrogen) and the plasmids used were transformed into One Shot<sup>®</sup> Chemically competent *E. coli* cells (Invitrogen) again. The entire open reading frame of the desaturase was digested with HindIII/XhoI and ligated into HindIII/XhoI sites of the pYES2 vector (Invitrogen) to yield plasmid pYES2-DES5. Its sequence was verified by DNA sequencing. The plasmids pYES2-DES5 and pYES2 were transformed into the *S. cerevisiae* YPH499 (ATCC, Manassas, VA) by *S.c.* Easy-Comp<sup>™</sup> transformation kit (Invitrogen). After uracil selection on minimal medium agar plates, cells containing the yeast plasmids were cultivated in complete supplement mixture dropout uracil medium (CSM-URA) (Krackeler Scientific) containing 1.0% (w/v) raffinose as the exclusive carbon source and 0.4% Tergitol Nonidet P-40 (Sigma) for the solubilization of fatty acids.

For gene expression experiments, the cultures were grown to an optical density (600 nm) of  $\sim 0.3$  in CSM-URA medium (Krackeler Scientific). Expression of the PPDES5 coding region was induced by the addition of galactose to 2.0% (w/v). Fatty acids were added to 0.7 mM and cultures grown for 24 h at 28 °C.

**Targeted Gene Disruption of PPDES5 in *P. patens***—For disruption of PPDES5 by gene targeting, the coding sequence of a genomic clone of PPDES5 was replaced by a <sup>35</sup>S-*hph-nos* terminator, hygromycin-resistance cassette (23), so that the selection cassette was flanked by 1536 and 1234 base pairs (bp) genomic locus homologous to 5'- and 3'-arms of PPDES5, respectively (see Fig. 2). Two specific primer pairs (Primer 1, 5'-TAGGGC-CACACCTGTTGCTTCG-3', and Primer 2, 5'-ATGGGC-CCCCAGTTGCTTCGTCGCCAG-3'; Primer 3, 5'-ATGTC-GACCAGGCAAGTAAATAAGTGA-3', and Primer 4, 5'-ATATGCATGAACGAAAGTAGTCCTGTC-3') were synthesized based on the corresponding *P. patens* genome sequence from the U.S. Department of Energy Joint Genome Institute data base (see Fig. 2). After PCR amplification with these primers from a wild-type genomic DNA template, the PCR fragments of the expected length (1536 and 1234 bp) were separately cloned into pGEM<sup>®</sup>-TEasy vector (Promega), released by digestion with ApaI and SalI/NsiI, respectively and then ligated into rr1 vector containing hygromycin-resistance cassette. Subsequently, the disruption construct was digested with XhoI/HindIII, resulting in a linear fragment with the cassette in its center flanked by genomic sequences of 1252 and 554 bp. This linear DNA was precipitated and used for the transformation without separation from the vector. Polyethylene glycol-mediated direct DNA transfer into protoplasts was performed as described by Schaefer *et al.* (24) with modifications. The protonemata were regenerated for 7 days on solid medium containing hygromycin (25  $\mu$ g/ml), transferred to medium without antibiotic for 7 days, and retransferred to selective medium for

a further 7 days. Strongly growing plants that survived this selection regimen were defined as stable transformants.

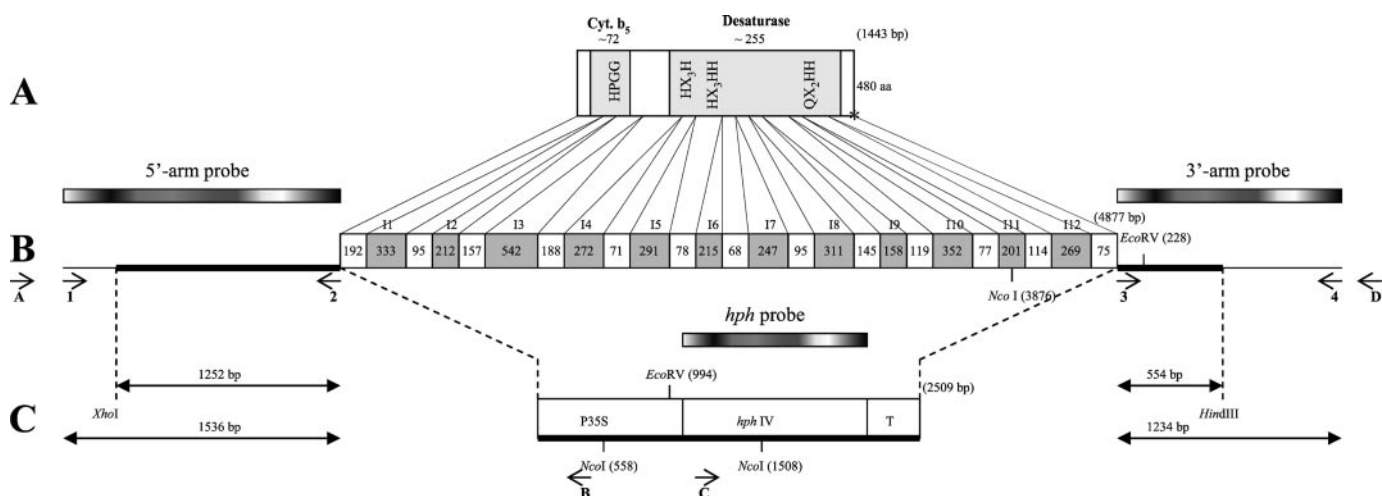
**Molecular Analysis**—Targeting events were analyzed by PCR and Southern blotting. For this purpose, genomic DNA of wild-type and transgenic plants was extracted and the 5'-integration event was confirmed by PCR experiments with primer A (5'-GACCTACCGAACTTTTCGA-3') corresponding to genomic sequence of *P. patens* but 164 bp upstream of the 5'-end of replacement vector and primer B (5'-ACCATCTGTGGGT-TAGCGTCC-3') derived from the <sup>35</sup>S promoter region of the selection cassette. The 3'-integration event was also confirmed by PCR with primer C (5'-ATGAAAAAGCCTGAACTACCG-3') derived from the 5'-end of the *hph* coding region and primer D (5'-GAACACTCAACTGTAGTAGC-3') corresponding to genomic sequence of *P. patens* but 99 bp downstream of the 3'-end of the selection cassette (see Fig. 2 for location of primers). PCRs were carried out in a total volume of 50  $\mu$ l. Each reaction contained 0.2  $\mu$ M each of the primers, 200  $\mu$ M each of the dNTPs, PCR buffer, and 2 ml of Elongase<sup>®</sup> enzyme mix (Invitrogen). The thermocycling conditions were as follows: initial denaturation for 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 0.5 min at 50 °C, 5 min at 72 °C, and terminated by a 10-min final extension at 72 °C.

For Southern blot analysis, 1- $\mu$ g aliquots of genomic DNA from wild-type and transgenic plants were digested with the appropriate restriction enzymes and separated on a 0.6% agarose gel electrophoresis. The final washing steps were performed in 0.1 $\times$  SSC with 0.1% SDS at 65 °C. Detection was accomplished with a chemiluminescent substrate (CSPD; Roche Applied Science).

**Transient Gene Expression of PPDES5**—The primers with PPFOR2, 5'-CACCATGGCGCCCCACTCTG-3', and PPREV2, 5'-TCAAGACCAGCCGCTCGCATCTTTCCAAGAGCCAT-CGAGCCGAAACT-3', were used for PCR amplification of pYES2-DES5 with KlentaqLA.<sup>5</sup> PCRs were carried out in a total volume of 50  $\mu$ l. Each reaction contains 0.4  $\mu$ M each of primers, 100  $\mu$ M each of dNTPs, PCR buffer, 1.5 mM MgCl<sub>2</sub>, and 0.5  $\mu$ l of KlentaqLA.<sup>5</sup> The thermocycling conditions were as follows: initial denaturation for 4 min at 94 °C followed by 28 cycles of 1 min at 94 °C, 0.5 min at 54 °C, 2.5 min at 72 °C, and terminated by a 10-min final extension at 72 °C. Amplification products were incorporated directly into pENTR<sup>™</sup>/D-TOPO<sup>®</sup> vector (Invitrogen). The resulting recombination plasmid has the PPDES5 sequence flanked by *attL* recombination sequences. It was then recombined with *attR* sites using the Gateway<sup>®</sup> LR Clonase<sup>™</sup> II Enzyme Mix (Invitrogen). This reaction transferred the PPDES5 sequence into a desired destination vector (tk1). Destination vector containing a gene, PPDES5, driven by the <sup>35</sup>S promoter, was transformed into protoplasts of the targeted strain (24). The regenerated protoplasts were cultured in liquid BCD medium supplemented with 5 mM di-ammonium tartrate and 6% mannitol for 2 days before fatty acid analysis.

**Fatty Acid Analysis**—Total fatty acids extracted from yeast and moss cultures were analyzed by gas chromatography (GC) of methyl esters. Lipids from yeast and moss tissue were trans-

<sup>5</sup> Patented by W. Barnes.



**FIGURE 2. Structure of the desaturase cDNA (A), the desaturase gene flanked by two arm-genomic sequences (B), and the fragment used for gene disruption (C).** A, PPDES5 cDNA (1443 bp) and schematic representation of the protein PPDES5 (480 amino acids) with two domains, a cytochrome  $b_5$ - and a desaturase-like region with typical amino acid blocks (light gray). The stop codon is symbolized by a star. B, corresponding genomic sequence PPDES5 (4877 bp) flanked by two arm genomic sequences (1536 and 1234 bp). The position and lengths of the twelve introns (i1-i12, gray shade) are marked. C, replacement fragment for gene disruption. The whole desaturase gene was substituted by the *hph* cartridge, which contained the *hph* coding region between the  $^{35S}$  promoter (*P35S*) and terminator (*T*). The transformation was carried out after linearization with *Xho*I and *Hind*III resulting in a linear fragment (underlined in bold) with the *hph* cartridge replacing the desaturase gene. The numbered and lettered arrows below the blocks indicate the binding sites of primers used for construction of the replacement vector and subsequent PCR analyzes, respectively. The localization of both 5'- and 3'-arm probes or *hph* probe and the restriction enzyme sites used for Southern blotting are marked with the gradient-gray blocks above the sequences and indicated in the parentheses, respectively (see Fig. 8).

methylated with 1% sodium methoxide in methanol and 2.5% sulfuric acid in methanol, respectively, at 85 °C for 30 min. Fatty acid methyl esters (FAMES) were then extracted in heptane. GC analysis of FAMES was conducted using an Agilent 6890N Series gas chromatograph equipped with a 0.25 mm × 30 m × 0.25 mm HP-INNOWax capillary column and a flame ionization detector. Fatty acids were identified by comparison with retention times of standards. Relative percentages of the fatty acids were estimated from peak areas. The corresponding fatty acids were further characterized on diethylamide derivative by gas chromatography-mass spectrometry using the Agilent 6890N Series operating at an ionization voltage of 70 eV with a scan range of 50–500 Da.

## RESULTS

**Cloning of a *P. patens* Membrane-bound Desaturase**—The moss *P. patens* has received attention because of its ability to produce several PUFAs, including ARA (20:4  $\Delta^{5,8,11,14}$ ) and EPA (20:5  $\Delta^{5,8,11,14,17}$ ) (8). We were therefore interested in the molecular information underlying the biosynthesis of these fatty acids. To identify a gene coding for the desaturase involved in the final step of ARA (20:4  $\Delta^{5,8,11,14}$ ) and EPA (20:5  $\Delta^{5,8,11,14,17}$ ) biosynthesis, NCBI data base searches in *P. patens* were conducted and the sequence identified based on its limited identity with other  $\Delta^5$ -desaturases was used (27). cDNA from reverse-transcribed mRNA from 7-day-old protonemal tissue of *P. patens* was amplified by PCR. An amplification product containing the expected length was cloned and sequenced.

The open reading frame of the PPDES5 cDNA is 1443 bp from an ATG start to a TGA stop codon and codes for 480 amino acids with a molecular mass of 54.3 kDa. Data bank searches and alignments with this sequence indicated similarity

with acyl-lipid desaturases from residue 190–454, with a cytochrome  $b_5$ -like domain in the N terminus from residue 36–107.

Amino acid sequence comparison using the ClustalW program revealed the PPDES5 has the strongest homology to  $\Delta^5$ -desaturase from the liverwort *Marchantia polymorpha* (66% identity) (28) and also shares 36% identity with  $\Delta^5$ -desaturase from *Dictyostelium discoideum* (29, 30) and *Mortierella alpina* (31), which is required for the desaturation of PUFAs. Alignment with those sequences indicates that homology occurs mainly in the cytochrome  $b_5$ -like domain that serves as an electron donor (boxed with broken lines in Fig. 3) and in the three conserved histidine-rich motif areas (boxed in Fig. 3). The cytochrome  $b_5$ -related domain contained the eight invariant residues typical for the cytochrome  $b_5$  superfamily (marked with asterisks in Fig. 3) and the presence of a heme binding region characterized by the HPGG motif toward the N terminus (32). A heme-containing electron donor is required for fatty acid desaturation, and cytochrome  $b_5$  fulfills this function for the membrane-bound desaturase (16, 33, 34). Therefore, the whole sequence can be considered as coding for a fusion protein consisting of an N-terminal cytochrome  $b_5$  and a desaturase. The sequence motif QIEHH of the third histidine box starts with a glutamine instead of a histidine (marked with triangle in Fig. 3), which is the characteristic of the front-end desaturases but is not found in other desaturases such as  $\Delta^{12}$ - and  $\Delta^{15}$ -desaturases (35–37).

**Functional Analysis of PPDES5 in Yeast**—Fig. 4 shows the results of GC analysis of the fatty acid methyl esters of yeast strains, transformed with the PPDES5 expression construct as described under “Experimental Procedures”, to which fatty acid substrates had been supplied. An additional peak is apparent in the trace obtained from induced pYES2-DES5 grown in the presence of di-homo- $\gamma$ -linolenic acid (DHGLA) (20:3  $\Delta^{8,11,14}$ )

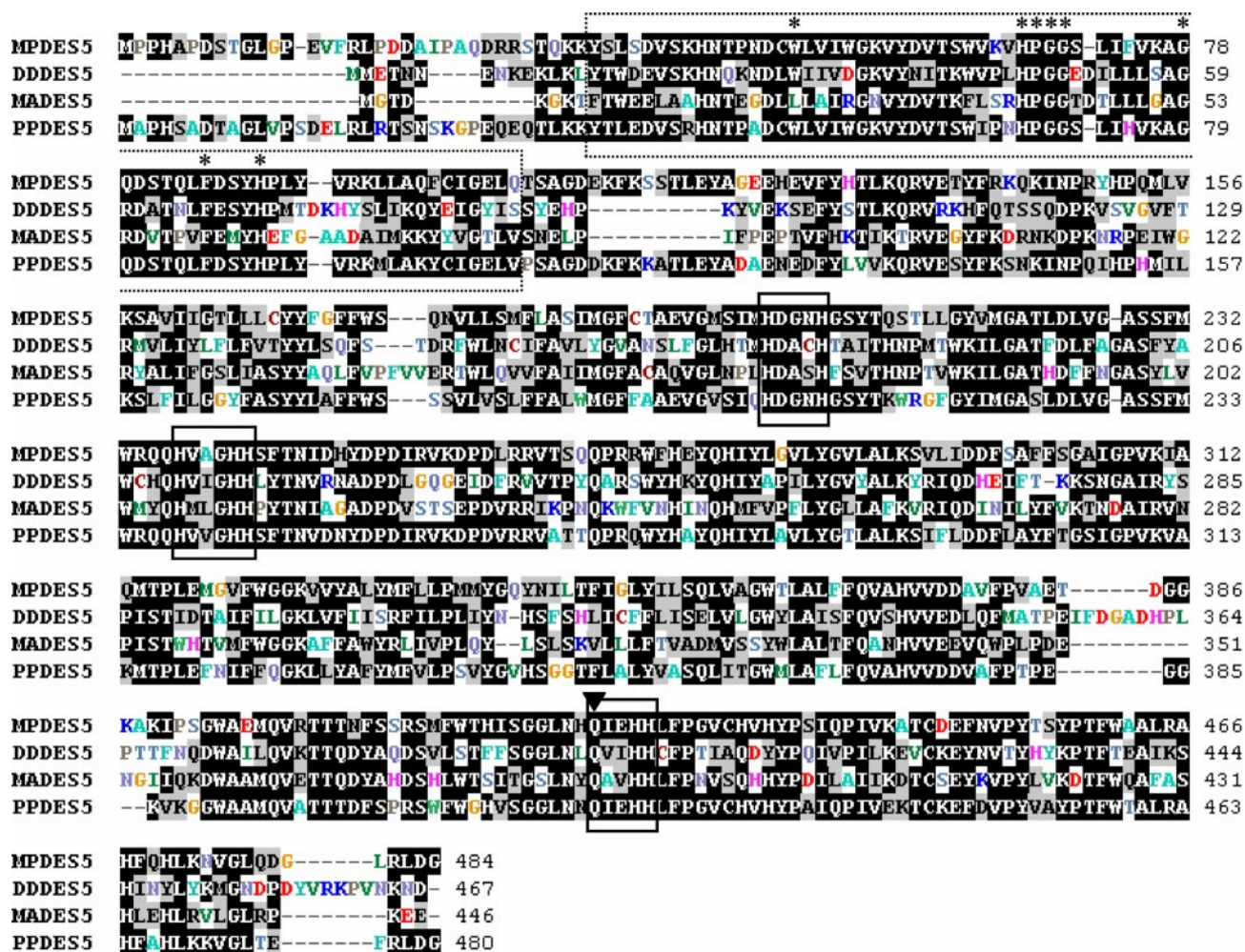


FIGURE 3. Alignment of amino acid sequences of PPDES5 and closely related proteins. The translation of the coding sequence of *P. patens*  $\Delta^5$ -desaturase is aligned with *M. polymorpha*  $\Delta^5$ -desaturase (MPDES5, NCBI accession number AAT85663), *Dictyostelium discoideum*  $\Delta^5$ -desaturase (DDES5, NCBI accession number BAA37090), and *Mortierella alpina*  $\Delta^5$ -desaturase (MADES5, NCBI accession number AAC39508). Identical or conserved residues are shaded. The cytochrome  $b_5$  domain is boxed with broken lines, and the eight amino acid residues that are highly conserved in the cytochrome  $b_5$  superfamily are marked with asterisks. Three histidine motifs are boxed. The conserved glutamine residue in the third histidine motif is marked with a triangle.

compared with an empty vector control. The retention time of the additional peak was identical to that of the methyl ester of authentic ARA (20:4  $\Delta^{5,8,11,14}$ ). This compound also displayed a molecular ion of 318  $m/z$  (the expected molecular ion for methyl ARA) as well as a fragmentation pattern identical to that of the authentic ARA methyl ester. The identity of this compound was further verified by obtaining the diethylamide derivative in order to produce a structure-specific mass spectrum. As shown in Fig. 5, the mass spectrum of the derivatized compound contained a molecular ion of 359  $m/z$  and a fragmentation profile consistent with the ARA diethylamide derivative. Yeast cells transformed with the plasmid pYES2-DES5 had therefore acquired functional specific  $\Delta^5$ -desaturase activity and were now capable of synthesizing ARA (20:4  $\Delta^{5,8,11,14}$ ) from the substrate DHGLA (20:3  $\Delta^{8,11,14}$ ). The specific  $\Delta^5$ -desaturase in the transformed yeast appeared to be an efficient catalyst, with 14.7% of the substrate converted to ARA under the conditions of the experiments.

To test whether the *P. patens*  $\Delta^5$ -desaturase was capable of desaturating other substrates, yeast cultures expressing PPDES5 were supplemented with several fatty acids (Table 1).

We were unable to detect any novel peaks when C18 and C22 fatty acids were fed to yeast; however, when 20:2  $\Delta^{11,14}$  and 20:3  $\Delta^{11,14,17}$  were provided as substrates, we detected novel peaks with mass spectra consistent with those of methyl 20:3  $\Delta^{5,11,14}$  (13.5%) and 20:4  $\Delta^{5,11,14,17}$  (14.5%), respectively (data not shown). Therefore, *P. patens*  $\Delta^5$ -desaturase appears to be specific for C20 fatty acids. In addition, we could exclude  $\Delta^8$ -desaturase activity because we could not detect any 20:3  $\Delta^{8,11,14}$  or 20:4  $\Delta^{8,11,14,17}$  from 20:2  $\Delta^{11,14}$  and 20:3  $\Delta^{11,14,17}$  substrates, respectively.

**Fatty Acid Profile in *P. patens* Wild Type**—Several  $\omega$ -6 and  $\omega$ -3 fatty acids were detected in wild-type tissue, including saturated, mono-, and polyunsaturated C16–20 fatty acids (Fig. 6). The principle fatty acid detected was ARA (20:4  $\Delta^{5,8,11,14}$ ), which is the  $\Delta^5$ -desaturation product from DHGLA (20:3  $\Delta^{8,11,14}$ ) in  $\omega$ -6 pathway (see Fig. 1). However, from our experiments we could detect very little  $\omega$ -3 eicosatetraenoic acid (ETA) (20:4  $\Delta^{8,11,14,17}$ ) despite the presence of its  $\Delta^5$ -desaturation product, EPA (20:5  $\Delta^{5,8,11,14,17}$ ).

**Targeted Gene Replacement**—For an alternative proof of the function of PPDES5, we analyzed the total fatty acids of wild

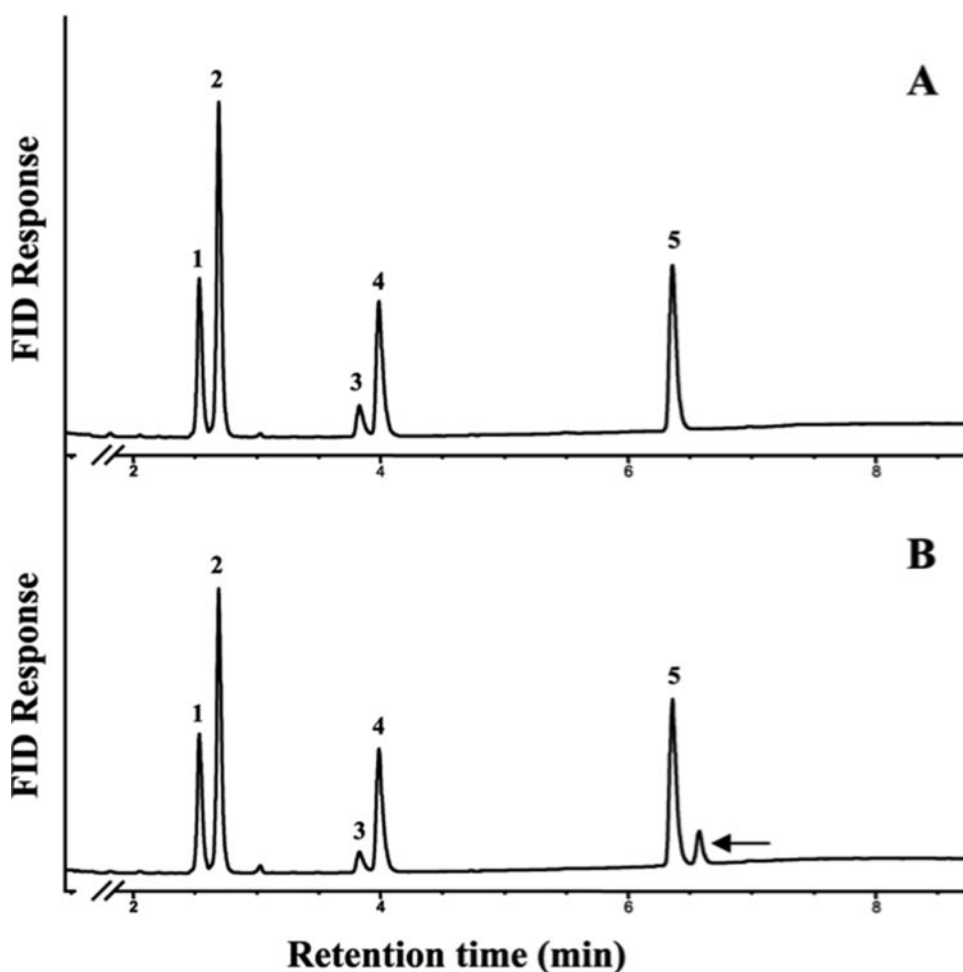


FIGURE 4. Identification of ARA (20:4  $\Delta^{5,8,11,14}$ ) synthesized in transgenic yeast by GC. The FAMES of total lipids of *S. cerevisiae* grown under inducing conditions in the presence of exogenously supplied DHGLA (20:3  $\Delta^{8,11,14}$ ) were analyzed by GC, using flame ionization detection. A, FAMES extracted from yeast transformed with control (empty) vector pYES2. B, FAMES extracted from yeast transformed with pYES2-DES5. The common peaks were identified as C16:0 (peak 1), C16:1 (peak 2), C18:0 (peak 3), C18:1 (peak 4), and C20:3 (peak 5 supplied exogenously). The additional peak (in panel B), which corresponds to the retention time of ARA (20:4  $\Delta^{5,8,11,14}$ ), is indicated by the arrow.

type and the three stable transgenic lines (K1–3) having the PPDES5 coding sequence replaced by a selection cassette (Fig. 6). In contrast to the wild type, the transgenic lines lacked the unsaturated fatty acids ARA (20:4  $\Delta^{5,8,11,14}$ ) and EPA (20:5  $\Delta^{5,8,11,14,17}$ ), products of the  $\Delta^5$ -desaturation step, and a clear increase in the level of its substrate DHGLA (20:3  $\Delta^{8,11,14}$ ) and ETA (20:4  $\Delta^{8,11,14,17}$ ), respectively.

**Molecular Analysis of the Transgenic Lines**—The specific integration of the transformed DNA into the PPDES5 gene was analyzed by PCR using genomic DNA from the three transgenic lines (K1–3) and the wild type. The locations of the different primers are presented in Fig. 2. The 5'-end of primer A and 3'-end of primer D bind 164 bp upstream and 99 bp downstream of the 5'- and 3'-genomic sequences flanking the selection cassette, thus excluding PCR signals resulting from contamination by DNA used for transformation.

PCR with primer pair A/B amplified a fragment of 2.0 kilobase pairs (kbp) and with primer pair C/D, a fragment of 2.5 kbp, from each of the three transgenic lines (K1–3), whereas experiments with the wild type gave negative results (Fig. 7).

The length of the bands agreed with successful targeted replacement of the PPDES5 gene.

To provide further evidence for targeted replacement of the PPDES5 gene in transgenic lines, the genomic DNA of a transgenic line (K2) and the wild type was analyzed by Southern blot hybridization with DIG-labeled 5'- plus 3'-arm probes or *hph* probe (Fig. 8). The 5'- plus 3'-arm probes hybridize with two fragments of EcoRV-digested genomic DNA from the wild type (*a* and *b*), and with two fragments digested with NcoI (*e* and *f*). Blotting of DNA from the transgenic line digested with EcoRV or NcoI generated strong signals of 6.7 (\*) and 7.5 (\*\*) kbp, respectively, consistent with the size of the targeting vector used for gene disruption (Figs. 2C and 8A). Because NcoI can cut within the resistance cassette, only the strong signal of 7.5 kbp (\*\*) was detected by *hph* probe (Fig. 8B).

In addition, hybridization of DNA from the transgenic line (K2) digested with EcoRV with the 5'- plus 3'-arm probes detected a fragment of *b* similar to that from the wild type but also detected two additional signals of *c* and *d*. With the *hph* probe only a weak signal of *d* was detected. Similarly, digestion with NcoI and hybridization with 5'- plus 3'-arm probes produced an *f'* signal similar in size to that from wild-type DNA. Additional signals were detected with 5'- plus 3'-arm probes (*g*) and *hph* probe (*h*). These results confirm that the K2 transgenic line has multiple copies of the targeting construct inserted at the PPDES5 genomic locus, but no insertion at ectopic sites.

**Transient Gene Expression of PPDES5**—Gateway-compatible plant destination vectors were used for protein overexpression of PPDES5 in the targeted strain (K2). In contrast to the control (K2), 2 days after protoplast transformation of the targeted strain with the PPDES5 expression construct (K2-PPDES5), regenerating plants were capable of synthesizing ARA (20:4  $\Delta^{5,8,11,14}$ ) (8.7%) but not EPA (20:5  $\Delta^{5,8,11,14,17}$ ) (Fig. 9).

## DISCUSSION

A cDNA isolated from *P. patens* has been characterized and found to encode a  $\Delta^5$ -desaturase protein with three histidine box motifs indicative of a microsomal fatty acid desaturase (19). The deduced protein sequence also contained the diagnostic features of heme-binding cytochrome *b<sub>5</sub>* domain located toward the N terminus. It suggests that the localization of this

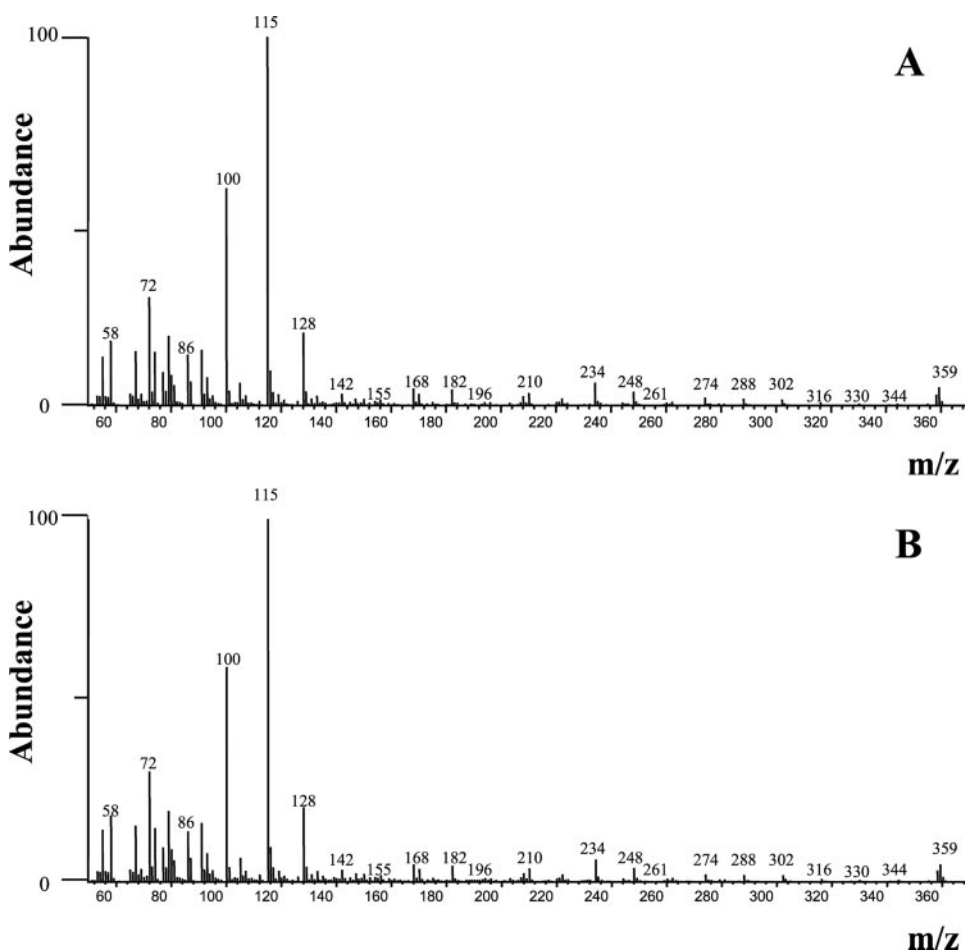


FIGURE 5. GC mass spectrometry analysis of diethylamide derivative of the novel peak identified in yeast carrying pYES2-DESS in the presence of DHGLA (20:3  $\Delta^{8,11,14}$ ). A comparison is shown of the mass spectra of the novel peak (A) and an authentic ARA (20:4  $\Delta^{5,8,11,14}$ ) standard (B).

**TABLE 1**  
Fatty acid substrate specificity of *P. patens*  $\Delta^5$ -desaturase (PPDES5)

Yeast cells transformed with pYES2-DESS were supplemented with different fatty acids. The FAMES of total lipids under inducing conditions were analyzed by GC, using flame ionization detection. Conversion was calculated as (product  $\times$  100)/(substrate + product) using values corresponding to the peak areas of the corresponding signals. Each value is the mean  $\pm$  S.D. from three independent experiments.

Fatty acid substrates	Percentage of substrate converted
18:2 ( $\Delta^{9,12}$ ), $\omega$ -6 linoleic acid	0.0 $\pm$ 0.0
18:3 ( $\Delta^{9,12,15}$ ), $\omega$ -3 $\alpha$ -linoleic acid	0.0 $\pm$ 0.0
20:2 ( $\Delta^{11,14}$ ), $\omega$ -6 eicosadienoic acid	13.5 $\pm$ 0.3
20:3 ( $\Delta^{8,11,14}$ ), $\omega$ -6 DHGLA	14.7 $\pm$ 0.5
20:3 ( $\Delta^{11,14,17}$ ), $\omega$ -3 ETA	14.5 $\pm$ 0.3
22:4 ( $\Delta^{7,10,13,16}$ ), $\omega$ -6 adrenic acid	0.0 $\pm$ 0.0
22:5 ( $\Delta^{7,10,13,16,19}$ ), $\omega$ -3 docosapentaenoic acid	0.0 $\pm$ 0.0

desaturase is in microsomes rather than in chloroplasts, because plastidial desaturases normally use ferredoxin as electron donor (38). This  $\Delta^5$ -desaturase may exist as a cytochrome  $b_5$  fusion protein. The fused N-terminal cytochrome  $b_5$  domain is essential for activity as demonstrated by site-directed mutagenesis studies in borage (*Borago officinalis*). Loss of the histidine residue of the HPGG conserved heme-binding domain of the cytochrome  $b_5$  region results in complete loss of enzyme activity (39). N-terminal cytochrome  $b_5$  domains have been identified in a number of other fatty acid desaturases (20,

28, 31, 40, 41). This may indicate that the N-terminal cytochrome  $b_5$  domain is restricted to the front-end class of the fatty acid desaturases. On the other hand, in yeast a similar cytochrome  $b_5$  domain was recognized in the  $\Delta^9$ -acyl-CoA desaturase (OLE1), but in this enzyme it was located at the C terminus. However, when the cytochrome  $b_5$  region was deleted from this gene, the yeast cells remained fatty acid auxotrophs, even in the presence of the endogenous yeast cytochrome  $b_5$  (33, 42). This suggests that the cytochrome  $b_5$  domain plays an essential role in the desaturation reaction. Whether the cytochrome  $b_5$ -desaturase fusion proteins are more efficient awaits further assessment. Another characteristic of the front-end desaturases is the substitution of histidine by glutamine in the third histidine box (35, 36). The most closely related protein sequence of PPDES5 identified was that of the liverwort *M. polymorpha*  $\Delta^5$ -desaturase involved in ARA (20:4  $\Delta^{5,8,11,14}$ ) and EPA (20:5  $\Delta^{5,8,11,14,17}$ ) formation (28), with which the fungal  $\Delta^5$ -desaturase showed 36% identity (29–31).

For functional characterization of the desaturase and confirmation of its positional and substrate specificity, heterologous expression of PPDES5 in *S. cerevisiae* was performed (Fig. 4). In contrast to other described  $\Delta^5$ -desaturases, PPDES5 did not desaturate any of the endogenous yeast fatty acids 16:0, 16:1, 18:0, 18:1 (Fig. 4). When exogenous substrates with different chain lengths and levels of unsaturation were added to the culture medium, only three were accepted as substrates for  $\Delta^5$ -desaturation, namely  $\omega$ -6 eicosadienoic acid (20:2  $\Delta^{11,14}$ ),  $\omega$ -6 DHGLA (20:3  $\Delta^{8,11,14}$ ), and  $\omega$ -3 ETA (20:3  $\Delta^{11,14,17}$ ), confirming that the PPDES5 clone encodes a  $\Delta^5$ -desaturase gene that is specific for C20 fatty acids without  $\omega$ -3 or  $\omega$ -6 preference. However, the enzyme from *Phaeodactylum tricornutum* (PtD5p) and all other  $\Delta^5$ -desaturases described so far do not have a specificity toward C20 fatty acids alone but also accept mono- and polyunsaturated C16 or C18 fatty acids (29, 30, 43). For PtD5p it has been shown that it also can desaturate 18:1  $\Delta^{11}$ , but only at the very low rate of 2% compared with the 25% conversion rate for DHGLA (20:3  $\Delta^{8,11,14}$ ) (43). In contrast, *D. discoideum*  $\Delta^5$ -desaturase genes are not involved in long-chain PUFA metabolism, because they can add a double bond at the  $\Delta^5$  position of C16 and C18 monoenoic acids only (29, 30). Recently a  $\Delta^5$ -desaturase from *Phytophthora megasperma* was described that specifically introduces an additional double bond only into C20 fatty acids

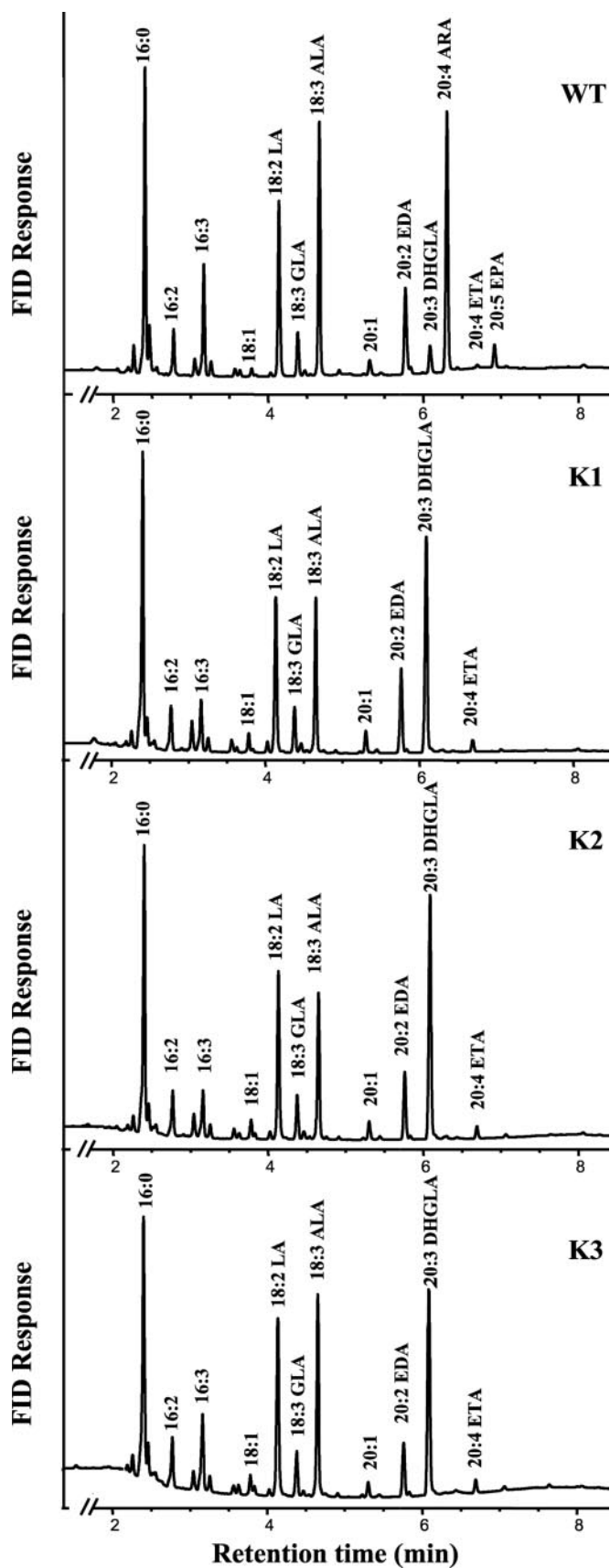


FIGURE 6. Fatty acid profiles of the *P. patens* wild type (WT) and the transgenic lines (K1–3). The FAMES of the total lipids were analyzed by GC. The chromatograms WT and K1–3 show the FAMES of protonemata grown on solid medium for 14 days.

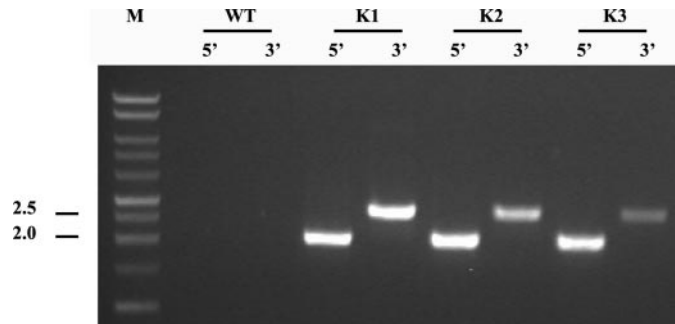


FIGURE 7. Verification of gene disruption by PCR amplification of the *P. patens* wild type (WT) and the transgenic lines (K1–3). The DNA sizes in kbp are indicated on the left.

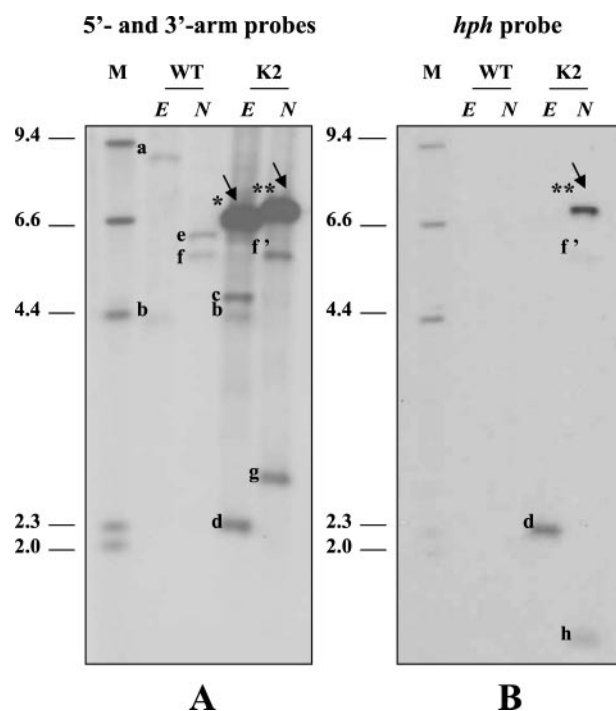


FIGURE 8. Southern blotting of *P. patens* wild type (WT) and the transgenic line (K2). Genomic DNA (1  $\mu$ g) from wild type and transgenic line (K2) was digested with EcoRV and NcoI and hybridized with the mixture of 5'- and 3'-arm probes (A) and *hph* probe (B). The locations of the probes are described in Fig. 2. DNA sizes in kbp are indicated on the left. Restriction digestion: E, EcoRV, and N, NcoI. The letters indicate signals detected. Arrows marked with asterisks indicate the replacement vector detected.

containing a double bond at position  $\Delta^8$  (44). On the other hand, in meadowfoam (*Limnanthes douglasii*), a gene coding for  $\Delta^5$ -desaturase of another type has been identified (45). This enzyme catalyzes  $\Delta^5$ -desaturation of primarily saturated C16–20 fatty acids instead of trienoic and tetraenoic C20 fatty acids, such as DHGLA (20:3  $\Delta^{8,11,14}$ ) and ETA (20:4  $\Delta^{8,11,14,17}$ ), and is structurally unrelated to the cytochrome  $b_5$  fusion type  $\Delta^5$ -desaturases.

In this study, we have described the efficient inactivation of the PPDES5 gene using targeted gene replacement. PCR and Southern blot experiments show that the PPDES5 coding sequence is replaced by the hygromycin selection cassette in the targeted transgenic lines. This result provides further evidence of the value of targeted gene replacement in *P. patens* to analyze gene functions include fatty acid biosynthesis (20–21), protein

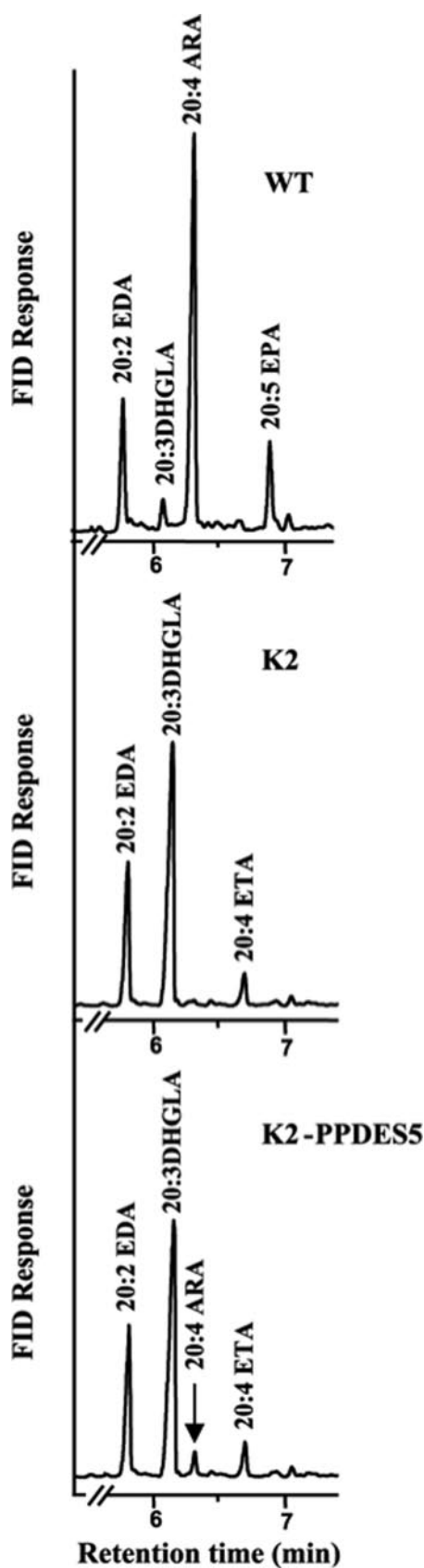


FIGURE 9. Fatty acid profiles of the *P. patens* wild type (WT), the targeted strain (K2) controls, and the targeted strain transformed by the PPDES5 expression construct (K2-PPDES5). The FAMES of the total lipids were analyzed by GC. The chromatograms WT, K2, and K2-PPDES5 show the FAMES of protoplasts regenerated in liquid medium for 2 days.

degradation (46), photomorphology (47), and sulfate assimilation (48). Disruption of the PPDES5 gene resulted in a dramatic alteration of the fatty acid pattern, with increased levels of the DHGLA (20:3  $\Delta^{8,11,14}$ ) and ETA (20:4  $\Delta^{8,11,14,17}$ ) substrates and an absence of ARA (20:4  $\Delta^{5,8,11,14}$ ) or EPA (20:5  $\Delta^{5,8,11,14,17}$ ), the products of  $\Delta^5$ -desaturase gene, confirming that PPDES5 codes for  $\Delta^5$ -desaturase. Overexpression of PPDES5 from a strong  $^{35}\text{S}$  promoter in the targeted line confirmed the  $\Delta^5$ -desaturase activity specific for C20 fatty acids. However, we could not detect EPA (20:5  $\Delta^{5,8,11,14,17}$ ) in protoplasts of the targeted strain, and so the absence of an  $\omega$ -3 product in the overexpression transgenics is likely explained by the low level of substrate present in *P. patens*.

We saw no major morphological changes in the phenotype of the disrupted lines. Regenerated protoplasts gave rise to normal filamentous growth and gametophore formation. However, changes in response to different stress conditions are now being tested in the disrupted lines compared with controls in order to determine any possible function(s) of the C20 PUFAs.

Currently, considerable effort is focusing on the combined expression in plants of the  $\Delta^6$ -desaturase with the  $\Delta^6$ -elongase and the  $\Delta^5$ -desaturase. Publication of these data is eagerly awaited. However, because the "reverse engineering" of any PUFA biosynthetic pathway requires the transgenic mobilization of multiple different enzyme activities, this will require the heterologous expression of a minimum of three transgenes (10). Therefore, this study of  $\Delta^5$ -desaturase in *P. patens*, which is an essential enzyme in the production of eicosanoid precursors, provides further evidence of important biotechnological applications involving the engineering of new plant oils to provide an economical alternative to naturally occurring oil and help the increasing demands of the chemical, pharmaceutical, and nutraceutical industry for therapeutic and prophylactic use (17, 49).

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*Addendum*—Previous work on this topic was reported by Sperling, P., Lucht, J. M., Tegener, T., Reski, R., Cirpus, P., and Heinz, E. (2003) in *Advanced Research on Plant Lipids* (Murata, N., Yamada, M., Nishida, I., Okuyama, H., Sekiya, J., and Hajime, W., eds) pp. 113–116, Kluwer Academic Publishers, Dordrecht.

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