Identification of a novel Δ6-acyl-group desaturase by
targeted gene disruption in Physcomitrella patens

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Summary

The moss Physcomitrella patens contains high levels of
arachidonic acid. For its synthesis from linoleic acid by
desaturation and elongation, novel Δ5- and Δ6-desaturases
are required. To isolate one of these, PCR-based cloning
was used, and resulted in the isolation of a full-length
cDNA coding for a putatively new desaturase. The deduced
amino acid sequence has three domains: a N-terminal
segment of about 100 amino acids, with no similarity to
any sequence in the data banks, followed by a cytochrome
b5-related region and a C-terminal sequence with low
similarity (27% identity) to acyl-lipid desaturases. To
elucidate the function of this protein, we disrupted its
gene by transforming P. patens with the corresponding
linear genomic sequence, into which a positive selection
marker had been inserted. The molecular analysis of five
transformed lines showed that the selection cartridge had
been inserted into the corresponding genomic locus of all
five lines. The gene disruption resulted in a dramatic
alteration of the fatty acid pattern in the knockout plants.
The large increase in linoleic acid and the concomitant
disappearance of γ-linolenic and arachidonic acid in all
knockout lines suggested that the new cDNA coded for a
Δ6-desaturase. This was confirmed by expression of the
cDNA in yeast and analysis of the resultant fatty acids by
GC-MS. Only the transformed yeast cells were able to
introduce a further double bond into the Δ6-position of
unsaturated fatty acids. To our knowledge, this is the first
report of a successful gene disruption in a multicellular
plant resulting in a specific biochemical phenotype.

Introduction

Compared to higher plants, many members of moss, algae
and fern families produce a wider variety of polyunsatur-
ated fatty acids (PUFA; Dembitsky, 1993; Jamieson and
Reid, 1975; Zhukova and Aizdaicher, 1995), and PUFA such
as arachidonic acid (AA) and eicosapentaenoic acid (EPA)
are produced only by lower plants. The function of these
long-chain PUFA in the membranes of lower plants is
still unclear, whereas in humans, they play a key role in
eicosanoid metabolism (Samuelsson, 1983).

The biosynthesis of AA and EPA generally starts with
linoleic acid (18:2), which is channelled into a widely
branching network of desaturation and elongation steps
(Arao and Yamada, 1994; Cohen et al., 1995; Shiran et al.,
1996). Key enzymes in this network are Δ5- and Δ6-desatur-
ases, which introduce the new double bond between the
first double bond and the carboxyl terminus of the fatty
acid, known as acyl-cleaved-directed desaturation. This mode
differs from the methyl-directed desaturation, which works
towards the methyl end of the unsaturated fatty acid.
Desaturases of both types belong to the membrane-bound
desaturases, which operate in microsomes or in plastids
(Heinz, 1993). All desaturases, including acyl-ACP,
(Ohlrogge et al., 1993), acyl-CoA (Enoch et al., 1976) and
acyl-lipid desaturases, are believed to catalyse an O2-
dependent reaction, in which either cytochrome b5 serves
as electron donor for the microsomal or ferredoxin for the
plastidial desaturases (Kearns et al., 1991; Schmidt and
Heinz, 1990; Smith et al., 1990).

In the last few years, extensive sequence information
from various desaturases in the methyl-directed group has
been accumulated, but only a few from the carboxyl-
directed group (Reddy et al., 1993; Sayanova et al., 1997)
have been cloned so far. A good source to clone new
desaturases is the moss Physcomitrella patens. Lipids of
P. patens contain high proportions of AA (up to 30% of
total fatty acids) indicating strong expression of Δ5- and
Δ6-desaturases (Grimsky et al., 1981). This moss can be
propagated vegetatively in the haploid state (Ashton and
Cove, 1977), which simplifies the phenotypic analysis after
mutation or transformation (Schaefer et al., 1991). Genes
of this organism can be specifically inactivated by gene
targeting, as shown by Schaefer and Zrídy (1997), who
demonstrated that integration of homologous DNA into
the genome of P. patens takes place by homologous
recombination with a relative efficiency of more than 90%
among transgenic plants.

In the present communication, we describe the isolation
of a new cDNA and its corresponding genomic sequence
from P. patens, using a PCR-based screening. The encoded
protein shared less than 27% sequence identity with known
desaturases and represents a fusion between a C-terminal

Received 2 January 1998; revised 30 March 1998; accepted 8 April 1998.
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desaturase with a cytochrome b5-related part and a N-terminal extension. Its function and importance for the biosynthesis of AA (20:4) was identified by disrupting the corresponding gene in P. patens. The biochemical phenotype of the null mutant and its subsequent complementation by feeding γ-linolenic acid (18:3 Δ6,9,12) demonstrated that the disrupted gene codes for a Δ6-desaturase, which plays a key role in the synthesis of 20:4.

Results

PCR-based cloning

For PCR experiments, different sets of degenerate primers, deduced from the three conserved histidine boxes of acyl-lipid desaturases, were synthesized (Avelange-Macherel et al., 1995; Shanklin et al., 1994). The template used was single-stranded cDNA from P. patens, which was reverse-transcribed from mRNA of 12-day-old protonema cultures. Bands of the expected length were cloned and sequenced. Data bank searches and alignments with these new sequences indicated similarities to acyl-lipid desaturases for seven cDNA fragments. Six of them were classified as putative members of the well-known Δ12- and Δ15-desaturases based on high identities of over 60%. In contrast to this, one sequence of 550 bp showed less than 27% identity to known desaturases. Since Physcomitrella was expected to express Δ5- and Δ6-desaturases, it was postulated that this sequence might be derived from one of those desaturases.

Isolation of a full-length cDNA

To isolate a full-length cDNA clone, the 520 bp PCR fragment was DIG-labelled, and used to screen a cDNA library of 12-day-old protonema. Of 3.0 \times 10^5 plaques screened, 19 positives were isolated. The restriction analysis of their inserts showed a similar pattern in all cases. The partial sequence analysis from six inserts revealed that they were identical to each other within their overlapping regions and also to the original 520 bp PCR fragment. The longest insert, designated PPDES6 cDNA, was sequenced on both strands. It had a length of 2012 bp excluding its poly(A) tail. An open reading frame stretched from position 319–1984, and several stop codons in the corresponding 5′ untranslated region indicated its full length (Figure 1). The protein PPDES6 translated from the PPDES6 cDNA contained 525 amino acid residues with a calculated molecular weight of 59.3 kDa. This is 7–20 kDa larger than all acyl-lipid desaturases known from higher plants and cyanobacteria. Data bank searches indicated similarity to cytochrome b5 sequences from residues 105–176 and to desaturases from residue 207 towards the C-terminus.

The desaturase domain showed the highest similarity to the cytochrome b5-containing fusion protein of Helianthus annuus (Sperling et al., 1995), a putative fusion protein from Caenorhabditis elegans encoded by cosmid T13F2 (Z81112) and the Δ6-desaturases of Spirulina platensis (X87094), Borago officinalis (Sayanova et al., 1997) as well as Synechocystis sp. PCC 6803 (Reddy et al., 1993). The identity values of PPDES6 to these proteins were low and ranged from 21% to 27% for the sequence between the first and third histidine boxes and from 12% to 23% over the entire length. The sequence motive QELRH of the third histidine box started with a glutamine instead of a histidine, which has also been found in Δ6-desaturases and the cytochrome b5 fusion protein of H. annuus, but not in other membrane-bound desaturases. The hydrophobicity plot (Kyte and Doolittle, 1982) after residue 200 showed the typical profile of membrane-bound desaturases (data not shown). The cytochrome b5-related domain contained the eight invariant residues typical for the cytochrome b5 superfamily (Lederer, 1994).

The N-terminal extension of about 100 residues did not share significant similarity to any sequence in the data banks, and computer analysis did not detect any motives for protein targeting or modification either for the extension or for the whole protein.

Structure of the gene

To knock out the PPDES6 gene, its genomic sequence was amplified by PCR with specific primers C and D. Primer C was deduced from the 5′ end and D from the middle of the 3′ untranslated region of the PPDES6 cDNA. PCR with these primers and genomic DNA of P. patens as template amplified a fragment that was 1578 bp longer than the distance between the binding sites of the primers on the cDNA. The genomic PCR fragment, denoted PPDES6, was cloned and sequenced on both strands (Figure 2). Apart from six putative introns (i1-i6) it was 100% identical with the cDNA, confirming its identity as the genomic locus of the PPDES6 cDNA. The 5′ splicing border of five introns was GT and the 3′ border of all six was AG. Only the fourth intron i4 contained the unusual 5′ splicing border GC, which has been found in genes of several plant species (Xue and Rask, 1995). The reliability of this intron sequence was confirmed by sequencing two other PCR-amplified clones over this region. The intron i4 was located between two triplets coding for residues 176 and 177. After residue 176 the detected similarity to cytochrome b5 sequences was terminated.

Gene targeting

For the disruption experiments, the first histidine box of the genomic clone was replaced by the npt II gene as a positive selection marker. The subsequent double digestion
Figure 1. Amino acid sequences of PFDE6 and closely related proteins.
For alignment the CLUSTAL X program was used (gap opening 10, gap extension 0.05). Conserved and invariant residues are grey. The approximate beginning of the three domains from PFDE6 are marked by arrows and their putative function. The eight invariant residues characteristic for the cytochrome b$_5$ superfamily and the three histidine boxes of the desaturase domains are framed. The underlined residues indicate the positions of introns 1–6 in the genomic sequence PFDE6. SYD6, SPDe6 and BODE6 refer to the $\Delta 6$-desaturases of Synechocystis (U79010), Spirulina (X87094) and Borago (U79010). NTCYTB5 and HAB5 refer to the cytochrome b$_5$ of Nicotiana (X71441) and the b$_5$ fusion protein of Helianthus (X87143), respectively.

with Saul/BstBI yielded a linear fragment with the npt II gene in its centre and the desaturase arms at both ends (Figure 2). This linear fragment was used to transform P. patens protoplasts by the PEG method (Schaefer et al., 1991). Seven transformation experiments with $3.0 \times 10^5$ protoplasts in each experiment resulted in the isolation of 56 independent and stably transformed lines. Five randomly selected transgenic lines (K1–K5) were used for detailed analysis regarding the molecular biology of gene disruption as well as its consequences for fatty acid biosynthesis.

Molecular analysis of the transgenic lines

The specific integration of the transformed DNA into the PPDES6 gene was analysed by PCR using genomic DNA from five transformed lines (K1–K5) and the wild type. The locations of the different primers are presented in Figure 2. It is important to point out that the 3' end of primer 4 binds 40 bp downstream of the cloned genomic sequence to exclude PCR signals resulting from contamination by the DNA used for transformation. Its sequence was derived from the 3' end of an incomplete cDNA clone, which showed the same sequence in the overlapping region with cDNA PPDES6, but contained a longer 3' end.

PCR with the primer pair 1/2 amplified fragments of 2.7 kbp, and with the primer pair 3/4 bands of 1.6 kbp, from all five transformants, whereas experiments with the wild type gave negative results. The length of the bands agreed with a substitution of the first histidine box of the PPDES6 gene by the npt II cassette. Both PCR fragments from two transformants (K2 and K3) were cloned and partially sequenced. The sequenced segments were identical with the corresponding regions of the transformed gene disruption construct. Most important, the fragments from primer pair 3/4 contained the downstream genomic element of 40 bp, which was absent in the transformed DNA. They lacked the first histidine box, and the transition regions of the npt II cassette to the PPDES6 gene, as well as the regions containing the restriction sites Aat II and Hpa I, were identical in their sequence with the disruption construct.

To provide evidence for a deletion of the first histidine box in the PPDES6 gene of the transgenic lines, the genomic DNA of the transformed lines and the wild type was digested with BglII, blotted and hybridized with the DIG-labelled deletion probe Del. This probe represents the Saul/BstBI fragment encoding the first histidine box, which had been deleted from the transformed disruption construct (Figure 3). Hybridization with the deletion probe Del showed one strong signal of 4.5 kbp and two very weak signals of 5.0 and 7.0 kbp with the wild type DNA. The transformed lines K1–K4 had lost the strong 4.5 kbp signal but not the two weak signals. Line K5 corresponded to the
wild type situation but contained an additional band of more than 21 kb.

To compare the expression of PPDES6 in the five transgenic lines with the wild type, we blotted total RNA of 14-day-old protonemata and hybridized it with a DIG-labelled RNA probe against the 3’ end of the PPDES6 cDNA (Figure 4). The wild type showed a strong signal of 2.0–2.2 kb, whereas the five transgenic lines had lost this transcript. Hybridization with a npt II-specific probe (blot not shown) detected a strong signal of 1.0–1.3 kb in all transgenic lines but not in the wild type.

Functional analysis of PPDES6 in P. patens

For the functional identification of the desaturase, we analysed the total fatty acids of the wild type and the five knockout lines. The fatty acid analyses presented in Figure 5 are confined to the wild type and to line K2, but the other four lines tested gave essentially the same results. Pathways [1] and [2] below show the sequences proposed for the biosynthesis of AA (20:4) and EPA (20:5) in P. patens,

and they are supported by our results (fatty acids are indicated as m:n^ab, c...; m refers to the number of carbon atoms, n to the double bonds and ^ab, c... to the position of the double bonds; desaturation and elongation steps are indicated by Δx and EL).

\[
\begin{align*}
18:2^{10,12} & \xrightarrow{\Delta \delta} 18:3^{16,9,12} \xrightarrow{\text{EL}} 20:3^{16,11,14} \xrightarrow{\Delta \delta} 20:4^{16,8,11,14} [1] \\
18:2^{10,12,15} & \xrightarrow{\Delta \delta} 18:4^{16,9,12,15} \xrightarrow{\text{EL}} 20:4^{16,11,14,17} \xrightarrow{\Delta \delta} 20:5^{16,8,11,14,17} [2]
\end{align*}
\]

Compared with the wild type, all transgenic lines showed a strong decrease in those unsaturated fatty acids, the formation of which involves a Δ6-desaturation step (Figure 5): 18:3^{16,9,12}, 18:4^{16,9,12,15}, 20:3^{16,11,14}, 20:5^{16,8,11,14,17} and most clearly 20:4^{16,8,11,14}. On the other hand, the possible substrates for a Δ6-desaturase, 18:2^{10,12} and 18:3^{16,9,12,15}, increased. Therefore, it is most likely that the reactions from 18:2^{10,12} to 18:3^{16,9,12} as well as from 18:3^{16,9,12,15} to 18:4^{16,9,12,15} were blocked, both of which are catalysed by a Δ6-desaturase (compare pathways [1] and [2]).

To provide further evidence for the function of the new
Δ6-desaturase, we supplemented the knockout line K2 and the wild type with 18:3\textsuperscript{16,9,12} (γ18:3). In K2 the feeding of this fatty acid resulted in the reappearance of 20:3\textsuperscript{16,9,12} and 20:4\textsuperscript{16,9,12}, whereas almost no change was observed in the wild type. This experiment indicates that the knockout line K2 is able to synthesize 20:4 from added 18:3\textsuperscript{16,9,12}, but not from 18:2\textsuperscript{16,12}, which increases in unsupplemented K2. However, the addition of 18:3\textsuperscript{16,9,12} did not result in a complementation of the almost complete disappearance of 20:5\textsuperscript{15,9,11,14,17} in K2.

The addition of 20:2\textsuperscript{11,14} and 20:3\textsuperscript{11,14,17} (data not shown) did not result in an increase of 20:4 and 20:5 in the wild type or in K2. Another interesting effect of the knockout was the completely different proportion of C20-fatty acids in K2 (7%) compared to the wild type (30%).

**Functional expression of PPDES6 in Saccharomyces cerevisiae**

To exclude the possibility that the loss of a Δ6-desaturase in the knockout lines is a consequence of a regulatory difference between the *Physcomitrella* wild type and knockout lines, *PPDES6* was functionally expressed in *Saccharomyces cerevisiae*. Plasmid pYESΔ6 containing the open reading frame of the *PPDES6* cDNA was transformed into the *S. cerevisiae* strain INVSC1. One clone transformed with pYESΔ6 and another with the empty vector pYES2 as control were grown for four to five generations after induction with 2% galactose in minimal medium. Since *S. cerevisiae* does not contain the dienoic fatty acid substrates required for a Δ6-desaturase, the expression was performed with supplementation of 18:2\textsuperscript{16,12} and 18:3\textsuperscript{16,12,15}, respectively. In subsequent analyses of total fatty acids, the following Δ6-desaturated products were detected in the strain expressing PPDES6: 18:2\textsuperscript{16,9}, 18:2\textsuperscript{16,9,12}, 18:3\textsuperscript{16,9,12} and 18:4\textsuperscript{16,9,12,15} (Table 1). In the control cells, none of these fatty acids were detected. The production of these fatty acids with an additional Δ6-double bond confirmed that cDNA *PPDES6* encodes a Δ6-fatty acid desaturase.

**Discussion**

**Structural properties**

The cDNA and the genomic sequence *PPDES6* encoding a novel Δ6-desaturase from *P. patens* were cloned using a PCR-based approach. The deduced protein shared less than 27% identity with the recently cloned Δ6-desaturase from *B. officinalis* and with the Δ6-desaturases from cyanobacteria (Reddy *et al.*, 1993; Sayanova *et al.*, 1997). This is a surprisingly low value, as until now all desaturases of the same regioselectivity and the same subcellular compartment have been more highly conserved, even between distantly related organisms. For example, six
Identification of a Δ6-desaturase by gene disruption

Furthermore, Southern blot experiments confirmed the deletion of a 200 bp segment encoding the first histidine box from the genome of four transgenic lines (K1–K4). It is likely that reciprocal exchange by double cross-over led to the integration observed in these four lines. Targeting experiments from Schaefer and Zryd (1997) demonstrated homologous integration into a locus but not a substitution. The blots with line K5 reveal an even more complicated situation. Nevertheless, K5 does not express the Δ6-desaturase activity any more. Two additional signals of low intensity in wild type and in all transgenic lines indicated that related genomic sequences were not involved in the gene targeting events. The presence of these sequences suggests that isoforms of other Δ6-desaturases could be expressed to some extent in the knockout lines.

In the Northern blots all transgenic lines showed a dramatically reduced expression of PPDES6 while this transcript was abundant in the wild type. Thus loss of desaturase activity, as evident from the fatty acid profiles most probably resulted from loss of transcription due to gene disruption.

Functional analysis of PPDES6 in P. patens and S. cerevisiae

The gene disruption of PPDES6 resulted in a dramatic alteration of the fatty acid pattern in the transformed lines. The knockout lines showed an increase of 18:2 and α18:3 and a decrease of Δ6-desaturated fatty acids. Therefore, it is likely that PPDES6 codes for a Δ6-desaturase, which desaturates 18:218,12 to 18:318,9,12 and 18:318,9,12 to 18:418,9,12,15. The Δ6-regioselectivity of PPDES6 was further verified by restoration of 20:4 biosynthesis upon feeding of 18:3 (Figure 5). The synthesis of 20:4 from 18:3 would not work if a Δ5-desaturase or the elongation system had been blocked. The Δ6-desaturation of 18:2 and α18:3 added to S. cerevisiae cells expressing PPDES6 confirmed these results and excluded the possibility that the loss of a Δ6-desaturase in the knockout lines was due to regulatory alterations, for example the loss of an activator for the Δ6-desaturase. On the other hand, we could not detect a Δ8-C20-desaturase in P. patens, since addition of 20:211,14 and 20:311,14,17 did not increase the content of 20:4 and 20:5. A Δ8-desaturase operating at the C20-level could theoretically replace the Δ6-C18-desaturase in the biosynthesis of 20:4 and 20:5. Such an enzyme has been suggested to be present in Euglena gracilis (Nichols and Appleby, 1969).

Based on the knockout effects and feeding experiments, we propose the two pathways [1] and [2] mentioned above for the biosynthesis of 20:4 and 20:5 in P. patens, which branch at 18:2. They are in agreement with the biosynthesis of 20:4 and 20:5 as suggested for Porphyridium cruentum (Shiran et al., 1996).

Table 1. Expression of the Δ6-desaturase in S. cerevisiae. The fatty acid methyl esters of the total lipids from cells transformed with pYES2 (WT control) and pYESΔ6 (Δ6-desaturase of P. patens) were analysed by GLC. The cells were cultured in minimal medium supplemented with 2% galactose for 24 h at 30°C. The last two columns show data from cultures supplemented with 18:218,12 (18:2) and 18:318,12,15 (α18:3).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>pYES2</th>
<th>pYESΔ6</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>16.4</td>
<td>16.1</td>
</tr>
<tr>
<td>16:115</td>
<td>54.0</td>
<td>55.5</td>
</tr>
<tr>
<td>16:218,9</td>
<td>-</td>
<td>4.2</td>
</tr>
<tr>
<td>18:0</td>
<td>3.2</td>
<td>2.4</td>
</tr>
<tr>
<td>18:115</td>
<td>24.9</td>
<td>19.7</td>
</tr>
<tr>
<td>18:218,9</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>18:318,9,12</td>
<td>-</td>
<td>8.5</td>
</tr>
<tr>
<td>18:318,9,12,15</td>
<td>-</td>
<td>4.0</td>
</tr>
<tr>
<td>18:418,9,12,15</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Molecular analysis of the transgenic lines

In this study, we have described the highly efficient knockout of the PPDES6 gene after transforming P. patens with a linear disruption fragment. PCR experiments proved the specific integration of the npt II cassette into the PPDES6 locus in all arbitrarily chosen transgenic lines. Other PCR fragments from P. patens, isolated in this screening, coded for putative Δ12- and Δ15-desaturases and displayed more than 60% identity to the corresponding desaturases of higher plants and cyanobacteria.

The presence of the cytochrome b5-related domain upstream of the desaturase suggests its localization in microsomes rather than in chloroplasts, because plastidial desaturases normally use ferredoxin as electron donor (Heinz, 1993). Besides this, PPDES6 contains a new N-terminal extension of about 100 amino acids, which is absent in other presently known desaturases. The function of this extension is unclear, since it shows no significant homology to any known protein, and targeting or modification signals were not detected. Interestingly, the three histidine boxes and the cytochrome b5 domain of PPDES6 are encoded by separate exons (Figure 2), implying that they may constitute separate evolutionary units. The fourth intron containing the unusual 5' splicing border GC is located directly after the last triplet for the cytochrome b5 domain. This organization could allow a differential splicing between the 5' border of the first and the 3' border of the fourth intron, resulting in a deletion of both the cytochrome b5 domain and the N-terminal extension from the desaturase domain of the PPDES6 transcript.
It should be noted that S. cerevisiae cells expressing PPDES6 produced not only 18:3Delta6,9,12 and 18:4Delta6,9,12,15, but also 16:2Delta4,9 and 18:2Delta6,9, which were not detected in P. patens. The reason for their absence in P. patens may be the low content and rapid turn-over of the putative precursors, 16:1Delta9 and 18:1Delta9, in the moss, whereas they are produced in high amounts by S. cerevisiae. Since the Delta6-desaturase converts 16:1Delta9 to 16:2Delta6,9, but does not introduce a Delta8-double bond into 20:2Delta11,14 and 20:3Delta11,14,17 (mentioned above), the insertion of the Delta6-double bond involves measuring from the carboxy terminus (and the Delta9-double bond) rather than from the methyl end. This classifies the desaturase as a Delta6-desaturase (Heinz, 1993).

Another interesting effect is the significant decrease in C20-fatty acids in the knockout lines. The decrease from more than 30% in the wild type to less than 7% in K2 indicates that the elongation system of P. patens prefers or even requires Delta6-desaturated C18-fatty acids. This elongation process is either very rapid or channelled and thus prevents the accumulation of y18:3 or 18:4 in lipids. In the other organisms, from which Delta6-desaturases have been cloned (B. officinalis and Synechocystis), elongation systems do not co-operate with this desaturase and therefore Delta6-desaturated fatty acids can accumulate. A detailed analysis of lipids and fatty acids in P. patens wild type and knockout plants, as well as in S. cerevisiae expressing the Delta6-desaturase, will be published elsewhere (T. Girke et al., manuscript in preparation).

In our present study, all knockout lines still contained small amounts of fatty acids, which were synthesized by a pathway requiring Delta6-desaturase. This indicates that at least one other functional gene for a Delta6-desaturase should exist. Possible candidates may be the two faint signals observed above the targeted 4.5 kbp fragment in Southern blots of wild type and transgenic lines (Figure 3).

Apart from these biochemical changes, we did not detect any visibly altered phenotype in the knockout plants, at least in their protonema or gametophore states at 25°C. Therefore, it was not possible at this point to evaluate the physiological importance of 20:4 for the moss. The appearance of a visible phenotype may also be prevented by residual 20:4. Deletions of several desaturases in Synechocystis became critical only if the Delta6- and Delta12-desaturase were knocked out together, whereas a reduction in trienoic acids without affecting dienoic acids was not critical (Tasaka et al., 1996).

Experimental procedures

Plant material and culture conditions

The protonemata of Physcomitrella patens (Hedw.) BSG were grown in liquid medium (Reski et al., 1994). For feeding experiments with fatty acids, 4-day-old cultures were supplemented with ammonium salts of fatty acids (dissolved in ethanol) to a final concentration of 50 μM and further cultivated for an additional 6–8 days.

Analysis of nucleic acids

DNA manipulations were performed according to standard protocols (Sambrook et al., 1989) unless otherwise stated. DNA sequences were determined on both strands by the dyeoxy chain termination method using Dye Primer as well as Dye Terminator sequencing kits.

PCR with degenerated primers and cDNA library screening

Poly(A)+ RNA was isolated with Dynabeads (Dynal, Oslo, Norway) from total RNA of 12-day-old P. patens protonema cultures, and reverse-transcribed into single-stranded cDNA. This ss-cDNA was used as template in the PCR-based cloning. A 550 bp PCR fragment was amplified with the degenerate sense primer A 5'-TGGTTGAA(A/G)TGGA(C/A)ATGTTG/CAAA-3' and antisense primer B 5'-GG(A/G)AA/A/T/G/C/A/G/I/G/G/AT/CTCG/C/TGC-3' derived from the amino acid sequence WWK/W (N/T)THN and EHHFLF, respectively. The PCR reactions were carried out with Taq DNA polymerase using an amplification programme of 3 min denaturation at 94°C, followed by 30 cycles of 20 sec at 94°C, 30 sec at 45°C, 1 min at 72°C and terminated by 5 min extension at 72°C. The PCR fragments of the expected length (500–600 bp) were cloned in pUC18 and sequenced. A digoxigenin-labelled DNA probe of the PCR fragment was synthesized by PCR and used to screen a lambda ZAPII cDNA library of 12-day-old protonema according to the manufacturer's protocols (Boehringer, Mannheim, Germany; Stratagene, La Jolla, CA). The longest insert (PPDES6 cDNA) was sequenced on both strands using overlapping subclones. The corresponding genomic sequence PPDES6 was isolated by PCR with specific primers C 5'-CCGAGTCGGAGTCAGCC-3' and D 5'-CAGTATCCCGGGGTCC-3' using the Expand High Fidelity PCR System (Boehringer) and the hot start PCR program described below. PPDES6 was cloned into the pCR-Script Amp SK(+) cloning vector (Stratagene), resulting in plasmid pPPDES6 and sequenced on both strands.

Transformation of P. patens

First the vector pRT101neo was constructed to obtain a npt II selection cassette, which could be excised by HindIII digestion. For this purpose the npt II coding region of pRT100neo (Topfer et al., 1993) was excised with HindIII (blunted)/Xhol and ligated between the CaMV 35S promoter and terminator of pRT101 (Topfer et al., 1987), which had been digested with XbaI (blunted)/Xhol. The gene disruption construct resulted from the substitution of a SauI/NotI fragment in the genomic clone pPPDES6 by the npt II selection cartridge. Subsequently, the disruption construct was digested with AatII and Hpal, resulting in a linear fragment with the npt II gene in its centre flanked by genomic sequences of 923 bp and 1159 bp. Fifteen micrograms of this linear DNA were phenol extracted, precipitated and used for the transformation without separation from the vector. PEG-mediated direct DNA transfer into protoplasts was performed as described by Schaefer et al. (1991). The regenerated protonemata were selected for 14 days on medium with G418 (50 mg l−1), released for 12 days under non-selective conditions and again grown for 14 days on

Identification of a Δ6-desaturase by gene disruption

experiments, the cultures were grown to an optical density (600 nm) of 0.5 in CMdum medium, then supplemented with 2% galactose (w/v) as well as 0.003% of the corresponding fatty acid (w/v; stock solution solubilized in 5% tergitol) and finally grown to saturation for 24 h at 30°C.

**Lipid analysis**

Lipids were extracted from promonema and yeast cells by chloroform-methanol extraction (Siebert et al., 1979) and purified from apolar components by TLC in diethyl ether. In this solvent all membrane lipids (triacylglycerols were not produced by promonema) remained at the start. The fatty acid methyl esters (FAME) were obtained by transmethylation of the lipids with 1 N H2SO4 in methanol and 2% dimethoxypropane at 80°C for 1 h. The extracted FAME were analysed by gas-liquid chromatography using a capillary column (Chrompack, WCOT Fused silica, CP-Wax52 CB, 25 m, 0.32 mm). Their identities were confirmed by comparison with appropriate FAME standards (Sigma). The corresponding fatty acid pyrrolidines were obtained as described elsewhere (Andersson and Holman, 1974) and analysed by GC-MS on a HP 5890 A instrument (Hewlett-Packard) equipped with an HP-5 column using a temperature gradient 150°C (3 min) → 320°C at 5°C min⁻¹. Electron impact (EI) was carried out at 70 eV and chemical ionization mass spectra (Cl-MS) were recorded with ammonia as reactant gas (0.1 MPa).

**Acknowledgements**

This work is based on a doctoral study by Thomas Girke in the Faculty of Biology, University of Hamburg. Support by the Deutsche Forschungsgemeinschaft (He 695/14-1) and the Fonds der Chemischen Industrie is gratefully acknowledged. Ralf Reski is indebted to the Deutsche Forschungsgemeinschaft for a Heisenberg Fellowship (He 8373-1). We thank Hermann Moll for skilful help in GC-MS analysis and our colleagues in the EC-funded project EUROMOSS, who shared unpublished results.

**References**


EMBL nucleotide sequence database accession numbers AJ222980 and AJ222981.