The Plant Journal (2002) **31**(3), 255–268

Cloning and functional characterisation of an enzyme involved in the elongation of Δ6-polyunsaturated fatty acids from the moss *Physcomitrella patens*

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The nucleotide sequence reported in this paper has been submitted to the GenBank™/EBI Data Bank with the accession number AF428243

Summary

The moss *Physcomitrella patens* contains high proportions of polyunsaturated very-long-chain fatty acids with up to 20 carbon atoms. Starting from preformed C_{18} polyunsaturated fatty acids, their biosynthesis involves a sequence of Δ6-desaturation, Δ6-elongation and Δ5-desaturation. In this report we describe for the first time the characterisation of a cDNA (*PSE1*) of plant origin with homology to the *ELO-genes* from *Saccharomyces cerevisiae*, encoding a component of the Δ6-elongase. Functional expression of *PSE1* in *S. cerevisiae* led to the elongation of exogenously supplied Δ6-polyunsaturated fatty acids. By feeding experiments with different trienoic fatty acids of natural and synthetic origin, both substrate specificity and substrate selectivity of the enzyme were investigated. The activity of Pse1, when expressed in yeast, was not sensitive to the antibiotic cerulenin, which is an effective inhibitor of fatty acid synthesis and elongation. Furthermore, the *PSE1* gene was disrupted in the moss by homologous recombination. This led to a complete loss of all C_{20} polyunsaturated fatty acids providing additional evidence for the function of the cDNA as coding for a component of the Δ6-elongase. The elimination of the elongase was not accompanied by a visible alteration in the phenotype, indicating that C_{20}-PUFAs are not essential for viability of the moss under phytotron conditions.

Keywords: fatty acid elongation, polyunsaturated fatty acid, *Physcomitrella patens*, gene disruption, cerulenin, β-ketoacyl-CoA synthase

Introduction

Very-long-chain fatty acids (VLCFA), i.e. fatty acids with more than 18 carbon atoms, are widely distributed in the plant kingdom and have a multitude of functions depending on chain length, degree of unsaturation and the alcohol with which they are esterified. Based on their distribution and function in plant organs and within plant cells, a general distinction can be made between saturated/monounsaturated and polyunsaturated VLCFAs.

Saturated/monounsaturated VLCFAs and their derivatives are the main constituents of the plant cuticle which is involved in protecting the plant from environmental impact including non-stomatal water loss (Post-Beittenmiller, 1996) and pathogen attack (Kerstiens, 1996). Monounsaturated VLCFAs are also accumulated in seeds of several plants as triacylglycerols or wax esters (Harwood, 1980; Miwa, 1971). In plant protoplasmic membranes saturated and monounsaturated VLCFAs are only minor components confined to phosphatidylserine (Bohn *et al.*, 2000; Haschke *et al.*, 1990; Murata *et al.*, 1984), the diacylglycerol moiety of glycosylphosphatidylinositol-

anchored proteins (Guillas *et al.*, 2001) and sphingolipids (Lynch, 1993). Despite the fact that VLCFA-containing sphingolipids are essential for *Saccharomyces cerevisiae*, their exact function is yet not well understood. They might be involved in sorting of proteins for the vesicular transport to the cell surface and for the stabilisation of highly curved membrane areas (Bagnat *et al.*, 2000; Kohlwein *et al.*, 2001; Schneiter *et al.*, 1996). Usually, saturated and monounsaturated VLCFAs are excluded from major membrane glycerolipids (Frentzen, 1993; Ståhl *et al.*, 1995). Only in transgenic *Arabidopsis* plants constitutively accumulating VLCFAs to high proportions, were they incorporated into all major glycerolipid classes (Millar *et al.*, 1998). Consistent with the observation that VLCFAs perturb bilayer structure (Hui *et al.*, 1984), dramatic alterations in chloroplast membrane structure and plant morphology were observed in these plants (Millar *et al.*, 1998).

Polyunsaturated VLCFAs do not occur in higher plants (except in some gymnosperms (Wolff *et al.*, 2000)), whereas many algae, ferns and mosses accumulate them to high proportions (Ackman *et al.*, 1968; Dembitsky, 1993; Jamieson and Reid, 1975). Unlike saturated and monounsaturated VLCFAs, they are found in all major glycerolipids and accordingly represent major constituents of membranes, but a precise function cannot be ascribed to them. On the other hand, polyunsaturated VLCFAs have received increasing attention in recent years since they are considered to have beneficial effects on human health and development when included in the diet (Horrocks and Yeo, 1999; Uauy *et al.*, 2000). Polyunsaturated VLCFAs are normally taken up by consumption of fish. To provide alternative and more sustainable sources of these fatty acids by genetic engineering of oilseed crops, various efforts are directed towards the identification and cloning of genes coding for the enzymes that control their biosynthesis (Abbadi *et al.*, 2001).

Several different pathways are realised for the biosynthesis of polyunsaturated VLCFAs in different organisms. In marine bacteria and the primitive eukaryote *Schizochytrium* they are synthesised anaerobically *de novo* by polyketide-like systems (Metz *et al.*, 2001; Takeyama *et al.*, 1997; Tanaka *et al.*, 1999). In other algae and in lower plants their formation occurs via alternating elongation and aerobic desaturation steps (Khozin *et al.*, 1997; Korn, 1964). Starting from linoleic ($18{:}2^{\Delta 9,12}$) or α-linolenic acid ($18{:}3^{\Delta 9,12,15}$), this sequence involves a Δ6-desaturase, a Δ6-elongase and a Δ5-desaturase resulting in arachidonic acid ($20{:}4^{\Delta 5,8,11,14}$) and eicosapentaenoic acid ($20{:}5^{\Delta 5,8,11,14,17}$), respectively. For the synthesis of docosahexaenoic acid ($22{:}6^{\Delta 4,7,10,13,16,19}$) additional Δ5-elongase and Δ4-desaturase are required. All these enzymes have been cloned during the last few years (Abbadi *et al.*, 2001; Leonard *et al.*, 2000; Qiu *et al.*, 2001). In mammals the so-called Sprecher pathway is operating, in which the elongation of $22{:}5^{\Delta 7,10,13,16,19}$ followed by a Δ6-desaturation leads to $24{:}6^{\Delta 6,9,12,15,18,21}$, which is α-oxidised in peroxisomes to the final product $22{:}6^{\Delta 4,7,10,13,16,19}$ (Mohammed *et al.*, 1995).

The moss *P. patens* contains high proportions of $20{:}4^{\Delta 5,8,11,14}$ and some $20{:}5^{\Delta 5,8,11,14,17}$ which are assumed to be produced by the desaturation/elongation pathway. So far, only the gene encoding the Δ6-desaturase has been cloned from this organism (Girke *et al.*, 1998). Its targeted disruption by homologous recombination resulted in a nearly complete loss of $20{:}4^{\Delta 5,8,11,14}$. Since no obvious alteration in the phenotype could be observed, possible functions of this polyunsaturated VLCFA remain unclear. The enzyme catalysing the following step in the reaction sequence is the Δ6-elongase, but none of the genes coding for its components had been cloned from the moss yet.

Based on biochemical data, fatty acid elongation can be divided into four different reactions: condensation of malonyl-CoA with a long-chain acyl-primer to form a β-ketoacyl-CoA, reduction to β-hydroxyacyl-CoA, dehydration to *trans*-2-enoyl-CoA and reduction of the *trans*-double bond resulting in the elongated acyl-CoA (Fehling and Mukherjee, 1991). It is assumed that the elongase complex consists of distinct membrane-bound enzymes, each catalysing only a single step of the whole sequence. Furthermore, several elongases occur within a plant differing in their spatial and temporal expression. It is thought that the substrate specificities and catalytic activities of elongase complexes are controlled by β-ketoacyl-CoA synthases (KCS), which are responsible for the initial condensation reaction (Millar and Kunst, 1997), whereas different condensing enzymes apparently share the same set of reductases and dehydratases (Kohlwein *et al.*, 2001; Millar and Kunst, 1997).

During the last decade several genes coding for β-ketoacyl-CoA synthases have been cloned from different plant species. These enzymes, named *KCS* or *FAE*, are specific for saturated and monounsaturated fatty acids, which are used for the biosynthesis of waxes and seed storage lipids. Interestingly, they do not share any sequence similarity with the *ELO* genes from *S. cerevisiae*, which code for the corresponding condensing enzymes of the yeast fatty acid elongation systems. *ELO1* is involved in the elongation of saturated and monounsaturated medium-chain fatty acids (C_{14}-C_{16}), whereas *ELO2* and *ELO3* are required for the elongation of saturated long-chain fatty acids leading to C_{24} or C_{26}, respectively, that are used for sphingolipid formation (Oh *et al.*, 1997; Toke and Martin, 1996). The reason for this obviously convergent development in the first step of fatty acid elongation is unclear and for a long time no biochemical data have been available, which could provide direct evidence for the function of an *ELO* gene product acting as a condensing enzyme. Only recently, Moon and

coworkers have shown that a β-ketoacyl-CoA is indeed the first intermediate resulting from the activity of a recombinant *ELO*-like enzyme from mouse (Moon *et al.*, 2001). The cloned fungal and animal genes (Beaudoin *et al.*, 2000; Leonard *et al.*, 2000; Parker-Barnes *et al.*, 2000) that are involved in the elongation of polyunsaturated fatty acids (PUFAs), share homology with the yeast *ELO* sequences and not with the plant *KCS/FAE* sequences, but no such gene of plant origin has been cloned yet. In the present report, we describe for the first time the isolation of a *P. patens* cDNA with homology to the yeast *ELO* sequences. We demonstrate that the encoded enzyme is involved in the elongation of Δ6-PUFAs by heterologous expression in *S. cerevisiae* and by disrupting the gene in the moss by homologous recombination.

Results

Identification and structural characterisation of PSE1

P. patens may serve as a good source for the isolation of desaturases and elongases required for the production of $20{:}4^{\Delta5,8,11,14}$, which is accumulated up to 30% of the moss fatty acids. To identify an enzyme involved in the elongation of Δ6-PUFAs, ESTs were generated by random sequencing of cDNAs from a library prepared from protonemata of *P. patens*. We focused our analysis on ESTs with sequence homologies to the plant *KCS/FAE* genes (i.e. *FAE1* from *Arabidopsis thaliana*, Acc. No. T05272) and the yeast *ELO* genes (i.e. *ELO1* from *S. cerevisiae*, Acc. No. NP_012339), which both are known to be involved in the elongation of fatty acids. One EST displayed a significant sequence similarity to the *ELO* genes. The corresponding cDNA clone was designated *PSE1* (*P*UFA *s*pecific *e*longase) since it was expected to encode a component of the Δ6-elongase. Among 30000 EST sequences the sequence of *PSE1* occurred only once. The full-length cDNA contained an ORF of 873 bp coding for a protein of 290 amino acids with a calculated molecular mass of 33.4 kDa. Amino acid sequence comparison using the ClustalX program revealed that *PSE1 has* the strongest homology to GLElo1p from the zygomycete fungus *Mortierella alpina* (31% identity, 47% similarity), which is required for the elongation of PUFAs (Parker-Barnes *et al.*, 2000). In addition, several conserved motifs that are characteristic for this protein family, could be identified (Figure 1). One of these motifs, the so-called histidine-box, also occurs in other enzymes such as desaturases, where it might be involved in the binding of iron. The Pse1 protein (Pse1p) is similar to the Elo proteins in being highly hydrophobic as shown by hydropathy analysis (Kyte and Doolittle, 1982). A membrane topology prediction program (Krogh *et al.*, 2001) reveals that Pse1p contains 7 putative transmembrane domains (Figure 2). According to the prediction of protein localisation in cells (Nakai and Horton, 1999), Pse1p is localised in the ER, which is in agreement with the microsomal localisation of fatty acid elongation. The sequence presented no similarity to the plant *KCS/FAE* family (Figure 2), and in particular no conserved cysteine was found among the *ELO* sequences, which is considered to be essential for the catalytic activity of Kcs enzymes (Ghanevati and Jaworski, 2001).

Functional expression of PSE1 *in* S. cerevisiae

For a functional identification, plasmid pY2PSE1 containing the ORF of *PSE1* downstream of the *GAL1* promoter and the empty vector pYES2 as control were transformed into *S. cerevisiae* strain INVSc1. Transgenic clones of both constructs were grown in minimal medium for 24 h after induction with 2% galactose. Since *S. cerevisiae* does not contain any PUFAs, the medium was supplemented with 500 μM of $18{:}3^{\Delta6,9,12}$ to provide a substrate for the synthesis of polyunsaturated VLCFAs. Figure 3 shows the fatty acid profile of the transgenic yeasts after expression of the moss clone. In the chromatogram of pY2PSE1 one additional peak showed up, which was identified as $20{:}3^{\Delta8,11,14.}$ by GLC-MS of its DMOX derivative. In contrast, in yeast cells harbouring the empty pYES2 vector, $20{:}3^{\Delta8,11,14}$ was present in very low proportion, which might result from non-specific elongation by the yeast elongases. Thus, the accumulation of $20{:}3^{\Delta8,11,14}$ at the expense of $18{:}3^{\Delta6,9,12}$ in the transgenic yeast indicates, that *PSE1* encodes a protein involved in the elongation of $18{:}3^{\Delta6,9,12}$.

Substrate specificity

To characterise the enzyme encoded by *PSE1* in more detail, we determined its substrate specificity. For this purpose, the expression in yeast was carried out in the presence of several other fatty acids that differ in number and position of double bonds as well as in chain length. In particular, we also wanted to know, whether the elongase differentiates between various trienoic C_{18} fatty acids carrying a block of methylene-interrupted *cis*-unsaturations at various positions in the chain starting at C4, C5, C6, C7, C8 or C9. Since only two of these ($18{:}3^{\Delta6,9,12}$ and $18{:}3^{\Delta9,12,15}$) are readily available, the others had to be prepared synthetically following a new strategy, which is shortly outlined in the following paragraph.

The synthesis of trienoic fatty acids with an ensemble of homoconjugated double bonds in different positions of the molecule described so far required lengthy multistep procedures. Utilising the recently published three-component Wittig approach (Pohnert and Boland, 2000), the carbon skeleton of individual fatty acids (Table 1) is

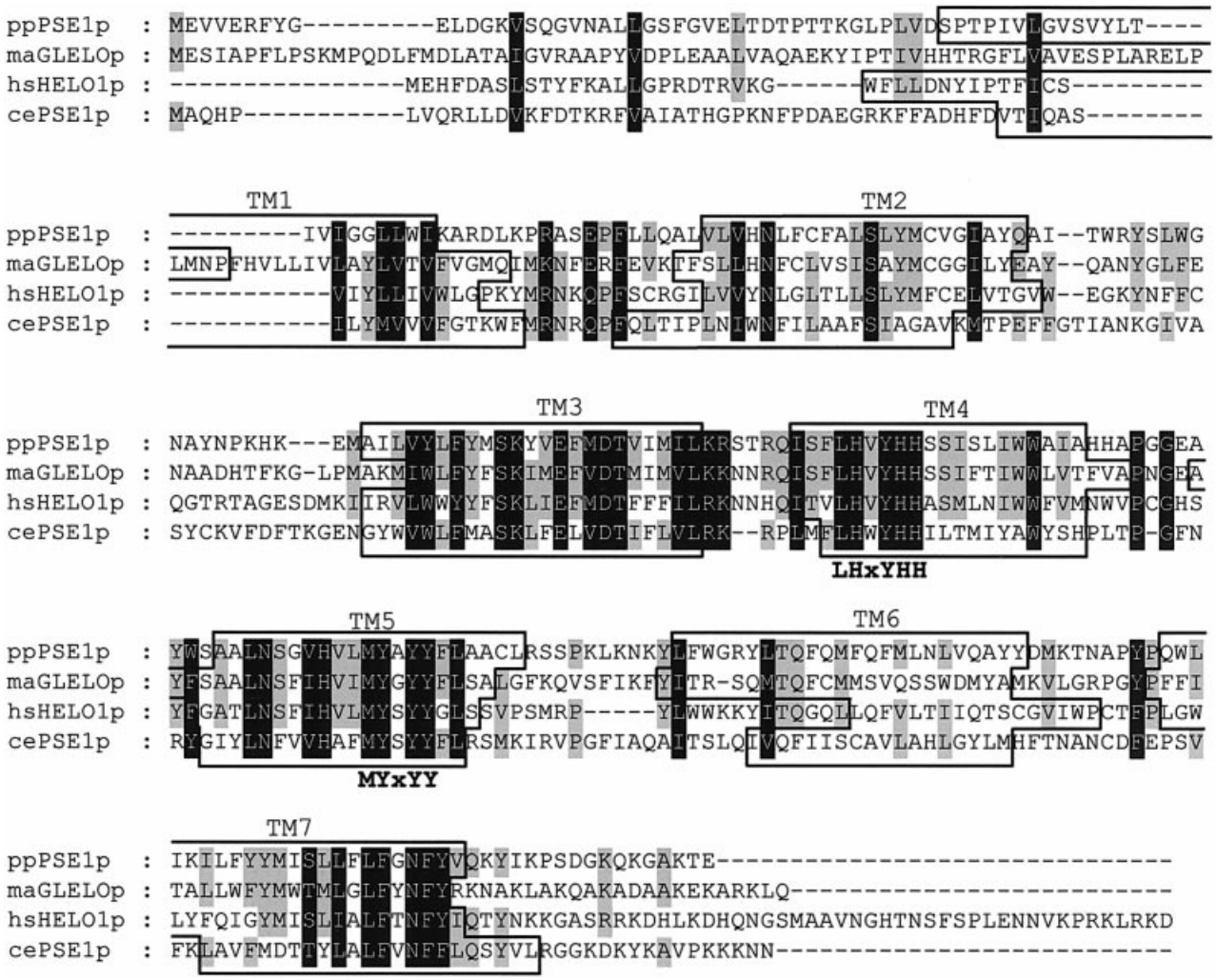

Figure 1. Sequence comparison of PUFA-elongation enzymes.
The derived amino acid sequences of *P. patens* (pp*PSE1*, Acc. No. AF428243), *Mortierella alpina* (ma*GLELO*; Acc. No. AAF70417), *Homo sapiens* (hs*HELO*; Acc. No. NP_068586) and *Caenorhabditis elegans* (ce*PSE1*; Acc. No. T22789) were aligned using the Clustal algorithm. All proteins have been shown to be involved in the elongation of PUFAs (Beaudoin *et al.*, 2000; Leonard *et al.*, 2000; Parker-Barnes *et al.*, 2000). Identical or similar amino acids in all four sequences are indicated by reverse contrast letters, those confined to three sequences are highlighted by a grey background. The sequence identities vary between 17 and 31%. The characteristic LHxxHH and MYxYY motifs of this protein family are indicated. Putative transmembrane helices (TM) of all sequences predicted by the TMHMM 2.0 server are boxed.

readily assembled in a single operation from simple precursors (Figure 4). The symmetric *bis*-ylide, obtained from deprotonation of the corresponding *bis*-phosphonium salt with two equivalents of potassium hexamethyldisilazide, is first reacted at −78°C with an aluminium alkoxide resulting from low temperature reduction of the corresponding ester (m = 2–6) with diisobutylaluminiumhydride (abbreviated as DIBAl-H in Figure 4). Slow release of the aldehyde from the aluminium alkoxide selectively yields a mono-ylide that can be directly reacted with the second carbonyl component (generally an ω-oxoester; n = 1–6) to yield the complete trienoic fatty acid ester. Careful control of the temperature conditions during the whole olefination sequence is, however, essential to warrant non-statistical reactions and high overall yields. Saponification of the methylesters with KOH in aqueous ethanol liberates the free acids. Overall yields are given in Table 1.

The results of expression experiments in the presence of different fatty acids are summarised in Figure 5. The highest elongation activity was detected with 18:3$^{\Delta6,9,12}$ and 18:4$^{\Delta6,9,12,15}$, of which 51 and 45% were elongated, respectively, indicating that Pse1p is responsible for the elongation of Δ6-PUFAs of both n-6 and n-3 structure. In contrast, the saturated and monounsaturated fatty acids present in yeast cells and the exogenously added petro-

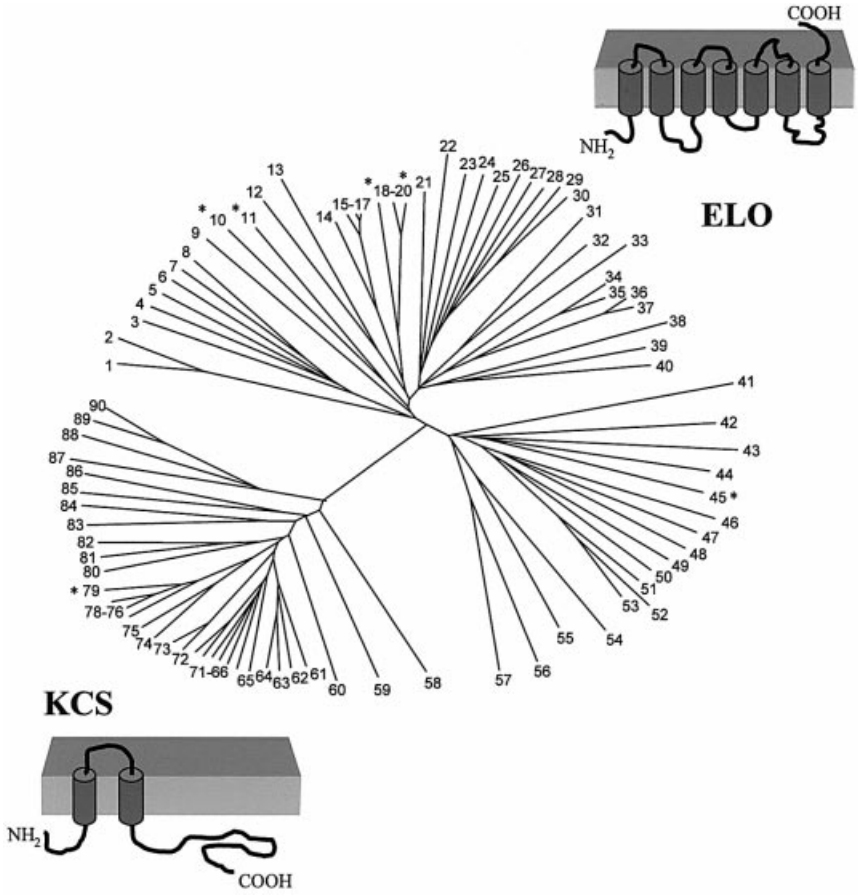

Figure 2. Phylogenetic relationship between Kcs- and Elo-like proteins.
The amino acid sequences of 90 proteins, expected or known to be involved in the elongation of fatty acids, were aligned with ClustalX 1.8. The phylogenetic tree was created subsequently using TreeView 1.5.2. The proteins diverge into two groups: one showing similarities with the yeast Elo proteins (1–57) and the other closely related to the plant Kcs proteins (58–90). Membrane topologies for all sequences were predicted by using several programs (TMHMM2.0, SOSUI, TMPred). The results obtained were reproducible with regard to the number of transmembrane helices (1–2 for Kcs, bottom left corner; 5–7 for Elo, upper right corner), whereas their actual orientation (N-termini inside/outside) varied with the program used. The GenBank accession numbers of the proteins can be seen in the supplementary material in appendix 1 (see URL for supplementary material above). Identified PUFA-elongases and the *FAE1.1* gene from *B. napus* used as *KCS* control in the cerulenin experiments are marked by stars.

selinic acid (18:1$^{\Delta 6}$) did not serve as substrates (data not shown). Next, we compared the conversion of 18:3$^{\Delta 6,9,12}$ with that of five other octadecatrienoic acids, in which the system of the double bonds was either shifted towards the methyl or the carboxyl end. 18:3$^{\Delta 5,8,11}$, 18:3$^{\Delta 7,10,13}$ and 18:3$^{\Delta 8,11,14}$ still served as good substrates, with 19%, 21% and 16% of elongation, respectively. In contrast, 18:3$^{\Delta 4,7,10}$ and 18:3$^{\Delta 9,12,15}$ were markedly poorer substrates. Interestingly, pinolenic acid (18:3$^{\Delta 5cis,9,12}$) was elongated at about the same rate as 18:3$^{\Delta 5,8,11}$, suggesting that Pse1p did not distinguish between this pair with methylene- and ethylene-interrupted double bonds. In contrast, columbinic acid (18:3$^{\Delta 5trans,9,12}$), the 5-*trans* isomer of pinolenic acid, was not accepted as substrate. Furthermore, the conversion of 16:3$^{\Delta 7,10,13}$ (36%) and 18:3$^{\Delta 7,10,13}$ (17%) suggests that Pse1p has no strict chain-length specificity. Since 16:3$^{\Delta 7,10,13}$ was also efficiently elongated by the yeast fatty acid elongation systems (29% conversion in control cells) the elongation was calculated as percentage conversion in pY2PSE1, corrected by subtracting the yeast background. Consistent with the fatty acid profile of *P. patens*, C_{20} PUFAs were not elongated, which is evident from the negative outcome of elongation experiments with 20:3$^{\Delta 8,11,14}$, 20:4$^{\Delta 5,8,11,14}$ and 20:5$^{\Delta 5,8,11,14,17}$.

Substrate selectivity

Whereas the substrate specificity describes the activity of an enzyme when faced with a single substrate, the substrate selectivity describes the selection of a particular substrate from a substrate mixture. To determine the substrate selectivity of Pse1p for different PUFAs, particularly for those occurring naturally in higher plants or in transgenic crops, the expression in *S. cerevisiae* was performed in the presence of an equimolar mixture of 18:2$^{\Delta 9,12}$, 18:3$^{\Delta 6,9,12}$, 18:3$^{\Delta 9,12,15}$ and 18:4$^{\Delta 6,9,12,15}$. Figure 6 shows that the yeast incorporated these fatty acids to similar proportions and that none of them was elongated in control cells harbouring the empty vector pYES2. In contrast, in yeast cells expressing *PSE1*, the proportions of 18:3$^{\Delta 6,9,12}$ and 18:4$^{\Delta 6,9,12,15}$ decreased to approximately 50% of the control and two new peaks appeared which were identified as 20:3$^{\Delta 8,11,14}$ and 20:4$^{\Delta 8,11,14,17}$ by comparison of their retention times with those of appropriate standards. The elongation rates were approximately the same as those observed in the experiments, in which each fatty acid was provided separately (41% for 18:3$^{\Delta 6,9,12}$ and 52% for 18:4$^{\Delta 6,9,12,15.}$). From these results we conclude that Pse1p is highly selective for Δ6-PUFAs and that it

discriminates Δ9-PUFAs, but that it does not distinguish between n-6 and n-3 fatty acids.

Gene targeting

For an alternative proof of the function of *PSE1*, we disrupted the gene in *P. patens* by homologous recombination. For this purpose, a 221-bp fragment of the cDNA, containing the conserved histidine box, was replaced by the *npt*II gene as a positive selection marker. The recombination event was initially verified by PCR experiments using primers binding at the 5′-untranslated region of *PSE1* and at the *npt*II cassette and by Southern blot analysis (data not shown). The Southern blot analysis revealed further that the moss has no additional gene in its genome hybridising with the ORF of *PSE1*. Only one DNA-fragment was detected on a blot obtained with wild-type genomic DNA cut with restriction enzymes that do not cut within the ORF of *PSE1* (data not shown).

The fatty acid profiles of the wild-type and of the knock-out line 46-2 of *P. patens* are shown in Figure 7. The wild-type plant contained high proportions of $20{:}4^{\Delta5,8,11,14}$ and some $20{:}3^{\Delta8,11,14}$ and $20{:}5^{\Delta5,8,11,14,17}$. On the other hand, in the transgenic knock-out line 46-2 these polyunsaturated VLCFAs were not detectable. Consistent with the proposed pathway for the biosynthesis of $20{:}4^{\Delta5,8,11,14}$ and $20{:}5^{\Delta5,8,11,14,17}$ in the moss, this effect was accompanied by an increase in the proportions of the possible elongase substrates $18{:}3^{\Delta6,9,12}$ and $18{:}4^{\Delta6,9,12,15}$. To investigate whether only the elongation step was specifically affected, we cultivated the knockout line in the presence of $20{:}3^{\Delta8,11,14}$ to complement the missing elongase. This resulted in the reappearance of $20{:}4^{\Delta5,8,11,14}$ indicating that the Δ5-desaturase was not affected. Thus, we could confirm the involvement of Pse1p in the elongation of Δ6-polyunsaturated fatty acids.

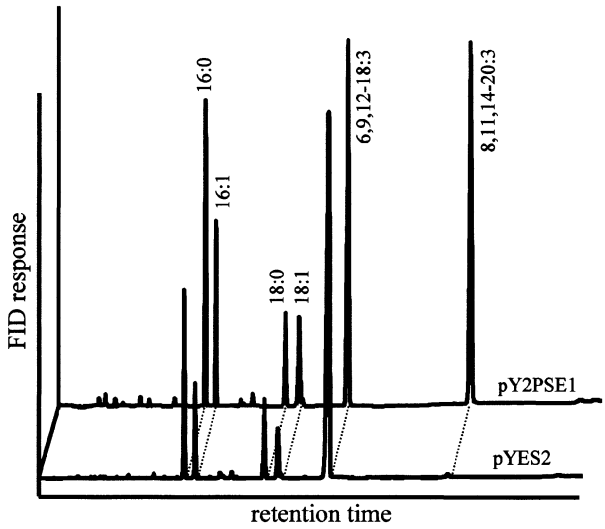

Figure 3. Functional identification of *PSE1* as a component of the moss Δ6-elongase complex.
The GLC traces show the fatty acid profiles of transgenic yeast cells transformed either with the control vector pYES2 (lower trace) or the *PSE1*-containing construct pY2PSE1 (upper trace). In both cases expression was performed in the presence of $18{:}3^{\Delta6,9,12}$. The expression of *PSE1* led to the accumulation of $20{:}3^{\Delta8,11,14}$ by elongation of $18{:}3^{\Delta6,9,12}$. In contrast, the saturated and Δ9-monounsaturated C_{16} and C_{18} fatty acids present in yeast cells were not elongated.

Investigation of the phenotype of the pse1 *mutant*

Interestingly, the *pse1* mutant differed neither in appearance nor in growth from the wild-type moss when grown on Knop or complete medium at 25°C. To elucidate possible functions of C_{20} PUFAs in the moss we subjected both the wild-type and the mutant to different stress conditions: decrease in temperature from 25°C to 15°C, increase in salt concentration in the growth medium or an exposition to drought. But none of the stress conditions resulted in an altered phenotype of the mutant as in growth and appearance the *pse1* mutant was identical to the wild-type moss.

Effect of cerulenin on S. cerevisiae *expressing* PSE1

To investigate the mechanism by which Pse1p is acting, the expression in yeast was conducted in the presence of cerulenin. Cerulenin is an irreversible inhibitor of several β-ketoacyl-ACP synthases of type II fatty acid synthases (Price *et al.*, 2001) and several β-ketoacyl-CoA synthases of fatty acid elongases (Schneider *et al.*, 1993). It is thought to act by forming a covalent bond with an active site cysteine, which accepts the acyl group transferred from acyl-ACP or acyl-CoA. Figure 8 presents the fatty acid profiles of yeast cells harbouring the empty vector pYES2, the plasmid pY2FAE1.1 containing *FAE1.1* from *B. napus* or pY2PSE1 containing *PSE1* from *P. patens*. The left chromatograms show expression experiments in the absence of cerulenin (Figure 8a,c,e), the right ones in the presence of cerulenin (Figure 8b,d,f). As reported earlier (Han *et al.*, 2001), the recombinant β-ketoacyl-CoA synthase from *B. napus* elongated saturated and monounsaturated fatty acids up to 26 or 22 carbon atoms, respectively (Figure 8c), but the activity was completely lost when the expression was

Table 1 Synthesis of positional isomers of octadecatrienoic acids

fatty acid	*m*	*n*	overall yield (%)
$18{:}3^{\Delta4,7,10}$	6	1	18
$18{:}3^{\Delta5,8,11}$	5	3	26
$18{:}3^{\Delta7,10,13}$	3	5	35
$18{:}3^{\Delta8,11,14}$	2	6	31

performed in the presence of cerulenin (Figure 8d). In contrast, the Pse1 enzyme was not affected at all. In the absence of cerulenin 61% of 18:3$^{\Delta 6,9,12}$ were elongated (Figure 8e). In the presence of cerulenin the activity even increased (68% elongation of 18:3$^{\Delta 6,9,12.}$, Figure 8f) showing that Pse1 is insensitive to cerulenin.

Discussion

The biosynthesis of VLCFAs in plants is catalysed by microsomal fatty acid elongation systems, which use as substrates pre-existing C_{16} and C_{18} fatty acids derived from the plastidial fatty acid synthase metabolon. In higher plants elongation is mainly restricted to saturated and monounsaturated fatty acids, which are used for the formation of cuticular waxes on the surface of all epidermal cells (Post-Beittenmiller, 1996) including the pairs of specialised guard cells of stomata (Gray *et al.*, 2000) and seed storage lipids, whereas PUFAs do not serve as substrates. In contrast, lower plants elongate PUFAs since they contain considerable proportions of polyunsaturated VLCFAs in their membrane lipids. The Kcs enzymes that have been cloned so far from higher plants share sequence similarities with enzymes that catalyse other condensation reactions, such as KasIII (Tai and Jaworski, 1993), resveratrol synthase (Schröder *et al.*, 1988) and chalcone synthase (Schröder and Schröder, 1990). But they are completely different from the *ELO* sequences that have been shown to be involved in the elongation of saturated and polyunsaturated fatty acids in yeast, fungi and animals.

In the present report we describe the isolation of a cDNA clone (*PSE1*) from the moss *P. patens* which has some sequence homology to the yeast, fungal and animal *ELO* genes. The encoded protein shows structural similarities to the *ELO* gene products, which are highly hydrophobic and have multiple transmembrane helices. Expression of *PSE1* in *S. cerevisiae* led to the elongation of the exogenously supplied Δ6-PUFAs 18:3$^{\Delta 6,9,12}$ and 18:4$^{\Delta 6,9,12,15}$, suggesting that *PSE1* codes for an enzyme component of the moss Δ6-elongase. The demonstration of the new activity in yeast depends on the ability of the *PSE1* gene product to interact with other components of the yeast elongase. Since the substrate specificity of the elongase complex is thought to reside in the condensing enzyme, it is likely that Pse1p catalyses the initial reaction of the 4-step elongation process. The genes coding for the other components of the elongase are not known yet. Only recently the enoyl-CoA reductase, which catalyses the last reaction in fatty acid elongation, was cloned from yeast (Kohlwein *et al.*, 2001), providing new possibilities to elucidate the molecular organisation of fatty acid elongases.

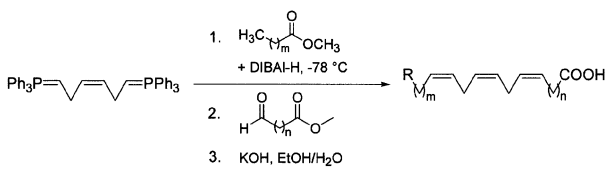

Figure 4. Overall strategy of the synthesis of homoconjugated fatty acids.

The recombinant *PSE1* gene product was also involved in the conversion of a wide range of other exogenously added C_{16} and C_{18} PUFAs, most notably of those that are structurally similar to 18:3$^{\Delta 6,9,12}$, i.e. 18:3 fatty acids, in which the double bond system is shifted by one position towards the carboxyl or the methyl end. Since these synthetic fatty acids do not occur in the moss (as well as 16:3$^{\Delta 7,10,13}$ or 18:3$^{\Delta 5,9,12}$), there is no need that *Pse1* discriminates them. In contrast, 18:2$^{\Delta 9,12}$ and 18:3$^{\Delta 9,12,15}$, which are actually present in *P. patens*, are only poor substrates for the Δ6-elongase. In agreement with this observation, 18:2$^{\Delta 9,12}$ and 18:3$^{\Delta 9,12,15}$ are hardly elongated in yeast expressing *PSE1*. We confirmed these results by a selectivity experiment in yeast, in which we tried to simulate the substrate supply present in seeds of transgenic crops containing Δ6- and Δ9-PUFAs. From an equimolar mixture of 18:2$^{\Delta 9,12}$, 18:3$^{\Delta 6,9,12}$, 18:3$^{\Delta 9,12,15}$ and 18:4$^{\Delta 6,9,12,15}$ only 18:3$^{\Delta 6,9,12}$ and 18:4$^{\Delta 6,9,12,15}$ were elongated in yeast expressing *PSE1*, indicating that Pse1p has a

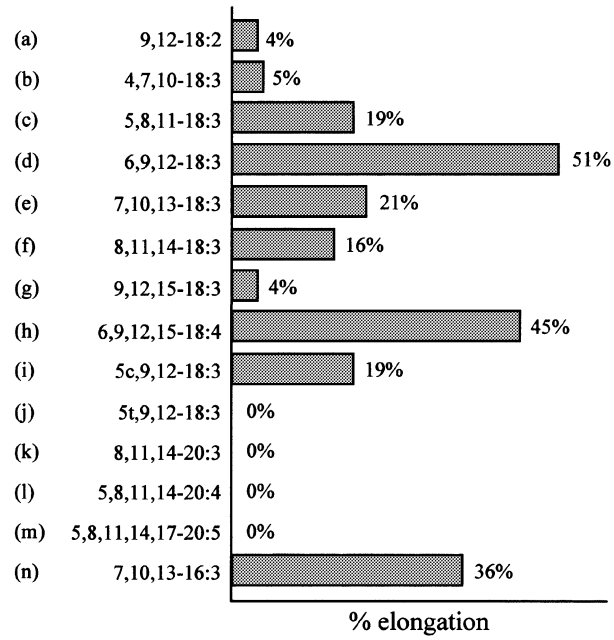

Figure 5. Substrate specificity of Pse1p.
Heterologous expression of Pse1p in yeast was performed in the presence of different polyunsaturated fatty acids (a-n), each provided separately. Fatty acid profiles of transgenic yeasts were analysed by GLC and percentage elongation was calculated as mol%$_{product}$ × 100/(mol%$_{educt}$ + mol%$_{product}$). The data show that among C18-fatty acids, Pse1p has a preference for Δ6-PUFAs (d and h).

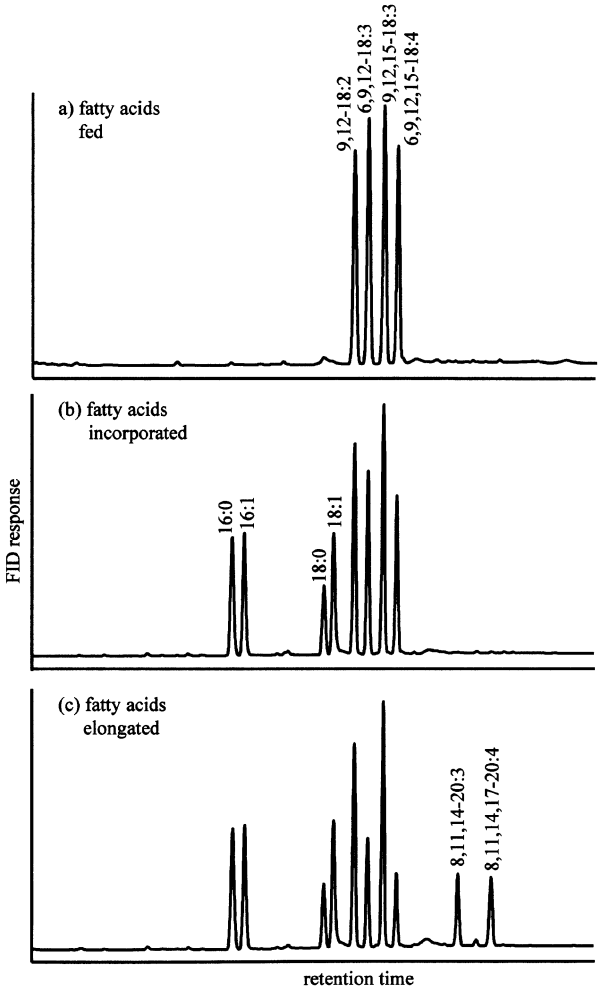

Figure 6. **S**ubstrate selectivity of Pse1p in *S. cerevisiae.*
Expression of *PSE1* was conducted in the presence of a nearly equimolar mixture of C_{18} PUFAs (a). After expression FAMEs were prepared from transgenic yeast cells and analysed by GLC. The chromatogram b shows the fatty acid profile of yeast cells containing the empty vector pYES2. All exogenously supplied PUFAs were incorporated to roughly the same proportion. From these fatty acids only $18:3^{\Delta 6,9,12.}$ and $18:4^{\Delta 6,9,12,15}$ were elongated in transgenic *S. cerevisiae* expressing *PSE1* (c) indicating that Pse1p is highly selective for Δ6-PUFAs and that Δ9-PUFAs, as well as Δ9-monounsaturated fatty acids (16:1Δ9·, 18:1Δ9·) are not accepted.

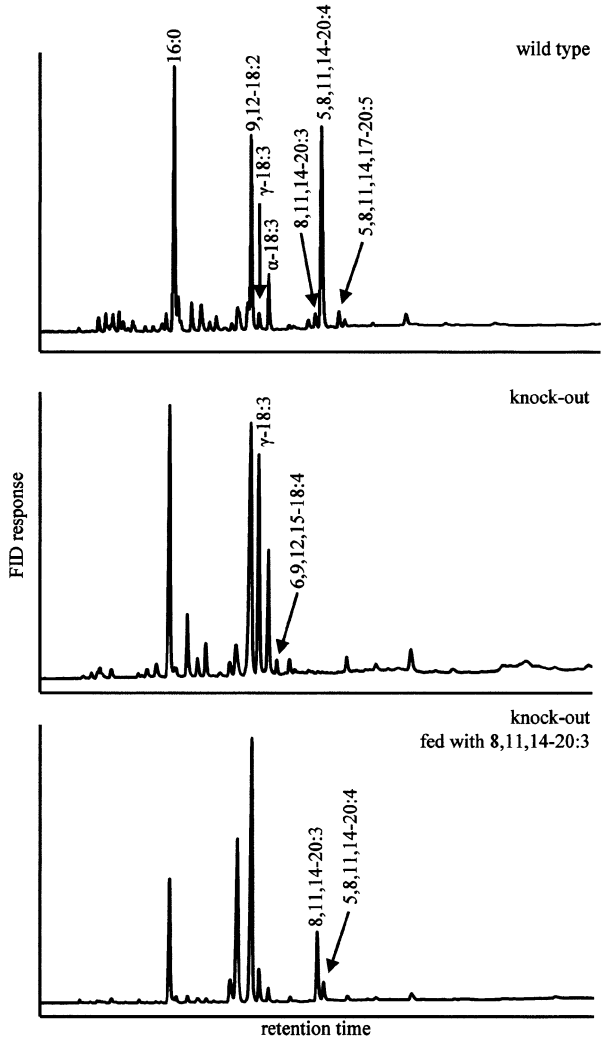

Figure 7. Disruption of the *PSE1* gene in *P. patens.*
FAMEs were prepared from 14-day-old protonemata of wild-type and the *PSE1* knockout line 46–2 and analysed by GLC. The wild-type moss contains a high proportion of arachidonic acid ($20:4^{\Delta 5,8,11,14.}$) as dominating C_{20} PUFA. In contrast, none of these polyunsaturated VLCFAs was detected in the knockout line indicating that *PSE1* is involved in the elongation of C_{18} PUFAs. The chromatogram at the bottom shows the fatty acid profile of the *PSE1* knock-out line cultured in the presence of $20:3^{\Delta 8,11,14.}$ The reappearance of $20:4^{\Delta 5,8,11,14}$ shows that only the elongation step is missing in the mutant plant but that the Δ5-desaturase is not affected, although the conversion of $20:3^{\Delta 8,11,14}$–$20:4^{\Delta 5,8,11,14}$ is much lower than in the wild-type.

high selectivity for Δ6-PUFAs. This selectivity is crucial for biotechnological purposes, as undesired side products accumulating during the production of transgenic oils should be kept at a minimum, when the engineered oil is to be used for human nutrition.

Additional evidence for the function of Pse1, acting as a component of the Δ6-elongase, was provided by the generation of a deletion mutant of *P. patens* through disruption of the *PSE1* gene with an antibiotic resistance gene cassette. It resulted in a dramatic alteration of the fatty acid composition in the knockout plant, which did not contain detectable C_{20} PUFAs anymore. This is the second PUFA mutant generated by homologous recombination in *P. patens.* In previous experiments the gene coding for the Δ6-desaturase was disrupted, which catalyses a preceding step of PUFA biosynthesis in this organism (Girke *et al.*, 1998). The mutant still had residual Δ6-desaturation activity since low proportions of C_{20} PUFAs could be detected, suggesting a redundant capacity of this step in the moss. In the case of the Δ6-elongation the situation is different: the moss has no additional *PSE1* homologue in its genome

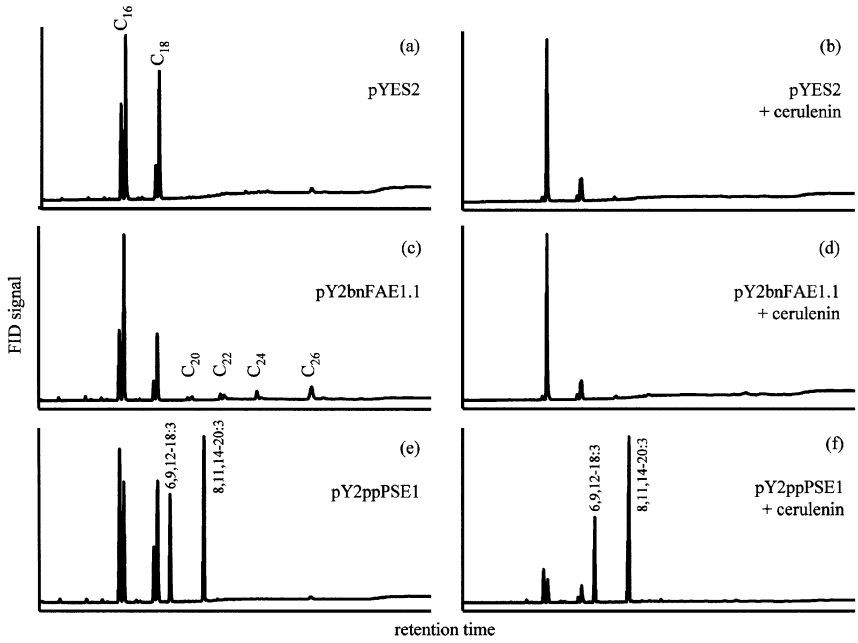

Figure 8. Fatty acid profiles of transgenic yeasts containing either the empty vector pYES2 (a+ b), pY2FAE1.1 expressing a β-ketoacyl-CoA synthase *FAE1.1* (Han *et al.*, 2001) from rapeseed (c+ d) or pY2PSE1 expressing *PSE1* from *P. patens.*
Expression experiments were performed in the absence (a, c, e) or in the presence (b, d, f) of 50 μM cerulenin. To ensure viability the culture medium was supplemented with a mixture of C_{16} and C_{18} fatty acids and in case of pY2PSE1 with a mixture of 16:0 and 18:3$^{\Delta 6,9,12}$. As previously demonstrated (Han *et al.*, 2001), the recombinant β-ketoacyl-CoA synthase readily elongated saturated and monounsaturated fatty acids up to C_{26} (c). This activity was completely lost when the expression was performed in the presence of cerulenin (d). In contrast, this inhibitory effect was not observed for Pse1p, which displayed high elongation activity with 18:3$^{\Delta 6,9,12}$ both in the absence (e) and presence (f) of cerulenin.

and consequently, the *Pse1* knockout mutant did not contain any C_{20} PUFAs at all. The disappearance of the C_{20} PUFAs was not accompanied by a visible change in the phenotype of the mutant plant grown under phytotron conditions. The complete loss of C_{20} PUFAs did also not result in a change of sensitivity of the moss towards different abiotic stress conditions such as cold, drought or high salt concentrations. Since the absence of a deviating visible phenotype cannot be ascribed to residual 20:4$^{\Delta 5,8,11,14}$, as possible in the Δ6-desaturase knockout mutant (Girke *et al.*, 1998), we conclude that C_{20} PUFAs are not essential for viability of the moss.

Complementation experiments, performed by providing 20:3$^{\Delta 8,11,14}$ to the knockout mutant, revealed that the Δ5-desaturase was not affected in the mutant plant, since the enzyme was able to convert exogenous 20:3$^{\Delta 8,11,14}$ to 20:4$^{\Delta 5,8,11,14}$. Despite good incorporation of 20:3$^{\Delta 8,11,14}$, its desaturation was much lower than in the wild-type, which may indicate substrate channelling from the Δ6-desaturase to the Δ5-desaturase via the Δ6-elongase. This is supported by the fact that almost no intermediates (i.e. 18:3$^{\Delta 6,9,12}$ and 20:3$^{\Delta 8,11,14}$) of the PUFA biosynthetic pathway can be detected in the wild-type moss. However, the nature of this substrate channelling remains to be elucidated.

We also isolated two *P. patens* cDNA clones with sequence similarities to the *KCS* genes. These clones did not result in any elongation activity during expression in *S. cerevisiae* (data not shown). This could simply be due to the fact that the *KCS* gene products are not able to interact with the endogenous yeast reductases and dehydratase but appears unlikely since the expression of *A. thaliana FAE1* and *B. napus FAE1.1* genes in yeast actually resulted in the elongation of fatty acids (Han *et al.*, 2001; Millar and Kunst, 1997). It is also possible that the substrates for the Kcs proteins (i.e. 22:0, 24:0, 26:0) are not available in high proportions in the yeast cells.

Both *KCS*-like and *ELO*-like genes are not only present in the genome of the moss but also in the genome of higher plants as evident from the *Arabidopsis* genomic and several plant EST-sequencing projects (corn, cotton, loblolly pine, soybean, tomato and wheat). This suggests that two different systems exist for fatty acid elongation in plants. It may well be that the *KCS* genes are involved in the supply of VLCFAs for waxes and seed storage lipids in plants, whereas the *ELO* genes might produce VLCFAs that are incorporated into membrane lipids, such as phosphatidylserine and sphingolipids (in the case of saturated fatty acids) and glycerolipids (in the case of PUFAs). This

hypothesis is supported by the lack of *KCS* genes in yeast (as evident from genomic sequencing), which do not contain any waxes. Furthermore, *KCS*-like genes are also absent from animals and fungi.

Elo proteins differ from Kcs proteins in different aspects. Elo proteins lack a clearly recognisable, active-site cysteine residue, and both families differ in their membrane topology. The highly hydrophobic Elo proteins form 5–7 transmembrane helices evenly spread between the N- and C-terminus, whereas in the Kcs proteins a small membrane anchor of 1–2 transmembrane helices is confined to the N-terminus. The TMHMM2.0 server places the active-site cysteine of most Kcs proteins to the luminal site of the ER membrane, which raises questions about the sidedness of VLCFA biosynthesis. On the other hand, this orientation changes with the prediction program used and thus requires experimental elucidation.

The absence of an inhibitory effect of cerulenin on the Pse1 activity in yeast may either be trivial or more significant. If it can be ascribed to differences in accessibility and hydrophobicity of an active centre shielding an active-site cysteine as actual target of cerulenin, then this observation would place Pse1 in line with other cerulenin-insensitive condensing enzymes. On the other hand, cerulenin-insensitivity and absence of an active-site cysteine may suggest a cysteine-independent reaction mechanism not requiring a transient transfer of the acyl group from a coenzyme A to an enzyme-bound cysteine thioester. Interaction of the CoA-thioester carbonyl group with appropriately placed active-site residues would increase its electrophilicity in such a way that attack by the malonyl-CoA derived carbanion could result in the condensation reaction with release of CoASH. In this context it should be pointed out that the actual state of the acyl-group required by the condensing enzyme (acyl-CoA or acyl-X) is still a matter of debate (Domergue *et al.*, 1999; Hlousek-Radojcic *et al.*, 1995), at least when using plant microsomal membranes as enzyme source. Therefore, it would be highly desirable to develop *in vitro* assays based on purified/recombinant enzymes to measure this first reaction of fatty acid elongation.

Experimental procedures

Materials

Restriction enzymes, polymerases and DNA modifying enzymes were obtained from New England Biolabs (Frankfurt A.M., Germany). All other chemicals were purchases from Sigma (St. Louis, MO, USA) unless indicated otherwise. Columbinic acid ($18{:}3^{\Delta 5 trans,9,12}$) and pinolenic acid ($18{:}3^{\Delta 5 cis,9,12}$) were isolated as methyl esters from seeds of *Aquilegia vulgaris* and *Larix decidua*, respectively, by preparative RP-HPLC on an ODS Hypersil column (5 μm) using methanol/acetonitrile/H_2O (9:1:2; 1 ml min^{-1}) as solvent. *all-cis*-Hexadecatrienoic acid ($16{:}3^{\Delta 7,10,13.}$) was purified from monogalactosyl diacylglycerol of parsley in the same way. The FAMEs were reconverted into free fatty acids by alkaline hydrolysis with 0.1 N KOH in methanol/H_2O (1:1).

Synthesis of homoconjugated trienoic fatty acids: general procedure

Trienoic fatty acids with an ensemble of homoconjugated double bonds in different positions of the molecule were prepared along a recently described 3-component Wittig approach (Pohnert and Boland, 2000). A symmetric *bis*-ylide is sequentially reacted with equivalents of two different aldehyde components yielding the required trienoic fatty acid in a single operation. The overall strategy is outlined in Figure 4. (i) Preparation of the *bis*-ylide: A cold (– 78°C) and well stirred suspension of the Wittig salt hex-3-enyl-1,6-*bis*-(triphenylphosphonium iodide) 1 (0.86 g, 1 mmol) in dry THF was gradually treated with $KN(SiMe_3)_2$ in hexane (4.4 ml of a 0.5-M solution, 2.2 mmol). The reaction mixture was allowed to warm to room temperature over a period of 30 min, stirred for 30 min and re-cooled to –78°C. (ii) Generation and reaction of the first aldehyde equivalent by ester reduction: In a second flask, a cold solution (–78°C) of the respective methyl ester (1 mmol) was gradually treated with pre-cooled (– 78°C) diisobutylaluminium-hydride (1 ml of a 1-M solution in hexane, 1 mmol). After being stirred for 10–60 min (progress of the reaction was monitored by GLC) the cold aluminate (– 78°C) was transferred quickly to the above solution of the *bis*-ylide using a pre-cooled cannula. The mixture was allowed to warm to room temp. over a period of 90 min, and stirring was continued for 30 min to 1 h (GLC-control) before re-cooling to –78°C. (iii) Reaction of the second aldehyde moiety: Then a solution of the aldehyde component (1.2 mmol in 1 ml THF) was added, the mixture was allowed to reach room temperature and stirred for 30 min. Hydrolysis with HCl (2 N), extraction with ether, drying over sodium sulphate and flash chromatography on silica gel (light petroleum:diethyl ether, 9:1, v:v) yielded the methyl ester of the homoconjugated trienoic fatty acid in 28–45% yield. Saponification was achieved by hydrolysis of the methyl ester (10 mg) with KOH (2 ml of a 1-M solution in ethanol/water, 95:5, v:v) for 60 min at 50°C. The free acid was purified by HPLC (RP 18) using a solvent gradient from MeOH/H_2O (90:10) to pure MeOH in 20 min. Elution was monitored at 215 nm. Yield: 90–95%.

Details of the synthesis of the individual fatty acids and their spectroscopic data are available as supplementary material in appendix 1 (see URL for supplementary material below).

Plant material and growth condition

P. patens (Hedw.) B.S.G. was grown axenically on agar plates or in agitated liquid Knop or complete medium (Reski *et al.*, 1994; Schween *et al.*, 2002) at 25°C or 15°C under long-day light (16 h) conditions. Feeding experiments with fatty acids were performed as described earlier (Girke *et al.*, 1998).

Stress treatment of Physcomitrella patens

Stress treatment of *P. patens* was performed as described elsewhere (Frank and Reski, 2002). For salt stress treatments plants cultured on solid Knop medium were transferred onto Knop medium supplemented with increasing concentrations of NaCl (200 mM to 1 M NaCl in 50 mM steps). After 3 days the plants were

re-transferred onto standard Knop medium and screened for survivors. The dehydration treatment was performed by drying the plants on solid Knop medium for 4 h, 8 h, 16 h, 24 h and 48 h, respectively, followed by a 1 h rehydration step in sterilised tap water and transfer of the plants onto standard solid Knop medium. After 2 weeks of recovery on Knop medium the plants were screened for survivors. During all dehydration treatments plants were weighed at designated time intervals to determine the loss of water.

cDNA library construction

Total RNA was isolated from 9-day-old protonemata from *P. patens* wild-type following the GTC-method (Reski *et al.*, 1994). Poly A+ RNA were obtained using Dynabeads^{R} (Dynal, Oslo, Finland) following the instructions of the manufacturer. For cDNA library construction first strand synthesis was achieved using Murine Leukemia Virus reverse transcriptase (Roche, Mannheim, Germany), second strand synthesis by incubation with DNA polymerase I and Klenow enzyme, followed by RNaseH digestion. The cDNA was blunted by T4 DNA polymerase (Roche, Mannheim), and *Eco*RI adapters (Pharmacia, Freiburg, Germany) were ligated to the cDNA by T4 DNA ligase. After phosphorylation with polynucleotide kinase and gel separation, DNA molecules larger than 300 bp were ligated into vector arms and packed into lambda ZAPII phages using the Gigapack Gold Kit (Stratagene, Amsterdam, The Netherlands).

DNA sequencing and computational analysis

The cDNA library was used for DNA sequencing by the chain termination method using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Weiterstadt, Germany). Random sequencing was carried out subsequent to plasmid recovery from the cDNA library via *in vivo* mass excision and retransformation of *E. coli* DH10B. Plasmid DNA was prepared on a (Qiagen, Hilden, Germany) DNA preparation robot (Qiagen, Hilden) according to the manufacturers' protocol. Sequences were processed and annotated using the standard software package EST-MAX commercially provided by Bio-Max (Munich, Germany). A more detailed description of the EST database of *P. patens* is given in Rensing *et al.*, in press.

This EST database was used to identify cDNA clones involved in the elongation of PUFAs. For this purpose the database were screened with sequences of the *ELO1* gene from *S. cerevisiae* (Acc. No. NP_012339) or the *FAE1* gene of *A. thaliana* (Acc. No. T05272) using the BLAST algorithm (Altschul *et al.*, 1990).

Expression of PSE1 *in* S. cerevisiae

For expression in yeast, the *P. patens* cDNA clone *PSE1* was cloned behind the galactose-inducible promoter GAL1 of the yeast expression vector pYES2 (Invitrogen, Leek, The Netherlands). For this purpose, the open reading frame (ORF) was modified by PCR to create a *Bam*HI restriction site and the yeast consensus sequence for enhanced translation (Donahue and Cigan, 1990) adjacent to the start codon and a *Bam*HI restriction site flanking the stop codon. The amplified DNA was first cloned into pUC18/*Sma*I using the SUREClone Ligation Kit (Pharmacia) resulting in pUC18/PSE1. The ORF was recovered by *Bam*HI restriction and cloned into the *Bam*HI site of pYES2 to yield pY2PSE1. The sequence of the cloned PCR product was confirmed by re-sequencing as described above. *S. cerevisiae* INVSc1 (MATa *his3Δ1 leu2 trp1–289 ura3–52*, Invitrogen, Leek, The Netherlands) was transformed with plasmid DNA by a modified PEG/lithium acetate protocol (Ausubel *et al.*, 1995). After uracil selection on minimal medium agar plates (Ausubel *et al.*, 1995), cells harbouring the yeast plasmid were cultivated in minimal medium lacking uracil but containing 2% (w/v) raffinose and 1% (v/v) Tergitol NP-40. The expression was induced by supplementing galactose to 2% (w/v) when the cultures had reached an optical density (at 600 nm) of 0.2–0.3. At that time, the appropriate fatty acids were added to a final concentration of 500 μM, unless indicated otherwise. All cultures where then grown for a further 24 h at 30°C and used for fatty acid analysis (see below). To analyse the effect of cerulenin on elongation activity in yeast cells expressing *PSE1*, the expression was performed as described above but in the presence of 50 μM cerulenin, 100 μM 16:0 and 400 μM $18{:}3^{\Delta 6,9,12}$, which were added at the time of induction. The same experiment was performed with a *FAE1.1* gene from *B. napus* cv. HEAR, that was previously shown to elongate saturated and monounsaturated fatty acids in *S. cerevisiae* INVSc1 (Han *et al.*, 2001). The only difference was, that the medium was supplemented with a fatty acid mixture to result in final concentrations of 100 μM 16:0, 200 μM 16:1, 100 μM 18:0 and 200 μM 18:1.

Targeted gene disruption of PSE1 *in* P. patens

For disruption of *PSE1* by homologous recombination, the ORF of *PSE1* was first modified by PCR as described above and subsequently blunt-end ligated into pBluescript/*Eco*RI/*Hin*cII. A 223-bp *Hin*dIII-fragment within the ORF of *PSE1*, containing the conserved histidine-box, was then replaced by the *npt*II cassette, which was obtained from the vector pRT101neo (Girke *et al.*, 1998) by *Hin*dIII digestion. The disrupted ORF was recovered from the plasmid by digestion with *Bam*HI. This resulted in a linear fragment with the selection cassette flanked by 256 bp and 411 bp homologous to the 5′- and 3′-ends of *PSE1*, respectively. The DNA was used for PEG-mediated transformation of moss protoplasts. Transformations were performed with 3×10^5 cells and 25 μg of DNA. Moss protonema culture, protoplast isolation, transformation, and time intervals for regeneration, selection, and release treatments were carried out as described (Strepp *et al.*, 1998). Selection plates were supplemented with 50 μg ml^{-1} G418.

Molecular analysis

The homologous recombination event was analysed by polymerase chain reaction (PCR) and Southern blot analysis. For this purpose, genomic DNA of wild-type and transgenic plants were extracted with cetyl-trimethyl-ammonium bromide (Rogers and Bendich, 1988). The 5′-integration event was confirmed by PCR experiments with primers derived from the *npt*II coding region and the 5′-untranslated region of *PSE1*. PCR incubations were run with a touch down program of 3 min denaturation at 96°C followed by 20 cycles of 30 s at 96°C, 1 min at 60°C (–1°C/cycle), 1 min at 72°C and a further 30 cycles of 30 s at 96°C, 1 min at 40°C, 1 min at 72°C and terminated by 10 min at 72°C. The origin of the PCR fragment was confirmed by sequencing. For Southern blot analysis 3 μg of genomic DNA of *P. patens* were digested with *Apa*LI. Separation, hybridisation and detection were performed as described earlier (Girke *et al.*, 1998). The DNA probe was labelled with digoxygenin by PCR using the PCR DIG probe synthesis kit (Roche) with the ORF of *PSE1* as template.

Fatty acid analysis

Fatty acid methyl esters (FAMEs) were obtained by transmethylation of yeast cell sediments or intact moss plants with 0.5 M sulphuric acid in methanol containing 2% (v/v) dimethoxypropane at 80°C for 1 h. FAMEs were extracted into petroleum ether and analysed by gas-liquid chromatography using a Hewlett-Packard 6850 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame-ionisation detector and a polar capillary column (ZB-Wax, 30 m × 0.32 mm i.d., 0.25 μm film, Phenomenex, Torrance, CA, USA). Data were processed using the HP ChemStation Rev. A 06.03. FAMEs were identified by comparison with appropriate reference substances or by GC-MS of their 4,4-dimethyloxazoline derivatives as described elsewhere (Sperling *et al.*, 2000). The percentage of elongation was calculated as percentage product × 100/(% substrate + % product).

Supplementary Material

The following material is available from http://www.blackwell-science.com/products/journals/suppmat/TPJ/TPJ1354/TPJ1354sm.htm

Appendix 1 Synthesis and spectroscopic data of homoconjugated trienoic fatty acid

Table S1 Accession-Numbers of proteins used for the sequence comparison shown in Figure 2

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