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Biosynthesis of C9-aldehydes in the moss *Physcomitrella patens* ☆

Michael Stumpe [a], Julia Bode [a], Cornelia Göbel [a], Thomas Wichard [b], Andreas Schaaf [c], Wolfgang Frank [c], Markus Frank [d], Ralf Reski [c], Georg Pohnert [b,1], Ivo Feussner [a,*]

[a] *Georg-August-University Göttingen, Albrecht-von-Haller-Institute for Plant Sciences, Department of Plant Biochemistry, Justus-von-Liebig-Weg 11, D-37077 Göttingen, Germany*
[b] *Max-Planck-Institut für Chemische Ökologie, Hans-Knöll-Strasse 8, D-07745 Jena, Germany*
[c] *Freiburg University, Plant Biotechnology, Schaenzlestrasse 1, D-79104 Freiburg, Germany*
[d] *BASF Plant Science GmbH, D-67056 Ludwigshafen, Germany*

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Abstract

After wounding, the moss *Physcomitrella patens* emits fatty acid derived volatiles like octenal, octenols and (2*E*)-nonenal. Flowering plants produce nonenal from C18-fatty acids via lipoxygenase and hydroperoxide lyase reactions, but the moss exploits the C20 precursor arachidonic acid for the formation of these oxylipins. We describe the isolation of the first cDNA (*PpHPL*) encoding a hydroperoxide lyase from a lower eukaryotic organism. The physiological pathway allocation and characterization of a downstream enal-isomerase gives a new picture for the formation of fatty acid derived volatiles from lower plants. Expression of a fusion protein with a yellow fluorescent protein in moss protoplasts showed that PpHPL was found in clusters in membranes of plastids. PpHPL can be classified as an unspecific hydroperoxide lyase having a substrate preference for 9-hydroperoxides of C18-fatty acids but also the predominant substrate 12-hydroperoxy arachidonic acid is accepted. Feeding experiments using arachidonic acid show an increase in the 12-hydroperoxide being metabolized to C8-aldehydes/alcohols and (3*Z*)-nonenal, which is rapidly isomerized to (2*E*)-nonenal. PpHPL knock out lines failed to emit (2*E*)-nonenal while formation of C8-volatiles was not affected indicating that in contrast to flowering plants, PpHPL is only involved in formation of a specific subset of volatiles.

Keywords: Nonenal; Cytochrome P-450; Lipid peroxidation; Oxylipin metabolism; Moss; Substrate specificity

1. Introduction

Oxylipin is a collective term for oxygenated metabolites derived from polyunsaturated fatty acids (PUFAs). Most oxylipins are bioactive compounds involved in signal and defense reactions in mammals, higher plants and algae [1–3]. The biosynthesis of many oxylipins is initiated by the conversion of PUFAs by a lipoxygenase (LOX) [4]. While in flowering plants C18-fatty acids like linoleic (18:2) and α-linolenic acid (α-18:3) are the main precursors of such compounds, in animals and algae oxylipins derive predominantly from C20-fatty acids [1,2,5].

In plants LOX-derived hydroperoxides serve as substrates for at least 7 enzyme families, including three subfamilies of *CYP74*-type of cytochrome P-450: hydroperoxide lyase (HPL), allene oxide synthase (AOS) and divinyl ether synthase (DES) [6]. In contrast to typical P-450 monooxygenases, *CYP74*-enzymes require neither O_2 nor NADPH as cofactors [4]. Instead, the hydroperoxide group of the fatty acid substrate serves as oxygen donor and as a source of reducing equivalents.

By trapping experiments Grechkin and Hamberg [7] have recently found that HPL catalyzes the isomerization of fatty acid hydroperoxide into an unstable hemiacetal, which is spontaneously

Abbreviations: AOS, allene oxide synthase; DES, divinyl ether synthase; DNPH, 2,4-dinitrophenylhydrazin; H(P)ETE, hydro(pero)xy-arachidonic acid; HPL, hydroperoxide lyase; H(P)ODE, hydro(pero)xy-linoleic acid; H(P)OTE, hydro(pero)xy-linolenic acid; LOX, lipoxygenase

☆ The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank with accession number CAC86920.

* Corresponding author. Tel.: +49 551 39 5743; fax: +49 551 39 5749.
E-mail address: ifeussn@gwdg.de (I. Feussner).
[1] Present address: Institute of Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland.

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decomposed into a short chain aldehyde and the corresponding ω-oxo fatty acid [7], whereas Noordermeer and co-workers [8] have proposed the formation of an allylic ether cation. In contrast, the AOS converts hydroperoxides to unstable allene oxides, which are hydrolyzed into α- and γ-ketols or can spontaneously undergo non-enzymatic cyclization [9]. In the presence of an allene oxide cyclase, the allene oxide is metabolized to 12-oxo phytodienoic acid. Subsequent reduction of 12-oxo phytodienoic acid by a reductase and three cycles of β-oxidation yields jasmonic acid [9]. DES produces divinyl ether which can be cleaved into short chain aldehydes and ω-oxo fatty acids under acidic conditions [10]. Several *CYP74*-enzymes have been cloned from different plant species. Some of them contain plastidic transit peptides, for instance an AOS from *Arabidopsis thaliana* [11]. A plastidic association was shown for the AOS and the HPL from tomato and for the AOS from barley [12,13].

In flowering plants, the basic substrates for *CYP74*-enzymes are 13- or 9-hydroperoxides of linoleic or α-linolenic acid. Based on their substrate specificity the enzymes may thus be classified into 13- or 9-hydroperoxide-specific enzymes, or unspecific enzymes. The short chain aldehydes of the HPL metabolization of 13-hydroperoxy linoleic (13-HPODE) or 13-hydroperoxy α-linolenic acid (13-HPOTE) are hexanal and (3*Z*)-hexenal, respectively [14]. The second resulting fragment, a C12 ω-oxo fatty acid may isomerize to traumatin, a wound hormone [15]. The volatile reaction products of 9-hydroperoxides (9-HPODE or 9-HPOTE) are (3*Z*)-nonenal and (3*Z*,6*Z*)-nonadienal, respectively. As in the case of traumatin the unsaturated (3*Z*)-aldehydes can further be metabolized by an isomerase into the (2*E*)-enal isoforms [16].

Short chain aldehydes are components of the green odor of plants and have an antimicrobial effect in vitro. The physiological function of aldehydes is mainly addressed to plant defense against microbes and herbivores [3,17]. This effect has, for example, been demonstrated upon inoculation of French bean with an avirulent variety of *Pseudomonas syringae* pv. *syringae*. Whereas this treatment resulted in production of C6-aldehydes, a virulent strain did not lead to such an increase [18]. Different C6-volatiles have also been shown to induce defense related genes and to stimulate phytoalexin accumulation [19,20]. In transgenic potato plants lacking *HPL* transcript an increase in aphid performance was observed [21]. Recently, the antimicrobial effect of C9-volatiles has been described as well [22].

In contrast to other eukaryotic organisms, the knowledge on the biosynthesis and function of oxylipins in mosses is limited. These organisms contain C18, C20, and longer chain length fatty acids [23]. In the moss *Physcomitrella patens*, for example, the main fatty acids are palmitic acid, linoleic acid and arachidonic acid (20:4) [24]. Certain mosses are known to emit aldehydes and their corresponding alcohols, like octenal, octenol but also hexanal and hexenal [23]. Recently, it was shown that the moss *P. patens* produces oct-1-en-3-ol, oct-2-en-1-ol and (2*E*)-nonenal after wounding. These volatiles are all derived from arachidonic acid [25]. While the octenols may be produced by the action of a novel LOX with a lyase activity that has been described recently [26], the enzyme responsible for C9-volatile production is still unknown.

Here, we present an in depth investigation of the second line of transformation of arachidonic acid hydroperoxides in *P. patens*. We introduce the isolation, characterization and localization of a new HPL from the moss and elucidate the further fate of the HPL products. The substrate specificity against hydroperoxides of different fatty acids was analyzed and we show that the enzyme is localized at a inner membrane of chloroplasts. Using a knock-out mutant for this HPL, we demonstrate that the release of (2*E*)-nonenal after wounding is mediated by this enzyme and is derived from 12-hydroperoxy arachidonic acid (12-HPETE).

2. Material and methods

2.1. Isolation, expression and purification of recombinant PpHPL

An EST-library from *P. patens* was provided by BASF Plant Science, and a partial EST-clone with a putative *CYP74*-sequence was identified based on sequence similarity to known *CYP74*-sequences from flowering plants. A 1470 bp PCR fragment was amplified with the sense primer 5′-CGT ACG GTT GTA GCC AGT CTT GGG-3′ and the antisense primer 5′-TCA ATC TGA TCG CGG CGT CAG TG-3′ using the partial EST-clone as a template. A ^{32}P-labeled cDNA probe of the PCR fragment was synthesized using HexaLabel DNA Labeling Kit from Fermentas and used to screen a lambda ZAPExpress cDNA library of moss gametophytes. The longest insert (*PpHPL*) was sequenced and used as a template in a PCR-based approach to construct a vector for recombinant expression of PpHPL: Forward primer (5′-GGA TCC ATG GAT CGC ACT TTA GTT C-3′) and reverse primer (5′-AAG CTT TCA ATC TGA TCG CGG CGT CAG TG-3′) for the amplification introduced *Bam*HI and *Hin*dIII restriction sites, respectively. To express a truncated protein, starting at amino acid 44, a primer was designed that introduced a *Bam*HI restriction site. Using these restriction sites it was possible to clone *PpHPL* and the truncated cDNA in-frame into the pQE30 expression vector. For heterologous expression, pQE30-PpHPLfull and pQE30-PpHPLtrunc was transformed into *E. coli* host strain SG13009 pREP4. Purification of recombinant PpHPL was performed as described previously [27].

2.2. Product Analysis of recombinant PpHPL

To produce fatty acid hydroperoxides, the corresponding fatty acids were incubated with recombinant cucumber lipid body LOX (production of 15-, 12-, 8-HPETE, 13-HPOD/TE, 13-γHPOTE) or potato tuber LOX (production of 11-, 5-HPETE, 9-HPOD/TE, 9-γHPOTE) [28]. Labeled fatty acid hydroperoxides were prepared by incubation of [1-^{14}C]-linoleic acid (Perkin Elmer Life Science, Boston, MA, USA; specific activity 2.0 GBq/mmol) with either soybean 13-LOX (Sigma, Germany) or recombinant potato tuber LOX [28]. Radiolabeled fatty acid was diluted with unlabeled linoleic acid in a mol-ratio 1:50.

For product analysis, recombinant PpHPL as well as 13-AOS (CAD29735), 9-AOS (CAI30876), 13-DES (CAI30435) and 9-DES (CAC28152), diluted in 2 ml of 100 mM sodium phosphate buffer was incubated with either 100 nmol [1-^{14}C]-13-HPODE or [1-^{14}C]-9-HPODE for 30 min at room temperature. The reaction was stopped by adding 40 μl of glacial acetic acid and extracted with methanol/chloroform [29]. The obtained samples were dissolved in HPLC solvent and analyzed as described [30]. For determination of kinetic parameters of the recombinant PpHPL, the initial reaction velocity was determined at 12 different concentrations. For substrate specificity assay recombinant enzyme was incubated with 30 μM of each hydroperoxide, and the decrease in absorbance at 234 nm was measured spectrophotometrically.

To identify the aldehydes produced, the reaction mixture was acidified to pH 3 with HCl and incubated with 2.5 ml ethanol containing 0.1% 2,4-dinitrophenylhydrazin (DNPH) for 1 h at room temperature. Heptanal was added as an internal standard. The hydrazones were extracted twice with hexane, evaporated under a stream of nitrogen and subjected to HPLC analysis [31]. ESI/MS analyses were performed by direct injection of derivatized aldehydes into ESI interface of an LCQ ion trap mass spectrometer (Thermo Electron,

Germany). The mass spectrometer was operated in negative ion mode with the source voltage set to 4.5 kV, and a capillary voltage of 40 V and 300 °C. In full MS mode, scans were collected between *m*/*z* values of 150 and 500.

2.3. Transient expression of YFP fusion constructs in P. patens and microscopy

The entire cDNA without the stop-codon was amplified containing *Nco*I restriction sites at the 5′ and 3′ ends. This fragment was ligated into the *Nco*I-restricted vector pCAT_YFP [32] in order to express PpHPL as YFP-fusion protein. Plasmid DNA from this construct and from pCAT_YFP carrying no insert were transiently transfected into *P. patens* protoplasts as described previously [33]. Localization of YFP and its fusions in transfected protoplast was analyzed after 3 days of expression by confocal laser scanning microscopy according to [34].

2.4. Creating and analyzing PpHPL-knock-out mosses

A region of the PpHPL cDNA comprising the nucleotides 372 to 1710 was amplified by PCR using the following primers: 5′-TGG GGC AAG AGA GCA ATC TG-3′ (forward primer) and 5′-AAA GCT CCG CCA GAA GAA GG-3′ (reverse primer). The PCR product was cloned into the *Eco*RV restriction site of pGEM-5Zf(+) (Promega). The selection marker cassette (*nos*-promoter::neomycin phosphotransferase::*nos*-terminator) derived from the vector pBIN19 [35] was cloned into the *Mls*I site of the *PpHPL* cDNA fragment. A resulting construct showing conformity of the selection cassette with the cDNA orientation was selected for plant transformation. Before transformation, the construct was digested with *Aat*II and *Not*I to release the disruption construct. 25 μg of DNA were used for plant transformation. The transformation of *Physcomitrella* protoplasts and selection of transgenic lines was performed according to [36]. Using gene-specific primers, transformants were screened in addition for lost of wild type HPL allele band and incorporation of the nptII cassette into the *PpHPL*-locus. Further analyses were performed with several independent transformants to exclude effects resulting from non-homologous recombination events.

2.5. Analytical methods

Moss cultures were grown under sterile conditions [37]. For determination of endogenous fatty acid, hydro(pero)xides 3 to 4 g of moss material was analyzed as described [38]. To analyze lyase products after feeding of different fatty acids, 4 ml of a suspension culture of each transformant was filtered to give 100–150 mg fresh weight biomass, which was resuspended in 1 ml fresh medium. The samples were cooled to 4 °C and treated with ultrasound for 2 min using a Labsonic L 1000 ultrasonic oscillator (B. Braun, Germany) and 200 μg of fatty acid was added. To characterize the emitted volatiles, 2 μl of internal standard (1 mM 2-decanon in methanol) was added, the vial was sealed with a Teflon septum, and a polydimethyldiloxane-coated fiber (Supelco, Germany) was inserted for 15 min in the gas phase [25]. Separation was performed on a Finnigan Trace GC/MS equipped with a 15 m EC5 column ID 0.25 m, 0.25 μm film thickness (Alltech, Deefield, Il). The temperature program started at 40 °C (4 min) and ramped to 120 °C with a rate of 5 K min^{-1}, then with 20 K min^{-1} to 280 °C (2 min).

Products were identified by comparison with commercial available (Figs. 7 and 8: 1,2,4) and synthesized (Fig. 7: 3,8) standards or tentatively assigned by

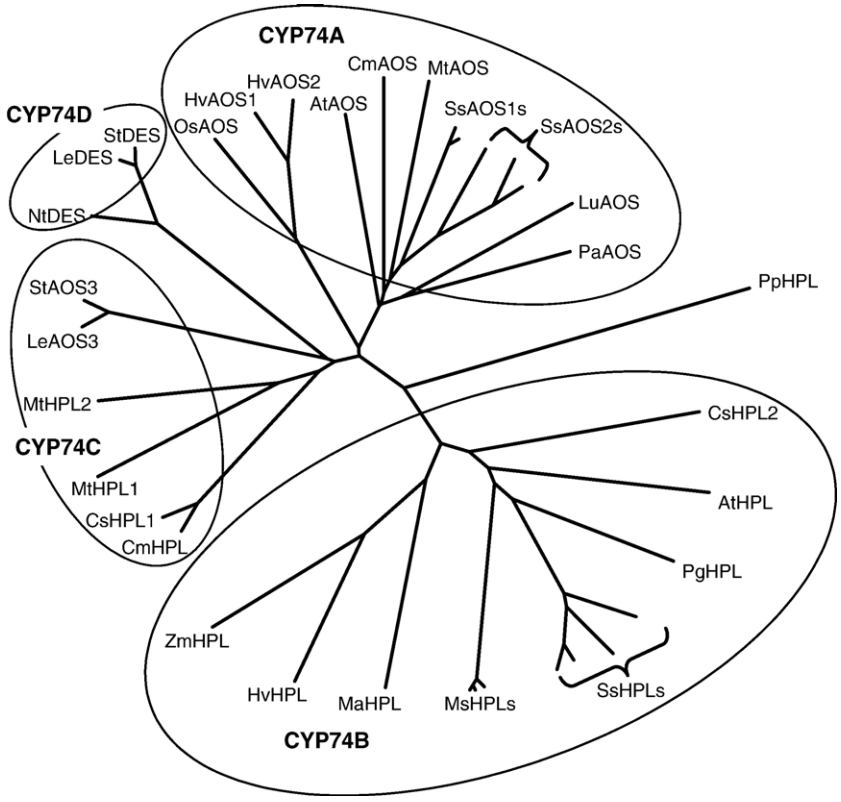

Fig. 1. Phylogenetic relationship of *CYP74* families. Amino acid sequences were aligned using ClustalX [58]. The phylogram was constructed using Treeview [59]. Based on sequence identity four *CYP74*-subfamilies (A to D) are indicated. Amino acid sequences corresponding to the following Acc.-No. were used for the analysis: *A. thaliana* (AtAOS: CAA63266, AtHPL: AAC69871), melon (CmAOS: AAM66138, CmHPL: AAK54282), cucumber (CsHPL1: AAF64041, CsHPL2: AF229812), barley (HvAOS1: CAB86384, HvAOS2: CAB86383, HvHPL: CAC82980), tomato (LeAOS3: AAN76867, LeDES: AAG42261), flax (LuAOS: AAA03353), banana (MaHPL1: CAB39331), *Medicago sativa* (MsHPLs: CAB54847, CAB54848, CAB54849), *Medicago truncatula* (MtAOS: TC22359, MtHPL1: CAC86898, MtHPL2: CAC86899), tobacco (NtDES: AAL40900), rice (OsAOS: AAL38184), guayule (PaAOS: CAA55025), *Psidium guajava* (PgHPL: AAK15070), *Physcomitrella patens* (PpHPL: CAC86920), different Solanaceae species (SsAOS1s: CAB88032, CAD29735, SsAOS2s: AAF67141, CAD29736, CAC82911, SsHPLs: AAF67142, AAA97465, CAC44040, CAC91565), potato (StAOS3: CAI30876, StDES: CAC28152), maize (ZmHPL: AAS47027).

MS-spectra (Fig. 9: 5,7) and UV absorption according to [22]. (3*Z*)-nonenal and 12-ODTE were synthesized according to [39]. For trapping of (3*Z*)-enal products and for structure elucidation of the ω-oxo fatty acids the filtered moss was resuspended in 1 ml derivatization reagent (25 mM pentafluorobenzyl hydroxylamine in 100 mM Tris/HCl, pH 7) and treated as described above after addition of 5 µl internal standard (1 mM benzaldehyde in MeOH). Extraction of oxime-derivates, subsequently derivatization treatments and analyses by GC/MS were done according to [40].

3. Results

3.1. Isolation and characterization

Previous studies suggested that the moss *P. patens* might contain a second 12-HPETE metabolizing activity besides a multifunctional arachidonate 12-LOX [26], which is involved in the formation of (2*E*)-nonenal [25]. As this conversion is rather unusual, we set out to clone the corresponding gene of a putative HPL, analyze the recombinant protein and gain insight into the fatty acid-derived aldehyde and alcohol formation in mosses. In an EST database we found a partial clone with similarity to known *CYP74*-enzymes from flowering plants. To isolate the full-length cDNA clone we screened a cDNA library of *P. patens* gametophytes. Of about 1×10^6 plaques screened, 12 positives were isolated and partially sequenced. The isolates were identical in the overlapping regions. The longest insert sequenced had a length of 1975 bp excluding its poly(A) tail. The coding sequence started at position 190 and ended at position 1599 of the isolated cDNA fragment. A stop-codon located 30 bp upstream of the start-Met indicated that the clone is full length. The deduced protein consists of 532 amino acids and has a predicted molecular mass of about 59.9 kDa. The amino acid sequence was most similar to that of AOS from *Arabidopsis thaliana* (61%; Acc.-No. CAA73184) and *Cucumis melo* (60%; Acc.-No. AAM66138). PpHPL contained typical *CYP74* motifs and amino acids within its sequence, however, the amino acid sequence did not reliable group into any existing subfamily (*CYP74*A to *CYP74*D). Currently, enzymes are grouped into subfamilies based on sequence identity higher than 55% (an arbitrary definition known from mammalian cytochrome P450 enzymes). PpHPL exhibits less than 50% amino acid identity to other *CYP74*s, and therefore cannot easily be categorized into the known subfamilies using the established criteria of sequence identity (Fig. 1). The N-terminal 39 amino acids of PpHPL were recognized as a plastidic transit peptide by the signal peptide prediction program TargetP (http://www.cbs.dtu.dk/services/).

When either the full length protein or the truncated protein lacking the plastidic transit peptide was expressed in *E. coli* only the truncated protein metabolized fatty acid hydroperoxides. Therefore, all further analyses were performed with the truncated protein. Using [1-^{14}C]-13-HPODE as well as [1-^{14}C]-9-HPODE as a substrate we tested the enzymatic activity of the recombinant enzyme. The only products found by radio-HPLC analysis were (9*Z*)-12-oxo-9-dodecenoic acid and 9-oxo nonanoic acid, respectively, the expected reaction products of a HPL enzyme. Ketols, products of AOS activity, or divinyl ethers resulting from DES activity were not observed (Fig. 2). We conclude from these results that the isolated cDNA encodes for an HPL. In order to characterize the range of accepted substrates for the novel HPL enzyme, different hydroperoxides of linoleic and α-linolenic acid were tested as substrates and the

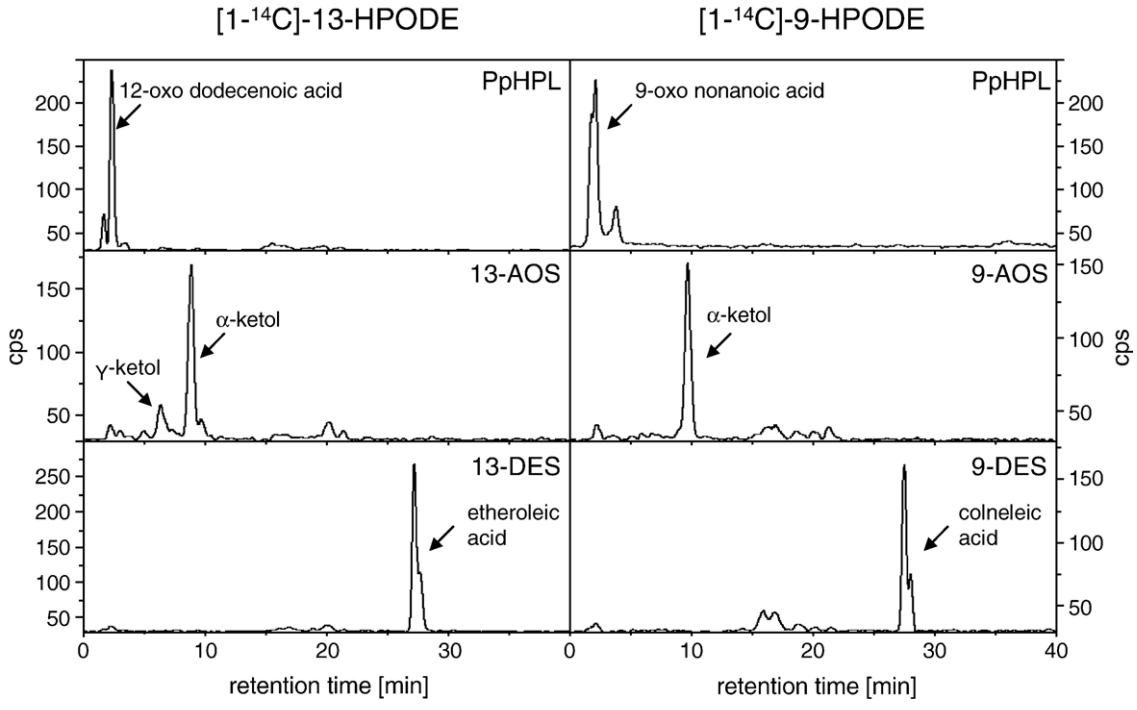

Fig. 2. Product analysis of PpHPL. Radiochromatograms of products formed after incubation of either [1-^{14}C]-13-HPODE or [1-^{14}C]-9-HPODE with recombinant enzymes as indicated.

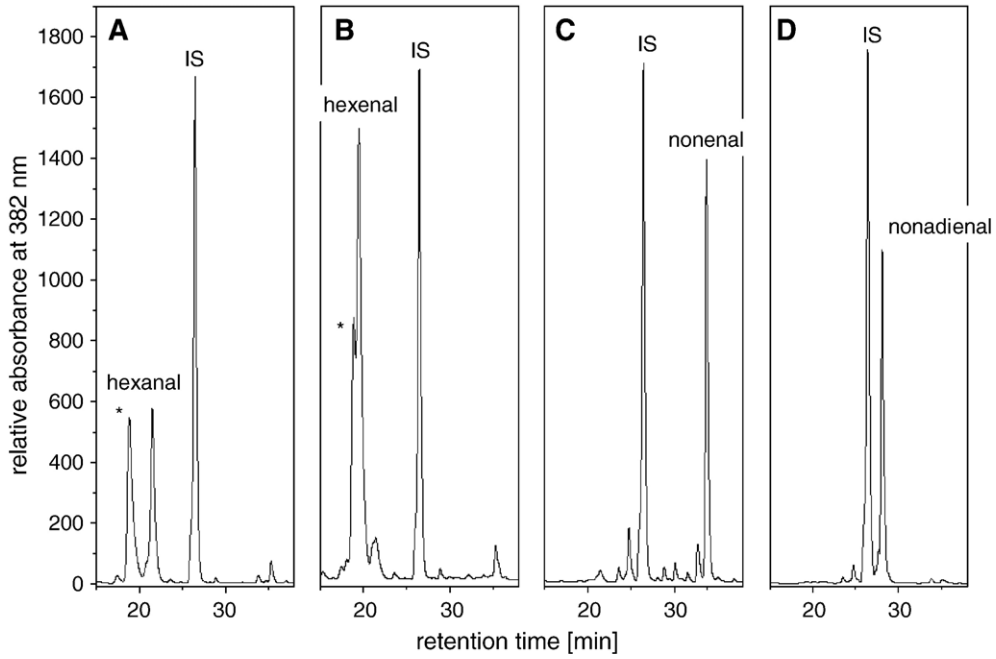

Fig. 3. Analysis of formed aldehydes. HPLC-analysis after incubation of recombinant PpHPL with 13-HPODE (A), 13-HPOTE (B), 9-HPODE (C) and 9-HPOTE (D). Samples were derivatized with DNPH before analysis. IS = internal standard (heptanal). * = 12-oxo-9-dodecenoic acid.

resulting aldehydes were detected as DNPH-derivatives in HPLC-analyses (Fig. 3). All tested fatty acid hydroperoxides of linoleic and α-linolenic acid were metabolized by the enzyme indicating that this HPL may belong to the group of unspecific 9/13-HPLs. Using 9-HPODE as substrate, we determined the pH optimum as well as kinetic parameters of the recombinant protein spectrophotometrically. PpHPL exhibited a pH optimum of about pH 8.0. Assuming the total protein content of purified recombinant PpHPL to be active, the specific activity was 532.3 U/mg and the K_m was 61.7 μM.

To gain further insight into the substrate specificity of the enzyme in addition to the previous substrates, the hydroperoxides of γ-linolenic and arachidonic acid were tested. The majority of hydroperoxides tested served as substrates and were

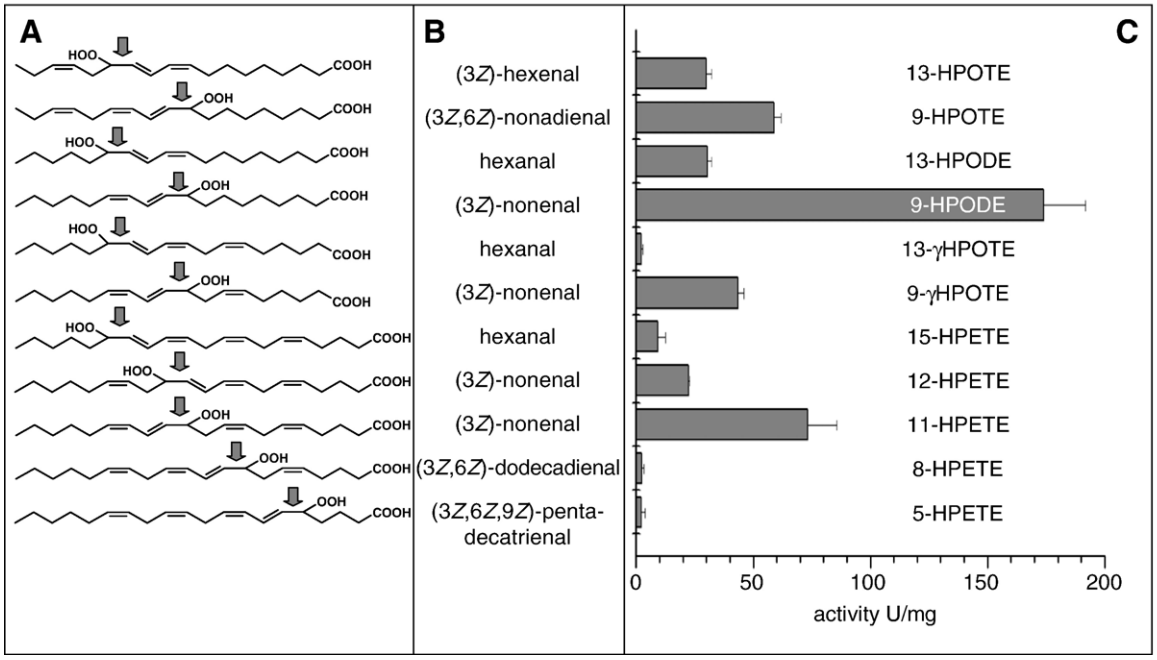

Fig. 4. Substrate specificity of recombinant PpHPL. (A) Structures of hydroperoxide substrates administered, the arrows indicate cleavage site by PpHPL; (B) Aldehydes formed after incubation with correspondent hydroperoxide. Aldehydes were identified as DNPH-derivatives by HPLC-DAD or HPLC-MS, respectively; (C) Relative activity of PpHPL against hydroperoxides; recombinant protein was incubated with hydroperoxide in 100 mM sodium phosphate, pH 8.0, and activity was measured spectrophotometrically at 234 nm. Error bars are based on S.D. (n = 3).

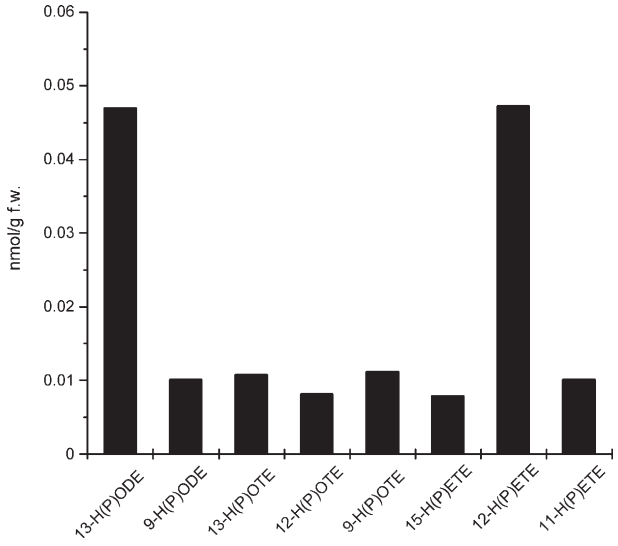

Fig. 5. Fatty acid hydro(pero)xides of wild type moss. The amount of hydro-(pero)xides was determined in protonema of wild type moss as described under Materials and methods. The figure shows mean values of two independent experiments.

converted to the corresponding aldehydes (Fig. 4). Fig. 4B shows the detected products with aldehyde functionality. In each case we found an additional aldehyde compared to the negative empty vector control. The identity of derivatized aldehydes was verified in HPLC runs by coelution with authentic standards. The aldehydes derived from 8- and 5-HPETE were separated on HPLC and analyzed directly by electrospray ionization/mass spectrometry. Their molecular weight and retention time matched those of C12- and C15-aldehydes. Based on biosynthetic considerations their structures were tentatively assigned to (3*Z*,6*Z*)-dodecadienal and (3*Z*,6*Z*,9*Z*)-pentadecatrienal. To analyze the substrate specificity on quantitative basis we used 30 μM of each hydroperoxide in a spectrophotometric assay where the consumption of the hydroperoxide was followed. The highest activity was observed using 9-HPODE as substrate, followed by 11-HPETE (40%), 9-HPOTE (35%), and 9-γHPOTE (26%). Using 13-γHPOTE, 8-HPETE and 5-HPETE the activity was not easily detectable (below 1% in comparison to 9-HPODE, Fig. 4C). Nevertheless the resulting aldehydes were reproducibly detected by HPLC analysis after 30 min of incubation time of the enzymatic reaction, whereas the negative empty vector control failed the produce such aldehydes.

3.2. Determination of fatty acid hydro(pero)xides

Fatty acid hydroperoxides are the direct precursors of volatile oxylipins. Since they are reactive substances which are not fully stable under our work up procedure their amount was expressed as a mixture out of hydroperoxy and hydroxy fatty acid derivatives. The amount of hydro(pero)xides was analyzed in protonema of wild type moss to get first hints for the possible endogenous substrates of *CYP74* enzymes. The predominant fatty acid hydro(pero)xides were 13-H(P)ODE and 12-H(P)ETE both accumulating to about 0.047 nmol/g fresh weight (Fig. 5). Other detected hydroperoxides were only found at a basal level of about 0.01 nmol/g fresh weight.

3.3. Intracellular localization

The enzymatic activity of *CYP74*-enzymes was previously found to associate with plastids, in particular with their envelope [30]. This observation is consistent with the prediction that some of the *CYP74*-enzymes carry a plastidic transit peptide. Import assays as well as immunocytochemical studies with different *CYP74*-enzymes support their plastidic localization [12,13]. We created an expression vector encoding a C-terminal fusion protein of PpHPL with YFP and transformed *Physcomitrella* protoplasts with this construct. After 3 days of expression

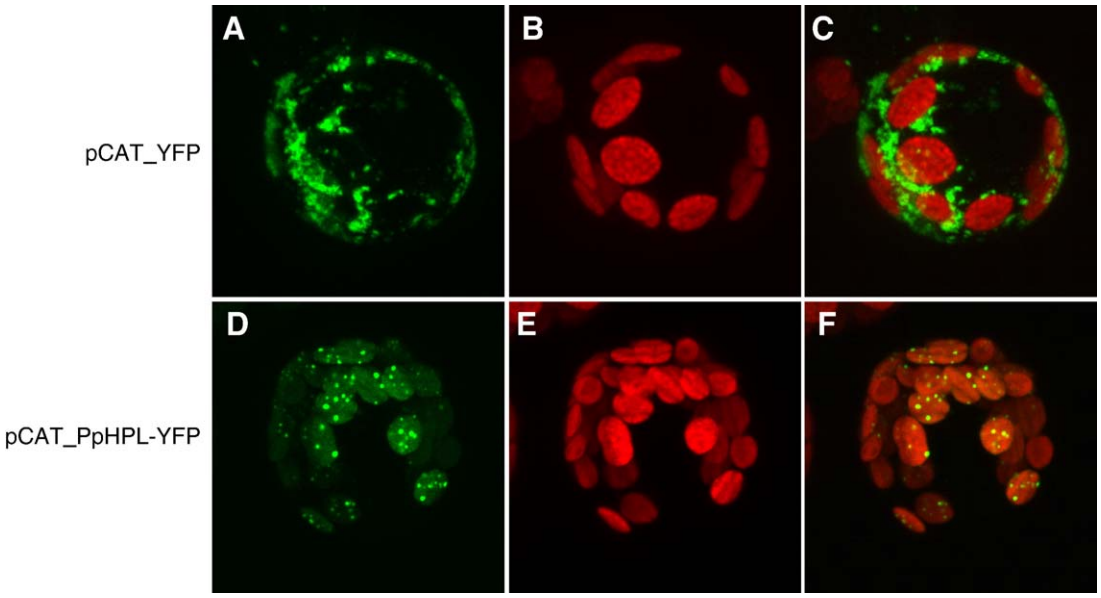

Fig. 6. Subcellular localization of PpHPL. *P. patens* protoplasts were transfected with pCAT_YFP and pCAT_PpHPL-YFP; (A, D) YFP fluorescence in a transfected protoplast. (B, E) Chlorophyll fluorescence of the same protoplast. (C, F) Merged image of A and B or D and C, respectively.

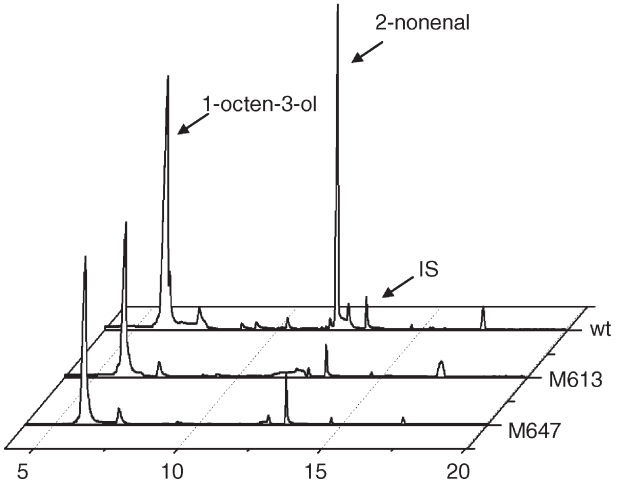

Fig. 7. SPME-analysis (GC/MS total ion chromatogram) of *P. patens* wild type and PpHPL-KOs volatile formation after wounding. Wild type (wt) and two knock-out lines (M613, M647) were wounded for 2 min. I.S., internal standard (2-decanon). Arrows indicate the emitted major volatiles.

YFP fluorescence was detectable. Visualization of the YFP fusion protein by fluorescence microscopy revealed a plastidic localization. To analyze it in more detail, we used confocal laser scanning microscopy. As seen in Fig. 6A YFP protein not fused to an HPL enzyme is distributed within the cytosol. In the case of PpHPL-YFP plastidic localization was obvious by colocalization with red chlorophyll autofluorescence. Interestingly, the distribution of the fusion protein in chloroplasts was not uniform, and a spotted pattern of localization was observed (Fig. 6D,F).

3.4. Analysis of PpHPL knock-out mosses

Recently it was described that after addition of arachidonic acid to wounded moss different volatiles like oct-1-en-3-ol, oct-3-en-1-ol and (2*E*)-nonenal were emitted [25]. The production of the octenols was caused by the recently cloned LOX of *P. patens* [26]. To analyze if the HPL cloned here is responsible for the formation of (3*Z*)-nonenal, which could be converted to (2*E*)-nonenal by an isomerase, we created knock-out mosses where the *PpHPL*-gene was disrupted by a cDNA coding for kanamycin resistance. We obtained 30 independent transgenic lines and the integration into the *PpHPL*-gene was verified by PCR as described in experimental procedures. Four independent lines were chosen for further studies. The knock-out lines showed normal development and analysis on minimal medium showed no visible phenotype. Since no pathogens for the moss are yet available, it was not possible to test whether these mutant lines were impaired in pathogen defense. However, all were

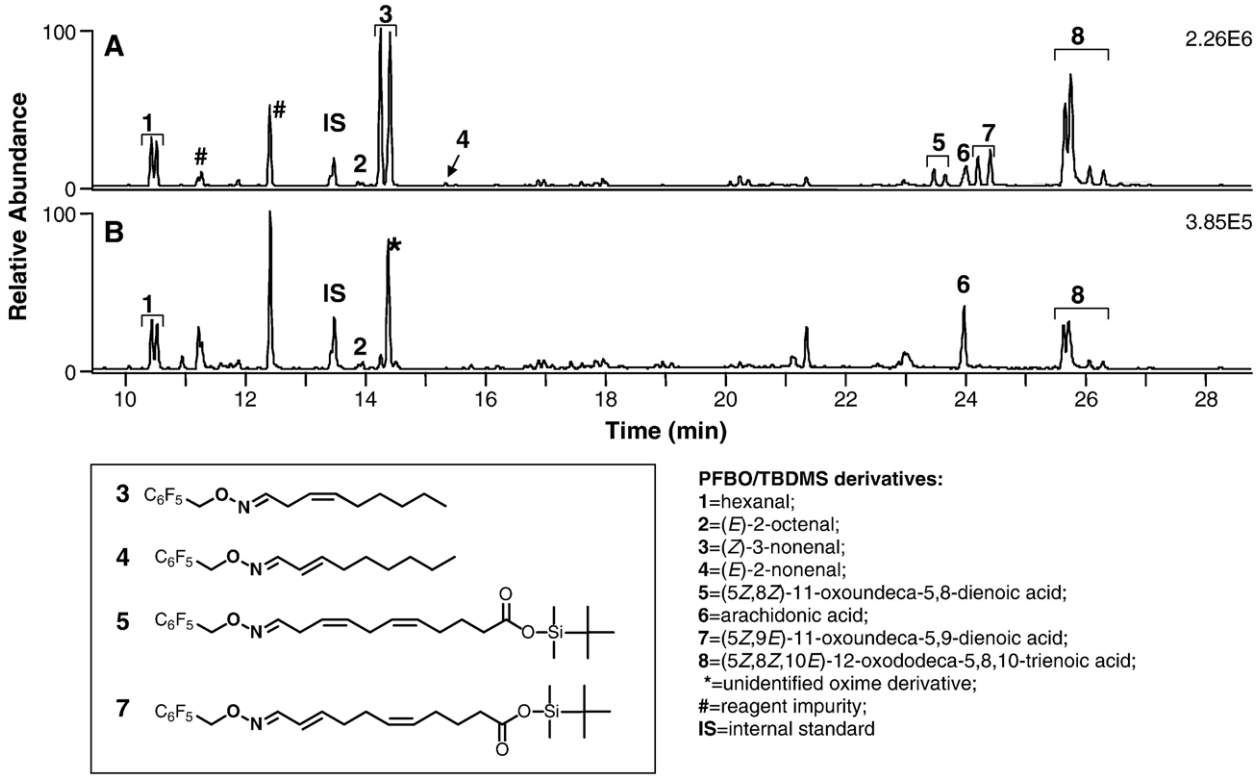

Fig. 8. In situ trapping of oxylipins harboring an aldehyde functionality after administration of arachidonic acid and pentafluorobenzyloxime to the medium of wounded WT (A) and PpHPL knock-out mutant (B). The chromatograms show the ion trace of the key ion of *m*/*z* 181. The reaction was stopped after 30 min and the extract was silylated with MTBSTA [40]. The picture shows the gas chromatographic separation of the derivates. While the WT produces elevated amounts of (3*Z*)-nonenal (3) and the 11-oxo fatty acid (5,7), the PpHPL knock-out mutant lacks these compounds. The 12-oxo fatty acid (8) is still found in elevated amounts in that knock-out.

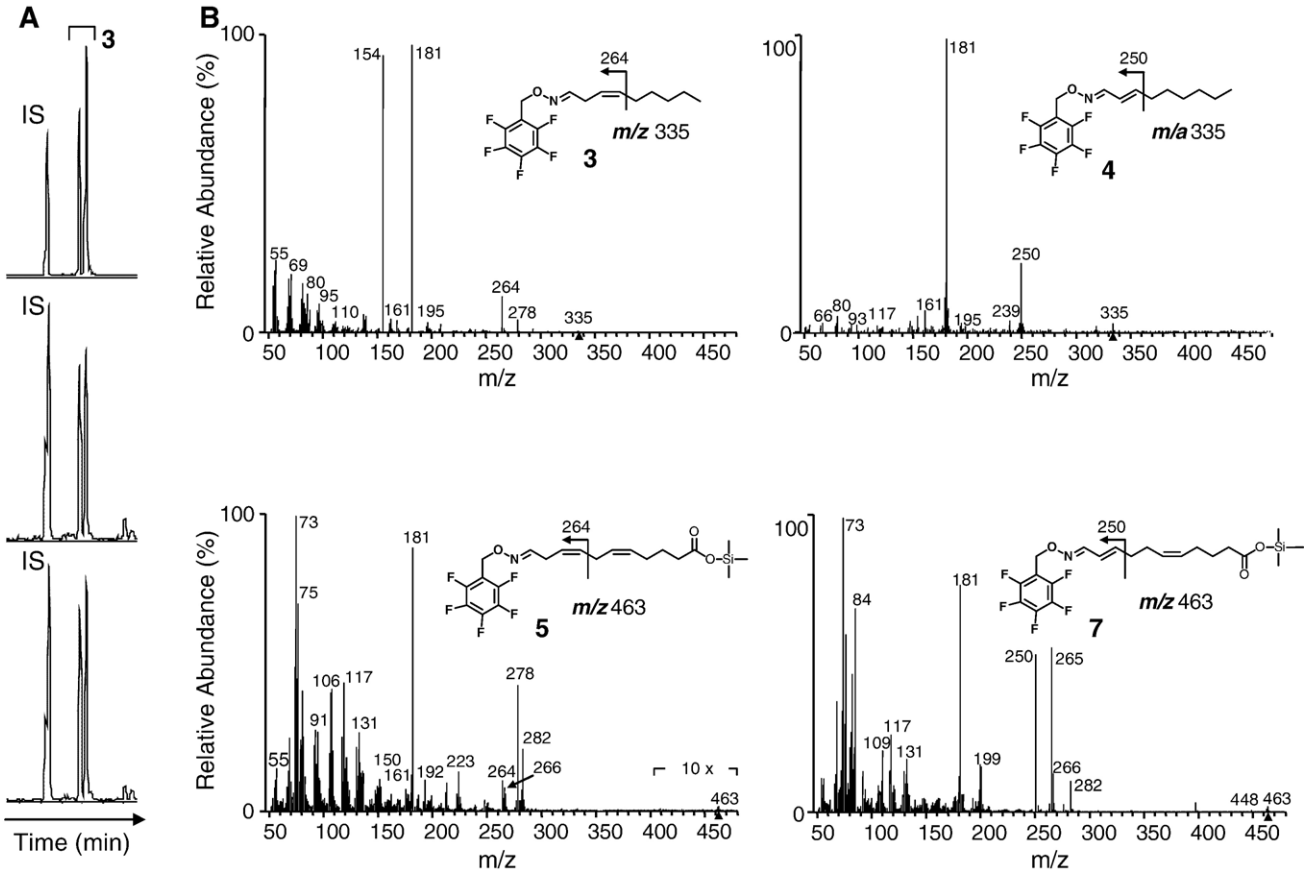

Fig. 9. Identification and structure elucidation of (3*Z*)-nonenal and 11-oxo fatty acids. (A) Co-injection of the derivatized standard of (3*Z*)-nonenal. The chromatograms show the ion trace of *m*/*z* 181 (IS = oxime derivative of benzaldehyde) 3. From top to bottom: Synthetic reference; extracted sample; co-injection at a ratio of 1:4. (B) Mass spectra of the derivatives of the nonenals 3 and 4 and the oxo acids 5 and 7. The structure of 4 has been previously documented by co-injection with commercial available standard [25]. Characteristic fragments of 264 (5) and 250 (7) allow the assignment of the double bond positions in the oxo acids (see Results).

deficient for HPL activity. Thus the formation of volatile oxylipins after exogenous addition of arachidonic acid was compared. As described for the wild type [25], we observed within the first 30 s the transient formation of (12*S*)-H(P)ETE and only trace amounts of racemic 11- and 15-H(P)ETE. The amount of (12*S*)-H(P)ETE declined then within the next 15 min and formation of oct-1-en-3-ol, oct-3-en-1-ol and (2*E*)-nonenal was observed. Addition of neither linoleic nor linolenic acid led to formation of volatiles under these conditions. The release of volatiles from moss lines sonicated for 2 min in an ice bath were analyzed by headspace analysis (Fig. 7). In the wild type moss we observed the production of octenols and (2*E*)-nonenal. In contrast no (2*E*)-nonenal was detected in two knock-out moss lines analyzed. The production of other volatiles was not affected in these knock-out lines. Interestingly, hexanal production was still observed in the knock out lines, even if the HPL is able to form this aldehyde from 13-HPODE in vitro (Fig. 8). The ω-oxo fatty acid (11-oxo undecadienoic acid), the other product of the HPL reaction, was also not detectable in the knock out mutants, whereas 12-oxo dodecantrienoic acid, the product of the reaction with the multifunctional LOX, was found (Fig. 8).

With pentafluorobenzyloxime trapping experiments, we could show that the wild type moss initially releases (3*Z*)-nonenal (Fig. 8), a product also formed with the recombinant enzyme. In contrast, investigations of the gas phase over the wounded moss with solid phase microextraction indicated the presence of (2*E*)-nonenal and no traces of the (3*Z*)-isomer were found. This indicates that an additional isomerizing activity existed in the crude moss preparations. Structures of (2*E*)- and (3*Z*)-nonenal were proven by co-injection of a commercial available or synthetic standard, respectively (Fig. 9A) [40]. The unsaturated ω-oxo fatty acids were initially detected as *tert*-butyldimetylsilyl-oxime-derivatives (Fig. 8), where the molecular mass can be easily determined by the detectable molecular ion and the M-57 fragment [40]. For further structure elucidation the carboxylic group was silylated with MSTFA and the aldehyde derivatized by pentafluorobenzyloxime treatment. The molecular ions of trimethylsilyl derivatives of the 11-oxo fatty acids are of low intensity. Nevertheless the molecular mass can be deduced from peaks which correspond to a loss of 181 u (*m*/*z* 282) and 197 u (*m*/*z* 266) (Fig. 9). The mass spectra allowed moreover the assignment of the double bond position in comparison with literature data of similar compounds [41]. The position of the α,β-double bond of the 11-oxo fatty acid 7 can be deduced from the diagnostic ion of mass 250 [42], a fragment also found in (2*E*)-nonenal 4 (Fig. 9). In case of the 11-

oxo fatty acid 5, where the double bonds are not conjugated, this key ion is not dominant, the same is true for the fragmentation pattern of (3*Z*)-nonenal 3 (Fig. 9). For these latter metabolites fragments with the masses of 264 are abundant, which are indicative for an α-fragmentation adjacent to the isolated double bond (Fig. 9). Presence of this fragment in the mass spectrum of the 11-oxo fatty acid 5 also excludes the presence of a conjugated double bond system. Together with biosynthetic considerations a (5*Z*,8*Z*)-double bond configuration of 5 is highly likely. Methylation instead of silylation of the carboxylic group supported this interpretation further, all key fragments according to [41] for α,β-unsaturated aldehydes could be detected.

4. Discussion

In contrast to flowering plants *P. patens* harbors high proportions of arachidonic acid (up to 30% of total fatty acids) besides C18 PUFAs [43]. *P. patens* exploits arachidonic acid for the formation of its major oxylipin-volatiles. We investigated details on how arachidonic acid, which is first oxidized to 12-HPETE [25], is further metabolized to a variety of different shorter chain oxylipins. 12-HPETE may be cleaved either by a typical plant-type HPL, belonging to *CYP74* enzymes (this report), leading to the formation of (3*Z*)-nonenal. Further fast isomerization of (3*Z*)-nonenal to the (2*E*)-isomer occurs by a separate isomerizing activity. An alternative source for the moss-volatiles is an unusual lyase activity of a LOX producing not only hydroperoxy fatty acids but also octenal and octenol isomers [26]. We describe here the isolation and characterization of the first HPL from a lower plant belonging like its homologues from flowering plants to the *CYP74* subfamily of P450 enzymes. By homology searches in EST-databases containing about 140,000 ESTs from *P. patens*, we found two ESTs with homology to *CYP74*-enzymes from flowering plants. One EST was completed by screening a phage library and the full length coding sequence was expressed in *E. coli*. The recombinant protein exhibited no activity, but an amino terminally truncated protein cleaved hydroperoxides into aldehydes (Fig. 3) and ω-oxo fatty acids. This property classified it as HPL belonging to *CYP74*-enzyme family [14]. The amino terminal region was identified as plastidic transit peptide using the prediction program TargetP. Several other *CYP74*-enzymes contain also such a transit peptide and show only as truncated versions enzymatic activity [11,44–46]. On amino acid sequence level, PpHPL shows a similarity of about 61% and identity of about 42% to an AOS from *Arabidopsis*, belonging to the *CYP74A* subfamily [11]. But in comparison to the other subfamilies (*CYP74B*-*CYP74D*), the values are nearly the same: CaHPL (*CYP74B*) 57% similarity and 40% identity, MtHPL (*CYP74C*) 59% similarity and 39% identity and NtDES (*CYP74D*) 57% similarity and 40% identity. These low and more or less equal levels may be due to the high phylogenetic distance between the moss and the flowering plants [47], thus making it difficult to decide to which subfamily PpHPL should belong to or whether it should belong to a new subfamily *CYP74E*.

Most HPLs cloned to date belong to the *CYP74B* subfamily. The substrate specificity of some recombinant enzymes of this subfamily has been investigated and found to have a high selectivity for 13-HPOTE [44–46]. Their activity against other hydroperoxides was very low (13-HPODE 7–15% and 9-hydroperoxides 0–9%). Similar results were observed when purified HPLs from plant tissues like green bell pepper fruits or tea leaves were analyzed [48,49]. In these examples unusual plant hydroperoxides like 9- or 13-γHPOTE were only poorly converted (0–2% relative to 13-HPOTE). Thus, it was proposed that the common feature of the substrates seems to be an ω6-hydroperoxide adjacent to a triene motif. Changes in *Z*–*E*-geometry [49] as well as elimination of the ω3-double bond lead to a dramatic decrease of the conversion rate [48]. The HPL from *Arabidopsis* is an exception, here 9-γHPOTE is converted with about 50% efficiency in comparison to the best substrate 13-HPOTE [50]. An explanation might be that both substrates contain the 4-hydroperoxy-(1*Z*,5*E*,7*Z*)-diene structure and only the orientation with respect to the carboxyl group differs. The influence of the length of the carbon chain was investigated using tea leaf HPL [48]. Here, hydroperoxides with carbon chain length ranging from C14 to C24 containing the ω6-hydroperoxide-triene motif were analyzed. Interestingly, the highest activity was detected using the C22-hydroperoxide, which is not present as a substrate in plants. In contrast to these 13-hydroperoxide-specific enzymes (13-HPLs), there were also HPLs having a broad substrate spectrum (unspecific or 9/13-HPLs). While the 13-HPLs belong to the *CYP74B* subfamily, unspecific HPLs are members of the *CYP74C* subfamily and have got the highest sequence similarity to AOS enzymes [4]. The 9/13-HPL from cucumber shows the highest activity towards 9-HPODE [51], whereas the HPL from melon prefers 9-HPOTE [52]. The activity against the other hydroperoxides ranged from 80% to 22%. Because of limited information, it is quite difficult to find conserved substrate motifs for this type of enzymes. The PpHPL shows the highest sequence similarity also to AOS enzymes and has also a slight preference to 9-HPODE but its activity against other hydroperoxides ranges from 5 to 40% (11-HPETE) (Fig. 4C). Thus we prefer to classify the enzyme as unspecific 9/13-HPL. In contrast to all other enzymes analyzed so far, the four best substrates of PpHPL shared an ω10-hydroperoxide-diene motif, whereas additional double bonds like in 9-HPOTE or 9-γHPOTE reduce drastically its activity.

Taken together, essential for optimal activity of HPLs is a *cis*–*trans*-configurated conjugated diene system with the *trans*-double bond beside the (*S*)-hydroperoxide bearing carbon atom. This system is always created by LOXs after metabolizing a (1*Z*,4*Z*)-diene system. Additional double bonds influence the activity rate: In case of 13-HPLs, a double bond at the other site of the hydroperoxide group leads to an increase in activity [6,14] whereas in case of cucumber HPL [51] and PpHPL the activity decreased. Analyses of the specificity of the tea leaf HPL, a 13-HPL, and our results indicated that the absolute position of the diene system in relation to the methylene end and not to the carboxyl end is an important factor for the substrate specificity. Nevertheless the carboxy group plays also a role, since methylation reduces often the activity whereas to some extent an acid-amine is a better substrate [50,53]. An influence of the pH is discussed in respect to amount of carboxylate anion in relation to the undissociated carboxyl group [54].

The enzymatic activity of *CYP74*-enzymes was often found associated with the plastid. Import assay studies with tomato AOS and HPL showed also an association with this organelle [12]. PpHPL also apparently contains a plastidic transit peptide and the PpHPL-YFP fusion protein was guided to the chloroplast (Fig. 6D,F). Since the enzyme is only active without its transit peptide we assume that PpHPL is imported into the chloroplast where a transit peptidase processes the protein. The pH optimum of PpHPL (around pH 8.0) supports also a plastidic localization. At neutral pH, there is still 80% of the activity suggesting that PpHPL could be active during photosynthetic and non-photosynthetic periods. During the purification of recombinant PpHPL, we observed an association of the protein with the *E. coli* membranes as it is described also for other *CYP74*s [55,56]. Because of that and the non-uniform distribution of PpHPL-YFP fusion protein in the chloroplast, we assume that the protein is somehow linked to special lipid domains within membranes that might be the inner envelope or the thylakoid.

It was recently described that after wounding the moss emitted a specific blend of volatiles that yet has not been found in flowering plants [25]. As main constituents we identified oct-1-en-3-ol, oct-3-en-1-ol and (2*E*)-nonenal, which were exclusively derived from arachidonic acid. While the production of the octenols is caused by the recently cloned LOX of *P. patens* [26], the HPL described here is responsible for the formation of (3*Z*)-nonenal as could be shown by knock out experiments. The analysis of two independent knock out lines show that PpHPL is only involved in the generation of (3*Z*)-nonenal which is rapidly transformed by an isomerase activity to the (2*E*)-isomer in the wild type (Fig. 7). The formation of octenols and the formation of hexanal is still observed in the knock out lines (Fig. 8). In this context it is interesting to note that the recombinant HPL is capable of transforming the precursor of hexanal 13-HPODE in vitro (Figs. 3 and 4), a hydroperoxide found in elevated amounts in the moss (Fig. 5). Apparently, other resources are as well responsible for the formation of this shorter chain length aldehyde. The specific transformation of 12-HPETE by PpHPL, while 13-HPODE seems not to be accessible for the enzyme cannot be easily explained by different locations of both substrates. Analysis of location of precursor fatty acids linoleic and arachidonic acid revealed an equal distribution of both fatty acids in all lipid classes detected in the moss and these data supported earlier findings on analysis of fatty acids in crude lipid fractions of this moss [57]. Therefore it will be interesting to identify the additional hexanal forming activity in the future. It is likely to be different from that of flowering plants since the available EST collection lacks a readily identifiable additional sequence coding for another HPL.

According to our findings described here and in recent publications [25,26], a new picture for the wound activated formation of volatile oxylipins in *P. patens* arises that involves only one key enzyme required for the activation of arachidonic acid. This single LOX (PpLOX1) can account itself for high product diversity [26]. It either produces directly octenols from arachidonic acid or it provides the substrate (12-HPETE) for PpHPL described here, leading to formation of (3*Z*)-nonenal, which is subsequently isomerized. Although 12-HPETE is not the best substrate of the recombinant enzyme, incubation of the moss with different fatty acids showed the increased formation of volatiles only in case of arachidonic acid via the production of 12-HPETE [25]. Analysis of the content fatty acid hydro (pero)xides shows a high level of 13-H(P)ODE and 12-H(P)ETE. The resulting HPL-products may be hexanal and (3*Z*)-nonenal. Interestingly only one of these aldehydes (3*Z*)-nonenal is reduced in the PpHPL knock out lines, which indicates that only 12-HPETE may be the endogenous substrate of PpHPL. So the specific formation of 12-HPETE determines the specificity of the HPL reaction although the recombinant PpHPL has a substrate preference for 9-HPODE. Thus, the moss produces metabolites typical for animals, plants, algae, and mushrooms by new transformations of arachidonic acid, combining in an unique way metabolic themes from all these organisms. This might be the reason why mosses are known to be highly resistant to herbivores and pathogens in contrast to less resistant flowering plants where this type of resistance is often mediated only by a single LOX/HPL pathway. It will be interesting to test this hypothesis in the future as soon as bioassays testing the activation of defense-response become available for *P. patens*.

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