The moss *Physcomitrella patens* contains cyclopentenones but no jasmonates: mutations in allene oxide cyclase lead to reduced fertility and altered sporophyte morphology

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Received: 26 May 2010
Accepted: 20 June 2010

**New Phytologist** (2010) **188**: 740–749

**Key words:** (9,13S)-12-oxo-phytodienoic acid, allene oxide cyclase, lipid peroxidation, moss, oxylipin metabolism.

**Summary**

- Two cDNAs encoding allene oxide cyclases (PpAOC1, PpAOC2), key enzymes in the formation of jasmonic acid (JA) and its precursor (9,13S)-12-oxo-phytodienoic acid (cis-(-)-OPDA), were isolated from the moss *Physcomitrella patens*.
- Recombinant PpAOC1 and PpAOC2 show substrate specificity against the allene oxide derived from 13-hydroperoxy linolenic acid (13-HPOTE); PpAOC2 also shows substrate specificity against the allene oxide derived from 12-hydroperoxy arachidonic acid (12-HPETE).
- In protonema and gametophores the occurrence of cis-(-)-OPDA, but neither JA nor the isoleucine conjugate of JA nor that of cis-(-)-OPDA was detected.
- Targeted knockout mutants for PpAOC1 and for PpAOC2 were generated, while double mutants could not be obtained. The ΔPpAOC1 and ΔPpAOC2 mutants showed reduced fertility, aberrant sporophyte morphology and interrupted sporogenesis.

**Abbreviations:** 12,13-EOT, (9Z,13S,15Z)-12,13-epoxy-9,11,15-octadecatrienoic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; cis-(+)-OPDA, (9S,13S)-12-oxo-phytodienoic acid; dn-OPDA, (7S,11S,13Z)-dinor-10-oxo-8,13-phytodienoic acid; H(P)ETE, hydro(pero)xy eicosapentaenoic acid; H(P)ODE, hydro(pero)xy octadecadienoic acid; H(P)OTE, hydro(pero)xy octadecatrienoic acid; JA, jasmonic acid; LOX, lipoxygenase; OPTA, oxo prostatrienoic acid; WT, wild type.

**Introduction**

During plant development or in response to stress a variety of signal molecules are generated, some of them derive from fatty acids (Browse, 2009a). For example, jasmonic acid (JA) and its precursor (9S,13S)-12-oxo-phytodienoic acid (cis-(+)-OPDA) derive from oxygenated polyunsaturated fatty acids that are collectively named oxylipins (Andreou et al., 2009). Their biosynthesis is initiated by formation of fatty acid hydroperoxides catalyzed mainly by lipoxygenases (LOxs) (Andreou & Feussner, 2009). Next allene oxide synthase (AOS) catalyses dehydration of fatty acid hydroperoxides to an unstable allene oxide, which may hydrolyse to a mixture of α-ketol, γ-ketol or racemic cyclopentenones. The allene oxide (9Z,13S,15Z)-12,13-epoxy-9,11,15-octadecatrienoic acid (12,13-EOT) is metabolized by an allene oxide cyclase (AOC) to an enantiomeric pure cyclopentenone, cis-(+)-OPDA (Fig. 1a). This AOS/AOC
reaction on 13-hydroperoxy octadecatrienoic acid (13-HPOTE) represents the first specific step leading to JA synthesis. While these steps were localized in the plastid (Browse, 2009a), the later steps of JA biosynthesis take place in peroxisomes. They include the reduction of \( \text{cis}-(+)-\text{OPDA} \) by \( \text{OPDA reductase 3 (OPR3)} \), activation to the coenzyme A (CoA) ester and three cycles of \( \beta \)-oxidation (Browse, 2009a).

\[ \text{JA and cis-(+)-OPDA are important signalling molecules in the coordination of the plants response to stress such as wounding, pathogen attack or water deficit. Mutants deficient in cyclopentenone formation or JA signalling are impaired in pathogen resistance, whereas mutants with constitutively active JA signalling show increased resistance (Browse, 2009b). In addition, JA plays a role in the regulation of developmental processes (Browse, 2009a). Leaf senescence as well as inhibition of growth and germination is induced by application of the methyl ester of JA. In Arabidopsis thaliana JA is important for the correct release of pollen and elongation of filaments and JA-deficient or JA-insensitive plants are male sterile. However, similar tomato mutants are female sterile (Browse, 2009b). Seed plants use mainly C18 fatty acids as precursors of oxylipins. In animals and algae such compounds may also derive from C20 and C22 fatty acids (Andreou et al., 2009). Similarly, the moss Physcomitrella patens can use arachidonic acid in addition to C18-fatty acids to form oxylipins (Wichard et al., 2005).

Genes encoding AOC have been isolated from various plant species. Hitherto all described AOCs are specific for the formation of two cyclopentenones: \( \text{cis}-(+)-\text{OPDA} \) or the roughanic acid-derived (7,11S,13Z)-dinor-10-oxo-8,13-phytodienoic acid (dn-OPDA). Both are precursors of JA. So far, all characterized AOCs carry a putative plastidic transit peptide and are located in plastids (Browse, 2009a).

Here, we isolated two cDNAs encoding AOCs from this moss. Characterization of both AOCs included substrate specificity of the recombinant enzymes and formation of a novel cyclopentenone derived from 12-hydroperoxy arachidonic acid (12-HPETE). Finally we could not detect any JA and we show that single knockout of an AOC-encoding gene already leads to reduced fertility, malformed spore capsules and to aberrant sporogenesis.

**Materials and Methods**

**Isolation, expression and purification of recombinant enzymes**

Based on sequence similarity to known AOC-sequences of flowering plants, partial expressed sequences tag (EST)-clones of two AOC were identified in an EST-library from \( P. \) patens (Stumpe et al., 2006a). To obtain full-length cDNA of all clones 5' rapid amplification of cDNA ends (RACE) using a lambda ZAPII cDNA library of moss protonema were performed. The PCR fragments were cloned into pGEM-T (Promega, Mannheim, Germany) and sequenced. Subsequently, primers were designed for amplification of full-length cDNA for expression of \( \text{PpAOC1} \) (sense 5'-GGA TCC ATG GGA AGA GGC GCC GCG-3'; antisense 5'-AAG CTT CTA ATT NGT GAA GTT GGG GG-3'). The underlined sequences represent restriction sites that were used for cloning the open reading frames in-frame into a pQE30 expression vector (Qiagen, Hilden, Germany) to produce the proteins with an \( N \)-terminal His\textsubscript{6} tag. The resulting plasmids were transfected into \( \text{Escherichia coli} \) strain SG13009 pREP4, expression and purification of

![Fig. 1 Allene oxide synthase (AOS)/allene oxide cyclase (AOC) pathways. (a) 13-Hydroperoxy octadecatrienoic acid (HPOTE) is converted by AOS to an allene oxide (12,13-EOT) which may either hydrolyse to ketols and a racemic cyclopentenone (OPDA) or the AOC reaction leads to specific formation of \( \text{cis}-(+)-\text{OPDA} \). (b) 12-Hydro(pero)xy eicosapentenoic acid (HPETE) is converted by AOS to an allene oxide (11,12-EETE) which is either hydrolysed to ketols and a racemic cyclopentenone or AOC2 is capable of catalysing formation of 11-oxo prostatrienoic acid (OPTA).](image)
recombinant proteins were performed as described in Stumpe et al. (2006b).

**Product analysis of recombinant PpAOC/2**

The fatty acid hydroperoxides were produced by incubation of corresponding fatty acid with recombinant cucumber lipid body LOX (production of 15-, 12-, 8-hydroperoxy eicosapentenoic acid (HPETE), 13-HPO(D/T)E, 13γ-HPOTE) or potato tuber LOX (production of 11-, 5-HPETE, 9-HPO(D/T)E, 9γ-HPOTE) and purified by HPLC as described in Stumpe et al. (2006b). Recombinant AOS1 from barley was used as helper enzyme (Maucher et al., 2000). The reaction products were analysed by high-pressure liquid chromatography (HPLC)/mass spectrometry (MS) as described in Hughes et al. (2006). Among them the methyl esters were characterized by gas chromatography (GC)/electron impact (EI)-MS analysis (Stumpe et al., 2006b). Determination of enantiomeric composition of OPDA ((9S,13S)- to (9S,13R)-ratio) was done according to Stenzel et al. (2003).

**Analytical methods**

Analysis of oxylinps was performed as described in Stumpe et al. (2006a). For measurement of cyclopentenones a mixture of fresh protonema and gametophytes was dried using filter paper and immediately frozen in liquid nitrogen. The ture of fresh protonema and gametophytes was dried using a chip ion source (TriVersa NanoMate; Advion BioSciences, Ithaca, NY, USA). Reversed-phase quadrupole coupled to an Applied Biosystems 3200 hybrid triple linear ion trap mass spectrometer (MDS Sciex, Ontario, Canada). Nanoelectrospray (nanoESI) analysis was achieved using a chip ion source (TriVersa NanoMate; Advion BioSciences, Ithaca, NY, USA). Reversed-phase HPLC was performed on an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) coupled to an Applied Biosystems 3200 hybrid triple quadrupole/linear ion trap mass spectrometer (MDS Sciex, Ontario, Canada). Nanoelectrospray (nanoESI) analysis was achieved using a chip ion source (TriVersa NanoMate; Advion BioSciences, Ithaca, NY, USA). Reversed-phase HPLC was performed on an EC 50/2 Nucleodure C18 gravity 1.8 μm column (50 × 2 mm, 1.8 μm particle size; Macherey and Nagel, Düren, Germany). The binary gradient system consisted of solvent A, acetonitrile : water : acetic acid (20 : 80 : 0.1, v : v : v) and solvent B, acetonitrile : acetic acid (100 : 0.1, v : v) with the following gradient program: 10% solvent B for 2 min, followed by a linear increase of solvent B up to 90% within 6 min and an isocratic run at 90% solvent B for 2 min. The flow rate was 0.3 ml min⁻¹. For stable nanoESI, 50 μl min⁻¹ of 2-propanol : acetonitrile : water : formic acid (70 : 20 : 10 : 0.1, v : v : v) delivered by a 515 HPLC pump (Waters, Milford, MA, USA) were added just after the column via a mixing tee valve. By using another post column splitter 740 nl min⁻¹ of the eluant were directed to the nanoESI chip. Ionization voltage was set to −1.7 kV. Phytohormone conjugates were ionized in a negative mode and determined in multiple reaction monitoring mode. Mass transitions were as follows: 141/97 (declustering potential (DP) −45 V, entrance potential (EP) −7 V, collision energy (CE) −22 V) for JA-Ile/JA-Leu, 352/135 (DP −80 V, EP −4 V, CE −30 V) for D3-JA-Leu, 356/164 (DP −80 V, EP −4 V, CE −30 V) for JA-Val, 296/170 (DP −70 V, EP −8.5 V, CE −28 V) for D3-cis (+)-OPDA, 291/165 (DP −70 V, EP −8.5 V, CE −28 V) for cis (+)-OPDA, 404/130 (DP −80 V, EP −10 V, CE −34 V) for cis (+)-OPDA-Ille, 263/59 (DP −70 V, EP −8.5 V, CE −28 V) for cis (+)-OPDA-Ille, 317.3/128 (DP −65 V, EP −4 V, CE −22 V) for 11-oxo prostatetnienoic acid (OPTA), and 430/130 (DP −65 V, EP −4 V, CE −22 V) for 11-OPTA-Ile. The mass analysers were adjusted to a resolution of 0.7 amu full width at half-height. The ion source temperature was 40°C, and the curtain gas was set at 10 (given in arbitrary units). Quantification was carried out using a calibration curve of intensity (m/z) ratio of [unlabelled]/[deuterium-labelled] vs molar amounts of unlabelled (0.3–1000 pmol).

**Chemical conjugation of cis-(+) OPDA with L-isoleucine**

The synthesis was described recently in Fonseca et al. (2009).

**Targeted gene knockout mutants**

The transfection of gene disruption constructs into P. patens protoplasts and the regeneration of transgenic lines was performed according to Frank et al. (2005). Disruption of the PpAOCI locus was verified by PCR with 5′-GGACCTTGTGCCTTCCTTAAC-3′ and 5′-GTTTGGGGAAGACACTGCTT-3′. The 5′- and 3′-integration of the disruption construct was analysed with the primers 5′-TGAGGTGAGGAGGGAGCTTG-3′ and 5′-ACGTGACTCCCCCTATCTC-3′ and 5′-CAGCCAGATGTTACATTACATTATTAG-3′ and 5′-TGCTTTGACCACACTGCTTT-3′, respectively. Reverse-transcription polymerase chain reaction (RT-PCR) with 5′-ATGATGGAGTTCGAGGAGGAGC-3′ and 5′-GGCTACCGAG-
CTAGGATGACAT-3′ was carried out as internal control for successful reverse transcriptase reaction, the confirmation for a disrupted wild type (WT) locus was obtained by PCR with primers used for a disrupted PpAOC1 locus. PpAOC2 mutant lines generated with an nptII selection cassette were screened by PCR with 5′-ATGATGGATC- GTGCAGAGGAG-3′ and 5′-GGCTACCCAGCTAGG- ATGACAT-3′ for a disrupted WT locus. The primer pairs 5′-TGACCATGCTTGCAATAAG-3′ and 5′-ACGT- GACTCCATTATCTCC-3′, and 5′-GAAAGATTGGA- GTTCACATGG-3′ and 5′-CCCCGAATTACATTAT- TAATCG-3′, respectively, were used to confirm precise 5′- and 3′-integration of the disruption construct. Loss of PpAOC2 transcript was verified with the same primers that were used for WT locus specific PCR. Southern blots were done using a nptII hybridization probe (35S promoter: neomycin phosphotransferase:35S terminator) which was released by HindIII digestion from the vector pRT101neo. The mutants are deposited in the International Moss Stock Centre with the accessions IMSC 40468 (∆PpAOC1 line 1), IMSC 40469 (∆PpAOC1 line 2), IMSC 40470 (∆PpAOC1 line 3) and IMSC 40471 (∆PpAOC2).

Growth conditions

Gametophores were grown under standard growth conditions, resulting in the formation of colonies within 4 wk. Thereafter, the plants were grown under sporophyte inducing conditions (culture conditions are detailed in Hohe et al., 2002).

Immunofluorescence analysis

Leaves of adult gametophores were fixed with 4% (w : v) paraformaldehyde and 0.1% (v : v) Triton X-100 in PBS (135 mM NaCl, 3 mM KCl, 1.5 mM KH2PO4, 8 mM Na2HPO4). After dehydration in a graded series of ethanol, material was embedded in polyethylene glycol (PEG) and cut as described (Hause et al., 2000). Cross-sections of leaves (2 μm thickness) were immunolabelled with polyclonal rabbit-antibodies raised against cucumber LOX (Ziegler et al., 2000), diluted 1 : 500 in PBS containing 1% (w : v) BSA, or against tomato AOC (Ziegler et al., 2000), diluted 1 : 1000 in phosphate-buffered saline (PBS) containing 1% (w : v) BSA. The use of pre-immune serum at the same dilutions served as control. All following steps were as described in Hause et al. (2000).

Microscopy of reproductive tissue

Bright-field and ultraviolet (UV) fluorescence microscopy was performed using the inverse microscope Axioplan (Zeiss, Jena, Germany) and an AttoArc UV lamp (Zeiss, manufactured by Atto Instruments Inc. Rockville, MD, USA). The images were taken with a charge-coupled device (CCD) camera (AxioCam MRc5 or ICc1, Zeiss, Jena, Germany).

Results

Isolation and characterization of PpAOC1/2

To analyse the formation and function of cyclopentenones in the moss, we isolated cDNAs coding for AOC enzymes. In an EST library of P. patens two sequences were identified with similarity to AOC sequences. Full-length cDNAs were obtained by 5′-RACE with a cDNA library as template. Stop codons upstream of putative start-Met indicated that the clones were full length. The isolated AOC-cDNAs were named PpAOC1 and PpAOC2 and had a length of 570 bp and 567 bp, respectively. They encoded for proteins of 189 and 188 amino acids, respectively, both with a predicted molecular mass of c. 20.6 kDa. Both sequences are c. 60 amino acids shorter than the AOCs from flowering plants (see the Supporting Information, Fig. S1). The protein sequences shared a sequence similarity of 87% to each other and of 74% to the AOC of Nicotiana tabacum (Acc. No. CAC83765). A screen of the annotated genome sequence with both sequences revealed that the P. patens genome contains only these genes coding for AOC enzymes (Rensing et al., 2008). Using the CHLOROP v 1.1 program (Emanuelsson et al., 1999) the sequences were analysed and both PpAOCs seem to lack the typical N-terminal plastidic transit peptide. However, the same program detected typical N-terminal plastidic transit peptides only for three of the seven recently published plastid-localized LOX enzymes from P. patens (Anterola et al., 2009).

Overexpression of the full-length sequences in E. coli resulted in additional bands of c. 23 kDa in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). In a coupled enzyme assay using 13-HPOTE as substrate and recombinant AOS1 of barley as a helper enzyme, formation of a cyclopentenone with mass and retention time of cis-(+)-OPDA was observed in presence of either PpAOC1 or PpAOC2 (Fig 2e,f). In presence of either PpAOC1 or PpAOC2 we observed the preferential formation of cis-(+)-OPDA (> 95% of (9,13,15)-enantiomer) but without any of the two AOCs a nearly 50% ratio of (9,13,15)-OPDA to cis-(+)-OPDA was found, indicating that both cDNAs encode for proteins with AOC activity.

In addition, PpAOC1 and PpAOC2 were incubated with the allene oxides derived from 9-hydroperoxy linoleic acid (9-HPODE), 13-HPODE, 9γ-HPOTE, 13γ-HPOTE, 9-HPOTE, 8-HPETE, 12-HPETE and 15-HPETE. Only with 12-HPETE and PpAOC2 was a remarkable increase in cyclopentenone formation observed compared with control (Fig. 2l vs j). The newly formed substance showed an absorption maximum at 224 nm and a molecular mass
of 317 Da (Fig. S2a). To further characterize this compound derivatization and analysis by GC/EI-MS was performed (Fig. S2b). The EI-MS spectrum showed a typical loss of one side-chain. Based on biosynthetic consideration and experimental data the structure was assigned to (5Z,8S,12S,15Z)-11-oxo-5,9,15-phytotrienoic acid (11-OPTA; Fig. 1b).

Analysis of the ratio of putative cis/trans-isomers of 11-OPTA according to the procedure for cis-(+)-OPDA measurements could not be performed because the cis/trans-isomers of 11-OPTA could not be separated by HPLC.

Subcellular localization of PpLOX and PpAOC

The first three enzymes of JA biosynthesis, the LOX, AOS and AOC, were repeatedly localized in the plastids of flowering plants (Wasternack, 2007; Browse, 2009a). As both cloned moss AOCs contained no predictable transit peptide we performed immunolocalization on sections of leafy shoots using antibodies raised against cucumber lipid body LOX and tomato AOC, respectively. All seven LOX enzymes identified group in phylogenetic tree analysis together with plastidic LOX enzymes from flowering plants, but only three of the seven isoforms harboured the typical N-terminal plastid transit peptides (Anterola et al., 2009). However, the label indicative for LOX is localized in the chloroplasts of leafy shoot cells (Fig. 3a), whereas use of the pre-immune serum resulted in brownish autofluorescence only (Fig. 3c). The green fluorescence indicative for AOC protein occurred at the same distinct organelles (Fig. 3b). The corresponding staining with 4,6-diamidino-2-phenylindole (DAPI) (Fig. 3d–f) as well as differential interference contrast images showed that these organelles contained starch granules, revealing that these structures are indeed chloroplasts (Fig. S3). Moreover, nuclei that were strongly stained by DAPI showed no label (Fig. 3a–e, arrows).

Analysis of ΔPpAOC1 and ΔPpAOC2

Targeted knockout lines were generated for PpAOC1 and PpAOC2 making use of the homologous recombination system in P. patens (Strepp et al., 1998). After transfection of the respective gene disruption constructs into P. patens protoplasts, the transgenic lines were screened by PCR to identify lines with a disrupted WT locus (data not shown). We identified three ΔPpAOC1 and two ΔPpAOC2 mutants, while we were unable to obtain double mutants. All single mutants showed disruption of the corresponding genomic locus and were unable to accumulate the respective mRNA, thus confirming the generation of null mutants for both genes. One of the two ΔPpAOC2 mutants was lost immediately after its generation. Therefore, the rest of the analysis was performed with the remaining four lines.

First we analysed whether the mutants obtained exhibited altered amounts of metabolites of the JA pathway by measuring precursors of the LOX and AOS/AOC reaction as well as products of the AOC reaction in a mixture of proth active and gametophores. For all other organs, that is sporophytes, the amount of cell material obtained was not sufficient for analysis. It may be assumed that JA biosynthesis one may expect an increase in metabolites that are upstream of the AOC reaction in the ΔPpAOC1 and ΔPpAOC2 mutants: substrates of LOX (polyunsaturated fatty acids),
AOS (H(P)ODE, H(P)OTE, H(P)ETE) and AOC. However, no significant differences were detected between WT and mutants in this respect. The profiles from WT protonema are shown in Fig. S4. As products of the AOC reaction we analysed cyclopentenones again in a mixture of protonema and gametophores of WT and mutants. We detected \textit{cis}-(+)-OPDA and 11-OPTA, whereas \textit{dn}-OPDA was below the detection limit (Fig. S5). For amount of \textit{cis}-(+)-OPDA again no statistical significant differences were detected between WT and mutants. However, for 11-OPTA we observed a twofold increase (\(P\)-value 0.018 in Student’s \(t\)-test) in \(\Delta PpAOC1\) line 1. For all other lines we observed no statistical significant differences against WT. The increase in 11-OPTA may be explained by the substrate preference of \(PpAOC2\) for the allene oxide of 12-HPETE when this isoform is compensating the lack of \(PpAOC1\). Neither JA and JA amino acid conjugates nor the Ile conjugate of \textit{cis}-(+)-OPDA and 11-OPTA could be detected.

During vegetative growth at standard conditions the phenotype of \(\Delta PpAOC1\) and \(\Delta PpAOC2\) mutants did not differ from WT. However, we observed differences in sporophyte development between the \(\Delta PpAOC1\) and \(\Delta PpAOC2\) mutants and WT. After 14 wk the WT produced spore capsules containing mature spores that were able to germinate. All lines of \(\Delta PpAOC1\) and \(\Delta PpAOC2\) were able to develop sporophytes within the same period of time, but the number of capsules was >10-fold reduced compared with the WT. In addition, the spore capsules formed by \(\Delta PpAOC1\) and \(\Delta PpAOC2\) differed from WT concerning the shape of the capsule itself and the shape of the seta. The seta of the mutant capsules was broader and the colour of the mutant sporogenic tissue was darker during capsule matura-
tion (Fig. 4a). In \(\Delta PpAOC\) mutants the malformed capsules failed to dehisce and meiospores were not released. The sporogenic tissue was analysed with bright-field and UV microscopy. In contrast to the well-separated WT spores, the mutant capsules contained sporocytes in different developmental stages. Here, mainly tetrads (Fig. 4b) together with a very small number of spores (Fig. 4c) were found. The sporangia of the mutants did not dehisce and the sporogenic tissue inside the capsules died as shown by its darker colour. Nevertheless, the very few spores of the \(\Delta PpAOC\) mutants were able to germinate when transferred to solid medium. They germinated and developed branched protonema filaments and leafy shoots similar to the WT.

In order to complement the aberrant sporophyte morphology and the interrupted sporogenesis in \(\Delta PpAOC1\) and \(\Delta PpAOC2\), 50, 100 or 150 nM \textit{cis}-(+)-OPDA, respectively, was repeatedly added to the growth medium at sporophyte-inducing conditions. As a second approach, young sporophytes were treated with 1 mM \textit{cis}-(+)-OPDA by applying the solution directly to the capsules weekly. Both complementation experiments did not restore sporophyte morphology and sporogenesis in the mutants.

In order to analyse the reduced fertility of the \(\Delta PpAOC1\) and \(\Delta PpAOC2\) mutants, gametangia development was analysed with bright-field and UV fluorescence microscopy. Archegonia and antheridia development was the same in the mutants as in the WT.

**Discussion**

Numerous studies describe the occurrence and function of cyclopentanones and cyclopentenones as important group of oxylipins in flowering plants (Browse, 2009a,b) but knowledge on occurrence and function of oxylipins in non-flowering plants is still scarce (Andreou \textit{et al.}, 2009).
Therefore, we aimed to analyse the formation of cyclopentanones and cyclopentenones by analysing recombinant AOCs and putative function(s) of these AOC products via targeted knockout mutants of *P. patens*. This moss serves as a model system for nonflowering plants (Charron & Quatrano, 2009). Recently, we found that it is able to metabolize arachidonic acid to form oxylipins (Wichard et al., 2005). One major hydro(pero)xide that was formed endogenously in *P. patens* is 12-H(P)ETE (Fig. S4) which serves as precursor of volatiles such as octenols, octenals and nonenals (Stumpe et al., 2006a). These volatiles are formed by at least two LOX enzymes with an unusual lyase activity or a classical plant-type hydroperoxide lyase-derived pathway (Wichard et al., 2005; Anterola et al., 2009).

Another important enzyme in oxylipin metabolism is AOC which catalyses the formation of cyclopentenones. In this step the enantiomeric structure is established which occurs in the naturally occurring jasmonates. The available EST library from *P. patens* harboured two sequences with similarity to AOCs. This allowed us to isolate full-length cDNAs. Both cloned AOCs did not contain a predictable transit peptide (Fig. S1), but the immunocytological approach showed location of both AOCs in chloroplasts (Figs 3 and S3). Proteins lacking a transit peptide may be imported via a transit peptide-independent sorting route, as observed for the AOS of barley (Maucher et al., 2000).

Both corresponding proteins of the full-length cDNAs showed AOC activity (Fig. 2). We tested fatty acid hydroperoxides in a coupled AOS/AOC assay and the typical reaction with 13-HPOTE leading to enantiomeric pure cis-(+)-OPDA was catalysed by both AOCs. Common features of substrates for AOC were identified by analysis of potato and corn AOC (Ziegler et al., 1999; Stumpe et al., 2006b). There, an epoxy group at position n-6,7 and a β,γ-double bond relative to the epoxy group at position n-3 was found to be essential. In case of C20 fatty acids only the allene oxide derived from 15-HPETE was tested as substrate of the corn AOC (Ziegler et al., 1999). Here, we used 12-HPETE as substrate which leads to formation of an allene oxide with an epoxy group at position n-9,10 and the β,γ-double bound at position n-6. The recombinant PpAOC1 and PpAOC2 formed the corresponding cyclopentenone (Fig. 2). Based on HPLC/MS and GC/EI-MS data as well as the UV spectrum and biosynthetic considerations we

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**Fig. 4** Spore capsules, spores and sporogenic tissue of WT, ΔPpAOC1 and ΔPpAOC2 mutant lines. (a) Spore capsules of wild type (WT) compared with ΔPpAOC1 (line 2) and ΔPpAOC2 (line 1). (b) Photographs of spores from WT and tetrads from ΔPpAOC1 and ΔPpAOC2 mutant lines under brightfield and UV-fluorescence. (c) A single spore from ΔPpAOC1 indicated by an arrow surrounded by sporogenic tissue. Bar, (a) 500 μm, (b) 20 μm, (c) 20 μm.
assign the structure as 11-OPTA (Figs 1 and S2). This cyclopentenone cannot be a precursor of JA because it has an octenyl instead of a pentenyl side-chain (Fig. 1). Interestingly, the formation of a similar eicosanoid was observed by incubation of arachidonic acid with extracts of corals such as Plesiota homomalla (Brash, 1989). Here, arachidonic acid was oxidized by an (8R)-LOX-AOS fusion protein first to (8R)-HPETE. This is further metabolized by the same fusion protein to an unstable allene oxide, which hydrolyses to ketols and undergoes nonenzymatic cyclization to racemic (5Z,14Z)-9-oxo-prosta-5,10,14-trienoic acid, called pre-calvulone A. This cyclopentenone was discussed as putative precursor of prostaglandins, calvulones and other marine eicosanoids (Andreou et al., 2009). However, the novel cyclopentenone 11-OPTA shares the same \(\alpha,\beta\)-unsaturated carbonyl group in the cyclopentenone ring as cis-(+) -OPDA. It is important to note that in flowering plants cis-(+) -OPDA has gene regulatory activity which is independent of JA (Stintzi et al., 2001; Farmer et al., 2003; Taki et al., 2005; Müller et al., 2008). Therefore, we analysed the endogenous amounts of cyclopentenones in protonema and gametophores first. An average amount of cis-(+) -OPDA of c. 0.5 nmol g\(^{-1}\) FW was detected corresponding to observed levels in Arabidopsis leaves (Stintzi et al., 2001; Müller et al., 2008). The amount of the novel cyclopentenone, 11-OPTA, in \(P\). \(P\). patens was c. 0.1 nmol g\(^{-1}\) FW. Furthermore, it was not possible to detect any amount of JA or amino acid conjugates of cis-(+) -OPDA or JA. This is in agreement with similar observations by other groups (Browse, 2009a), but contrasts with a recent report on infected moss cultures (Oliver et al., 2009). However, in the latter report \(J\). \(J\). was detected by head-space analysis only and was not confirmed by additional GC/MS experiments. Therefore, one may assume that in contrast to flowering plants \(P\). \(P\). patens harbours only the plastid-localized part of the LOX pathway (Westernack, 2007; Browse, 2009a). This is supported by the fact that seven out of nine putative LOX genes, were identified as plastidial 13-LOXs while the other two appeared to be pseudo genes (Anterola et al., 2009).

The next enzyme in the biosynthetic pathway from cis-(+) -OPDA to JA is the OPDA reductase, \(A\). \(A\). harbours at least five genes encoding proteins with activity and different substrate specificities but only OPR3 converts that enantiomeric form of OPDA, which is the precursor in \(A\). \(A\). biosynthesis (Breithaupt et al., 2006). Owing to undetectable levels of JA and its amino acid conjugates such as JA-Ile, JA-Leu and JA-Phe as well as cis-(+) -OPDA-Ile in \(P\). \(P\). patens, it is likely that the enzyme metabolizing cis-(+) -OPDA is missing or not active, although sequences were found with similarity to OPDA reductase genes (Li et al., 2009).

In respect to the JA deficiency and cis-(+) -OPDA accumulation \(P\). \(P\). patens is similar to the opr3 mutant of \(A\). \(A\). thaliana, which is a molecular tool to analyse cis-(+) -OPDA-specific gene activity and development (Stintzi et al., 2001; Browse, 2009a). However, it is a matter of debate how cis-(+) -OPDA perception takes place. In the case of JA perception, the F-box protein COI1 was recently identified as a JA receptor (Yan et al., 2009) which interacts with repressors of JA-responsive gene expression, the JAZ proteins, if (+)-7-iso-jasmonoyl-\(l\)-isoleucine is bound (Browse, 2009a; Fonseca et al., 2009). Consequently, JAZ proteins are directed to proteosomal degradation. JA or cis-(+) -OPDA are inactive in binding to COI1. As yet, there is no mechanistic explanation for cis-(+) -OPDA perception (Browse, 2009a), and a COI1-independent perception may be probable (Böttcher & Pollmann, 2009).

As \(P\). \(P\). patens is JA-deficient, but able to accumulate cis-(+) -OPDA, we investigated possible functions of cyclopentenones via targeted knockout mutants of \(PpAOC1\) and \(PpAOC2\). All our attempts to obtain double knockout mosses failed, revealing that both enzymes have overlapping functions in protoplast regeneration and that depletion of both enzymes in one \(P\). \(P\). patens plant may be lethal. The recombinant enzymes showed different substrate specificities as \(PpAOC2\) is capable to form 11-OPTA in addition to formation of cis-(+) -OPDA (Fig. 2). This is reflected in unchanged amounts of cis-(+) -OPDA in all lines analysed, at least in the gametophytic tissues protonema and gametophores, but in an increase of 11-OPTA in \(\Delta PpAOC1\) line 15 (Fig. S5). Under standard growth conditions both gametophytic tissues were WT-like in the mutants. Seed plants affected in cis-(+) -OPDA and JA biosynthesis or JA perception show differences from WT often only under specific stress situations, such as pathogen attack or at certain developmental stages (Westernack, 2007); for example, JAC-deficient and JA-insensitive mutants of \(A\). \(A\). are male sterile and show diminished resistance to herbivores and pathogens (Browse, 2009a). By contrast, the tomato mutant \(\text{aji}1\) affected in the COI1 gene is female sterile (Li et al., 2004). Such differences in action of JA in reproductive tissues even in two dicot species prompted us to inspect sporphyte formation in \(P\). \(P\). patens \(\Delta AOC\) mutants. This analysis revealed that targeted disruption of single members of the two \(PpAOC\) genes resulted in reduced fertility and in defective sporogenesis. Further, both mutants developed capsules (sporophytes) that did not release mature meiospores. The sporogenesis of the knockout mosses was interrupted in the post-meiotic stage of haploid tetrads. This leads to the conclusion that \(PpAOC1\) and \(PpAOC2\) are required for fertilization, for spore maturation and for a subsequent dehiscing of the capsules. The phenotype may be caused by a locally lowered cis-(+) -OPDA content or a yet unidentified metabolite derived there from, since our complementation experiments with cis-(+) -OPDA failed and the cis-(+) -OPDA amount was not reduced in mutant protonema or in gametophores. As the gametangia development of the \(PpAOC1\) and \(PpAOC2\) mutants is
comparable to WT we assume that the fertilization process itself is hampered leading to the reduced amounts of spore capsules in the mutants. As P. patens is a monoecious moss (Reski, 1998), we were unable to assess if this was caused by specific problems in the male or in the female part of the fertilization process.

The mutant phenotypes observed here differ from those obtained after knockout of another nuclear-encoded but plastid-localized metabolic enzyme, sulphite reductase (Wiedemann et al., 2010), arguing in favour of a specific requirement of AOCs in moss fertilization and sporophyte development. The phenotypes of the knockout mosses described here, suggests that a role of oxylipins in reproductive development of plants is evolutionary conserved but is specified differentially in different branches of the plant kingdom, as has previously found for auxin (Ludwig-Müller et al., 2009; Paponov et al., 2009; Sun et al., 2010) and for gibberellin signalling (Vandenbussche et al., 2007).

Acknowledgements

The technical assistance of A. Nickel and P. Meyer is gratefully acknowledged. The research was supported for IF by the German Research Foundation (DFG) and for RR by the Excellence Initiative of the German Federal and State Governments (EXC 294). AKB is supported by the DFG RTG 1305.

References


Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Alignment of protein sequences of PpAOC1 (CAD48752), PpAOC2 (CAD48753), NrAOC (CAC83765), Medicago truncatula AOC1, (MtAOC) (CAC83767) and Hordeum vulgare AOC (HvAOC) (CAC83766).

Fig. S2 Structural characterization of the 12-hydroperoxy eicosapentenoic acid (HPETE)-derived cyclopentenone.

Fig. S3 Subcellular structures of leafy shoot cells of Physcomitrella patens.

Fig. S4 Fatty acid and hydroxy fatty acid profiles of Physcomitrella patens protonema.

Fig. S5 Cyclopentenone profiles of protonema of Physcomitrella patens wild type, ΔPpAOC1 and ΔPpAOC2 knockout mosses.

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