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RESEARCH PAPER

Polyphenol oxidases in *Physcomitrella*: functional PPO1 knockout modulates cytokinin-dependent development in the moss *Physcomitrella patens*

Hanna Richter[1,2], Reinhard Lieberei[1], Miroslav Strnad[3,4], Ondrej Novák[3], Jiri Gruz[3,4], Stefan A. Rensing[5,6] and Klaus von Schwartzenberg[1,*]

[1] University of Hamburg, Biozentrum Klein Flottbek, Ohnhorststraße 18, D-22609 Hamburg, Germany
[2] University of Amsterdam, Swammerdam Institute for Life Sciences, Molecular Plant Pathology, PO Box 94215 1090 GE Amsterdam, The Netherlands
[3] Laboratory of Growth Regulators, Faculty of Science, Palacký University & Institute of Experimental Botany AS CR, v.v.i., Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic
[4] Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic
[5] University of Freiburg, Faculty of Biology, Schänzlestr. 1, D-79104 Freiburg, Germany
[6] University of Freiburg, BIOSS Centre for Biological Signalling Studies, Hebelstr. 25, D-79104 Freiburg, Germany

* To whom correspondence should be addressed: E-mail: kvschwartzenberg@botanik.uni-hamburg.de

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Abstract

Polyphenol oxidases (PPOs) are copper-binding enzymes of the plant secondary metabolism that oxidize polyphenols to quinones. Although PPOs are nearly ubiquitous in seed plants, knowledge on their evolution and function in other plant groups is missing. This study reports on the *PPO* gene family in the moss *Physcomitrella patens* (Hedw.) B.S.G. as an example for an early divergent plant. The *P. patens PPO* multigene family comprises 13 paralogues. Phylogenetic analyses suggest that plant PPOs evolved with the colonization of land and that *PPO* duplications within the monophyletic *P. patens* paralogue clade occurred after the separation of the moss and seed plant lineages. PPO functionality was demonstrated for recombinant *PPO6*. *P. patens* was analysed for phenolic compounds and six substances were detected intracellularly by LC-MS analysis: 4-hydroxybenzoic acid, *p*-cumaric acid, protocatechuic acid, salicylic acid, caffeic acid, and an ester of caffeic acid. Targeted *PPO1* knockout (d|ppo1) plants were generated and plants lacking *PPO1* exhibited only ~30% of the wild-type PPO activity in the culture medium, thus suggesting extracellular localization of PPO1, which is in contrast to the mostly plastidic PPO localization in seed plants. Further, d|ppo1 lines formed significantly more gametophores with a reduced areal plant size, which could be related to an increase of endogenously produced cytokinins and indicates an impact of PPO1 on plant development. d|ppo1 plants were less tolerant towards applied 4-methylcatechol compared to the wild type, which suggests a role of extracellular PPO1 in establishing appropriate conditions by the removal of inhibitory extracellular phenolic compounds.

Key words: Cytokinins, gene family, gene replacement, knockout, phylogeny, *Physcomitrella patens*, polyphenol oxidase, PPO.

Introduction

Polyphenol oxidases are copper-binding enzymes that oxidize various polyphenolic plant compounds to the corresponding quinones in the presence of molecular oxygen (Mayer, 2006). The extended group of polyphenol oxidases is widespread among all groups of organisms and can be divided into three subgroups based on their substrate preferences: *o*-diphenol oxidases (PPO,

EC 1.10.3.1), which oxidize *o*-diphenols to *o*-diquinones; laccases (LAC, EC. 1.10.3.2), which oxidize *p*-diphenols, and tyrosinases (TYR, EC 1.14.18.1), which are catechol oxidases with an additional function for hydroxylation of monophenols to *o*-diphenols prior to the diphenol oxidation.

TYRs occur typically in bacteria, fungi, and animals, but in plants they are rarely found (Mayer, 2006). PPOs and LACs are nearly ubiquitous in the plant kingdom, although for *Arabidopsis thaliana* no *o*-diphenol oxidase-encoding genes have been found in the genome (Sullivan *et al.*, 2004). For *Arabidopsis*, only the presence of a large LAC gene family has been reported (McCaig *et al.*, 2005).

PPOs are nuclear-encoded proteins (Lax *et al.*, 1984) and *PPO* cDNAs have been characterized for numerous seed plant species, including *Solanum tuberosum* (Hunt *et al.*, 1993; Thygesen *et al.*, 1995), *Solanum esculentum* (Newman *et al.*, 1993), *Prunus armeniaca* (Chevalier *et al.*, 1999), *Vitis vinifera* (Dry and Robinson, 1994), *Musa cavendishii* (Gooding *et al.*, 2001), *Triticum aestivum* (Demeke and Morris, 2002), and *Populus trichocarpa* (Tran and Constabel, 2011). In most plant species, PPOs are encoded by multigene families with no or a low number of introns.

Although many studies have been published for seed plants, the specific function of PPOs, which are in most cases located in plastids, is generally not well understood. *PPO* transcript levels are usually highest in young and meristematic tissues and decline during further development (Dry and Robinson, 1994; Thygesen *et al.*, 1995). In late developmental stages, *PPO* expression is often not detectable, as found for *Prunus armeniaca* (Chevalier *et al.*, 1999). Moreover, *PPO* gene family members from seed plants exhibit different temporal and spatial gene expression patterns in vegetative and reproductive organs, for example as described for the tomato *PPO* gene family (Thipyapong *et al.*, 1997). Also, in *Trifolium pratense*, *PPO* genes are differently expressed, each being predominant in a certain stage: for example *TpPPO1* in young leaves and *TpPPO2* in flowers and petioles (Sullivan *et al.*, 2004). Although being differentially expressed during plant development, it is unclear whether PPOs themselves are involved in the modulation of developmental processes. Galuszka *et al.* (2005) found evidence in maize that apoplastic laccases could play a role in enhancing cytokinin breakdown by reoxidation of the cytokinin catabolic enzyme cytokinin oxidase/dehydrogenase.

PPO transcript levels were found to be induced by biotic stress factors. In hybrid *Populus trichocarpa* × *Populus deltoides*, *PPO* expression was induced especially in young leaves by wounding, spraying with methyl jasmonate, and forest tent caterpillars (*Malacosoma disstria*) feeding on the plants (Constabel *et al.*, 2000). Promoter::GUS fusions revealed that the tomato *PPO* gene F was induced in young leaves in response to wounding and after infection by *Alternaria solani* and *Pseudomonas syringae*, presumably in order to protect juvenile tissues from subsequent attacks by pathogens and pests (Thipyapong and Steffens, 1997).

So far, little information exists on transcriptional changes in *PPO* expression following the onset of abiotic stress conditions. Tomato *PPO* genes B and D are transcriptionally upregulated in abscission zones of leaf petioles in response to water stress, and it was proposed that this upregulation is correlated with the onset of apoptosis during water stress (Thipyapong *et al.*, 2004). Moreover, tomato *PPO* genes B and F are ethylene inducible, and expression of the *PPO* gene F was found to be absent during water stress (Thipyapong and Steffens, 1997; Thipyapong *et al.*, 2004). Recently, analysis of tomato *PPO* B promotor by GUS fusions in transgenic tomato confirmed the ethylene responsiveness for various tissues and cell types (Newman *et al.*, 2011).

One widespread property of plastidic PPO activity is latency, meaning that PPOs are bound to thylakoid membranes in an inactive form and become active after tissue and membrane disintegration (Steffens *et al.*, 1994; Yoruk and Marshall, 2003), once contact with the vacuolar substrates is possible. PPOs can possess a pronounced persistence throughout growth and development: in apricot, PPO protein is still present and active at a late stage of fruit development, whereas its mRNA is no longer detectable (Chevalier *et al.*, 1999). In contrast, Thipyapong *et al.* (1997) suggested that PPO protein accumulation is primarily transcriptionally controlled by mRNA levels, because, in tomato, PPO accumulation patterns reflect *PPO* transcript patterns.

Known PPO proteins contain two highly conserved copper-binding domains, CuA and CuB, that are responsible for interaction with molecular oxygen and phenolic substrates. Each copper-binding domain harbours three histidines that bind one copper atom (Steffens *et al.*, 1994). The subdivision of the extended group of polyphenol oxidases into three subgroups is also supported by the structure of the copper-binding domains: *o*-diphenol oxidases (PPO) and tyrosinases (TYR) are type-3 copper proteins, whereas laccases (LAC) contain a combination of type-2 and type-3 copper centres (reviewed by Gerdemann *et al.*, 2002). Crystal structure of the active form of a catechol oxidase from *Ipomoea batatas* revealed that the secondary structure is dominated by α-helical regions and that the catalytic copper centre is situated within four α-helices in a hydrophobic pocket close to the enzyme surface (Klabunde *et al.*, 1998).

As PPO substrates, phenolic compounds play several and diverse roles in seed plants. They can be involved in defence mechanisms against herbivores and pathogens, UV radiation protection, and blossom pigmentation, and they also possess antibiotic activity against bacteria and fungi (reviewed by Hahlbrock and Scheel, 1989; Waterman and Mole, 1994). Interest in phenolic compounds has grown enormously in relation to human nutrition as the compounds have antioxidative properties as radical scavengers (Rice-Evans *et al.*, 1995) and are considered to exert protective effects against cancer and cardiovascular diseases. PPO and polyphenols contribute to flavour-generating processes, for example in the fermentation of wine, tea, coffee, and cocoa. In contrast, during the production of numerous vegetable products such as fruit juices and potato chips, PPO-mediated oxidation of phenolic compounds is undesirable and attempts are made to inhibit it.

With the scarcity of functional knowledge on plant PPOs outside of the group of seed plants, this study addressed the question of possible PPO functions in early divergent land plants. The PPO gene family of the moss *Physcomitrella patens* was phylogenetically characterized in the context of PPOs of other sequenced genomes. Functional conclusions on PPO1 as a major *P. patens* PPO isoform are drawn from a study of a targeted gene knockout and the effects on overall PPO activity, growth, and differentiation, content of endogenous cytokinins, and resistance

to an externally applied polyphenol are described. Results are discussed with respect to original PPO functions at the emergence of land plants.

Materials and methods

Plant material and culture/growth conditions

P. patens (Hedw.) BSG wild type (Bryophyta, Funariales, Funariaceae), derived from the strain 'Gransden 2004' (Rensing *et al.*, 2008), was used in all experiments. Moss tissue was cultivated axenically in growth chambers (RUMED Typ 1602) at 25 ± 1 °C and white light (fluorescent tubes, TLM 18W/840, Philips) under 16/8 light/dark conditions with a flux of 50 µmol m^{-2} s^{-1} (Schulz *et al.*, 2001). *P. patens* tissue grown in liquid medium (Wang *et al.*, 1980) was used for PPO activity determination, vitality tests, determination of cytokinins, and determination of isopentenyladenine depletion. For maintenance of liquid cultures, the tissue was disintegrated weekly using an Ultraturrax blender (IKA), separated from old medium, extensively washed with fresh medium, and subsequently transferred to fresh medium (day 0). Under standard conditions, protonema status was maintained by supplementation of medium with 5 mM di-ammonium tartrate. Tissue from liquid cultures transferred to agar plates (Knight *et al.*, 1988) was used for growth tests and phenotypical observations.

Application of phenolic compounds

A sterile filtered stock solution of 4-methylcatechol (4-MC) or caffeic acid (CA) was added to 5-day-old liquid cultures to a final concentration of 50 (4-MC) or 100 µM (4-MC/CA) and cultures were further cultivated under standard growth conditions. For growth inhibitory tests, CA was also added to solid culture medium to final concentrations of 50, 100, 500, and 1000 µM.

Application of tritiated isopentenyladenine

To determine *in vivo* cytokinin oxidase/dehydrogenase activity of liquid-cultured protonemata, feeding experiments were performed with tritiated isopentenyladenine ([2-^{3}H]-iP) at 25 °C and constant white light (c.30 µmol $m^{-2}s^{-1}$, fluorescent tubes, 15 W, Osram) according to Schwartzenberg *et al.* (2003, 2007). [2-^{3}H]-iP (specific activity 1.29 Tbq $mmol^{-1}$) was added at a final concentration of 5 pM to concentrated 6-day-old protonema suspensions (day 0). After 2, 4, and 8 hours, 50 µl samples of the culture medium were taken and overall radioactivity (disintegrations per minutes) was determined by liquid scintillation counting (liquid scintillation cocktail Optisafe 'HiSafe' 2, Tri-Carb 2800 TR, PerkinElmer).

Phylogenetic analysis

The *P. patens* PPO1 amino acid sequence was used in a BLAST query to retrieve PPO sequences from other completely sequenced plant genomes. Based on the length of the PFAM domain PF00264 (228 aa), which is considered necessary for a functional PPO, only sequences with at least 190 aa alignment length and 30% identity were taken into account. Upon initial alignment and phylogenetic tree inference, some species were removed for brevity: namely, from the Poales, the species with the highest (*Sorghum bicolor*, eight) and the lowest number of PPOs (*Oryza sativa*, three) were kept, while *Zea mays* (six), *Setaria italica* (five), and *Brachypodium distachyon* (five) were removed. The same scheme was applied to the Rosales, where *Malus domestica* (16) and *Fragaria vesca* (six) were kept and *Prunus persica* (seven) removed. No PPO could be detected in the genomes of the Brassicaceae *A. thaliana* and *Arabidopsis lyrata*. In the case of *P. patens*, the two PPOs that are considered pseudogenes were not included for phylogenetic inference. As an outgroup, three laccases each from *P. patens* and *A. thaliana* were added. The alignment was generated using MAFFT linsi (Katoh *et al.*, 2005) and contained 2093 columns. By manual curation using Jalview (Clamp *et al.*, 2004), all columns were removed that had a low quality score or contained only a single sequence, resulting in a clipped alignment of 489 positions. From this alignment, all sequences lacking continuous stretches >160 columns (1/3 of alignment length) were removed to avoid problems during phylogenetic inference. Such sequences are probably due to fragmentary gene models; in particular, this affected three sequences from *Malus domestica* and one each from *Eucalyptus grandis*, *Glycine max*, *Carica papaya*, and *O. sativa*. As an exception from this rule, the *P. patens* Pp1s559_8V6.1 (PPO5) sequence was kept, although it lacked ~220 positions from the C-terminus. It should be noted that some other remaining sequences lacked continuous stretches up to 100 columns from this core alignment. The minimum overlap between sequences remaining in the alignment was ~140 columns, i.e. ~30% of the alignment length. Low support of some nodes was probably due to these remaining fragmentary sequences. These sequences were kept in the alignment as otherwise some interesting information, e.g. about *C. papaya* and *V. vinifera*, would have been lost. The best-suited evolutionary model was selected using ProtTest (Abascal *et al.*, 2005) and was found to be WAG (Whelan and Goldman, 2001) with gamma distributed rates. Bayesian inference of phylogenetic relationships was carried out with MrBayes (Ronquist and Huelsenbeck, 2003) and visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Isolation of genomic DNA

A simplified CTAB method was used for isolation of genomic DNA from moss tissue. Part of a young green gametophore was transferred to a 1.5 ml tube containing 400 µl of 2× CTAB buffer (2%, w/v, CTAB, 100 mM TRIS-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0) and ground with a small plastic pestle. The homogenate was incubated for 1 hour at 60 °C in a water bath, subsequently extracted with an equal volume of chloroform/isoamylalcohol (25:1) and centrifuged at 16,000 *g* for 10 min. The upper aqueous phase was transferred to a new tube and an equal volume of 2-propanol (approx. 300–350 µl) was added, followed by a second centrifugation to precipitate the genomic DNA. The supernatant was discarded and the DNA pellet was washed with 70% ethanol and dissolved in 50 µl TE buffer containing 1 µl of 1 mg RNaseA ml^{-1}. For PCR, 0.5–2.0 µl DNA were used as a template in a total reaction volume of 25 µl.

Vector construction

The vector for heterologous expression of His-tagged PPO6 was created by amplification of the *PPO6* sequences encoding for the predicted mature protein PPO6 from a cDNA library (Reski *et al.*, 1995) with the primers *cPPO6_forw* and *cPPO6_rev* (Supplementary Material, available at *JXB* online) and ligation into the plasmid pTrcHis2_TOPO (Invitrogen) in frame to a sequence encoding for a C-terminal His-tag.

For cloning of a *PPO1* knockout construct, the entire coding region of *PpPPO1* (Richter *et al.*, 2005) was amplified with the primers *cPPO1_forw* and *cPPO1_rev* (Supplementary Material) from a protonema cDNA library with a proofreading polymerase, cut with *Sal*I and *Nco*I, and cloned into the *Sal*I/*Nco*I-cut plasmid pET28a (Novagen by Merck). In a second cloning step, an *nptII* resistance cassette (neomycin phosphotransferase II gene under control of the 35S promoter, terminated by the *nos* terminator) was released from the vector pHP23 (Paszkowski *et al.*, 1988) by digestion with *Eco*RI and blunt-end inserted into the coding sequence of *PpPPO1* digested with *Ecl*136II and *Bsp*1407.

Transformation of Physcomitrella

The *PPO1* knockout cassette (*nptII* cassette flanked by 730 and 853 bp of *PPO1* coding sequence) was amplified from the knockout construct using the primers *cPPO1_forw* and *cPPO1_rev* and used for PEG-mediated transfection of *P. patens* protoplasts. Generation of protoplasts from protonema tissue cultivated for 5 days in liquid medium and transformation was performed as described by Hohe *et al.* (2004) with slight modifications.

Heterologous expression of recombinant His-tagged PPO6 in Escherichia coli

To obtain recombinant PPO from *PPO6* expressing *E. coli* clones (strain TOP10, Invitrogen), 200 ml LB culture supplemented with the appropriate antibiotic was inoculated with a starter culture. Cultures were grown in 1 l flasks with 200 rpm shaking at room temperature to an OD_{600} of 0.4, and transcription was induced by the addition of 1 mM IPTG. Cultures were further supplemented with 20 μM $CuCl_2$ and grown for additional 6 hours at room temperature. Additionally, control expressions were performed with TOP10 clones expressing a His-tagged lacZ protein as well as with BL21(DE3) clones expressing His-tagged PPO1 of *Trifolium pratense* (TpPPO1) (Sullivan *et al.*, 2004). Protein extracts obtained from these expression systems served as positive controls for proper expression (lacZ:his and TpPPO1:his) and as negative (lacZ:his) as well as positive controls (TpPPO1:his) for PPO activity determinations from *E. coli* protein extracts.

Protein isolation/extraction and PPO enzyme activity assay

Crude protein extracts from *P. patens* tissue were prepared as described by Richter *et al.* (2005) with slight modifications. Sample tissue was frozen in liquid nitrogen and subsequently homogenized in a small volume of ice-cold phosphate buffer composed of KH_2PO_4 and Na_2HPO_4 (67 mM each) at pH 6.4 using the FastPrep FP120 homogenizer (MP Biomedicals). To remove cell debris, the homogenate was centrifuged for 10 min at 16,000 *g* and 4 °C.

Protein extracts from culture medium were prepared by lyophilization of the medium at –20 °C. The lyophilized powder was resuspended in ice-cold phosphate buffer and insoluble compounds were sedimented by centrifugation at 16,000 *g* for 5 min. The supernatant was subsequently desalted by application on an NAP 25 column (GE Healthcare), and the proteins were eluted in ice-cold phosphate buffer.

Protein extracts from *E. coli* TOP10 expressing *P. patens* PPO6 were prepared prepared by using the pTrcHis2-TOPO-TA expression kit (Invitrogen). Harvested bacteria were incubated for 15 min at room temperature with 100 mg lysozyme ml^{-1} and extract was further treated as described in Richter (2009). Recombinant His-tagged PPO was purified by column chromatography on His-bind Ni-NTA-columns (Novagen by Merck) following the manufacturer's instructions. The purified protein was desalted and finally eluted in phosphate buffer.

Protein contents were determined according to Bradford (1976) using bovine serum albumin as a standard. For quantification of low-concentration protein extracts (prepared from culture medium), the NanoOrange protein quantitation kit (Invitrogen) was used according to the manufacturer's instructions and fluorescence was determined with a luminescence spectrometer (LS 55, Perkin Elmer).

Total *in vitro* PPO activity was determined polarographically according to the method of Lieberei and Biehl (1976) as described in Richter *et al.* (2005).

Determination of cell viability

Cell vitality of *P. patens* was determined by fluorescein diacetate staining. Protonema culture (500 μl) was incubated for 5 min at 20 °C with 2 μl of fluorescein diacetate (FDA) stock solution (10 mg ml^{-1} in DMSO). Green fluorescence of living cells, resulting from de-esterification of FDA to fluorescein by esterase activity (Power and Chapman, 1985), was examined under blue light excitation.

Extraction of cytokinins from tissue and culture medium and analysis by ultra-performance LC-MS/MS

Concentration of endogenous cytokinins were determined by ultra-performance LC (UPLC)-MS/MS analysis as described in Schwartzenberg *et al.* (2007) including modifications described by Novák *et al.* (2008). Samples of both tissues and culture media were freeze-dried and stored at –20°C until analysis.

Extracts prepared with Bieleski's reagent were purified using a cation (SCX-cartridge), an anion exchanger (DEAE-Sephadex/C18 cartidge, and an immunoaffinity chromatography (IAC) based on wide-range specific monoclonal antibodies against cytokinins (Novák *et al.*, 2003). By comparison of the specific multireaction monitoring transition and chromatographic retention times with those of authentic standards, the identity of all measured cytokinin metabolites was verified.

Extraction of phenolic compounds from tissue and quantification by UPLC-MS/MS

Freeze-dried tissue samples were homogenized with 10% methanol using an oscillation ball mill (MM 301, Retsch, Haan, Germany). The extract was centrifuged for 10 min at 20,000 *g*, the supernatant was filtered through a 0.2 μm Micro-Spin nylon membrane filter (GRACE, Columbia, MD, USA) and directly analysed by UPLC-MS/MS as described by Gruz *et al.* (2008).

Video-based online growth monitoring

For online growth monitoring, protonemata were cultivated at 25°C in continuous light on agar plates containing standard medium supplemented with 500 μM caffeic acid (CA). Growth measurements took place in a growth chamber equipped with a camera for recording of two-dimensional growth based on increase of pixel number, as described by Roleda *et al.* (2004).

Statistical analysis

For statistical analysis, the software package Statistica version 9.1 (StatSoft, Tulsa, OK, USA) was used. Either t-test or ANOVA with post-hoc test according to Tukey was used with *P*-values <0.5, <0.05, or <0.01, as indicated.

Results

Physcomitrella *PPOs are encoded by a multigene family*

After the *P. patens* genome sequence became available (Rensing *et al.*, 2008), evidence was found that *P. patens* PPOs are encoded by a multigene family. BLAST analysis using the derived amino acid sequence of *PpPPO1* (Richter *et al.*, 2005) as a query for BLASTp in the *P. patens* V1.6 protein database and for tBLASTn in the *P. patens* V1.6 genome database (www.cosmoss.org), identified 15 loci possessing similarities to PPO1 (cut off 35% identity over a length of at least 80 amino acids). To complete the analysis, BLAST searches with PPOs from sequenced plant genomes were also performed and no additional loci were found. The 15 loci and their predicted gene models (version 1.6) with their intron/exon structure were evaluated according to the presence of the essential central domain binding two copper ions (PFAM Tyr PF00264), transcript evidence, and homology to published PPO sequences. PPO-related sequences that did not contain the complete CuA and CuB domains were excluded from experimental analysis. From 13 selected loci (Supplementary Table S1), PPO1 (Richter *et al.*, 2005) was found to be the most basal isoform of the *P. patens*-specific PPO (Fig. 1).

Sequence properties of the derived amino acid sequences of PPO1–PPO13

PPO5 and PPO8 were found to be located tail to tail on the same scaffold as a tandem array, separated by about 15 kbp. Despite their close localization, the protein sequences derived from the V1.6 gene models showed only 51% identity (not shown). PPO4 and PPO11 were found located head to head about 1.89 Mbp apart from each other and exhibited only 29% identity. The differences in the distance of these arrayed PPO loci was reflected by their relative distance in the phylogenetic tree. Percentage identities of pairwise alignment (not shown) of the overall amino acid sequences ranged from 27% (PPO11 and PPO13) to 72% (PPO1 and PPO2). Also PPO8 and PPO9 shared a high degree of identical amino acids (71%).

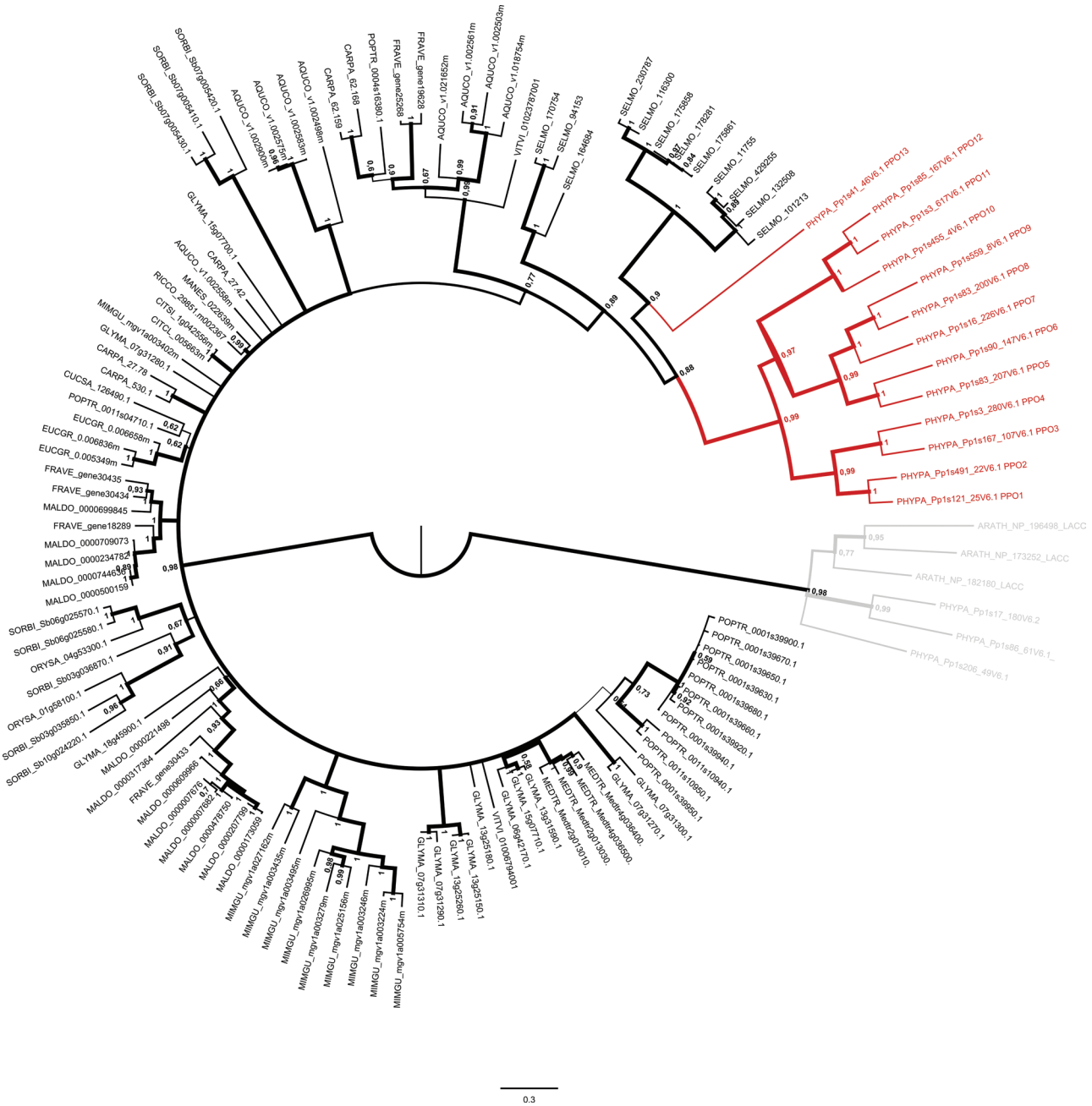

Fig. 1. Bayesian inference of the *PPO* gene family phylogeny. Branch width and numbers at nodes correspond to posterior probabilities. The tree is outgroup rooted with laccases (shown in grey). The *P. patens* PPOs are shown in red. Coding sequences were obtained from cosmoss.org version V1.6 (see Supplementary Table S1). AQUCO, *Aquilegia coerulea*; ARATH, *Arabidopsis thaliana*; CARPA, *Carica papaya*; CITCL, *Citrus clementina*; CITSI, *Citrus sinensis*; CUCSA, *Cucumis sativus*; EUCGR, *Eucalyptus grandis*; FRAVE, *Fragaria vesca*; GLYMA, *Glycine max*; MALDO, *Malus domesticus*; MANES, *Manihot esculenta*; MEDTR, *Medicago truncatula*; MIMGU, *Mimulus guttatus*; ORYSA, *Oryza sativa*; PHYPA, *Physcomitrella patens*; POPTR, *Populus trichocarpa*; RICCO, *Ricinus cummunis*; SELMO, *Selaginella moellendorffii*; SORBI, *Sorghum bicolor*; VITVI, *Vitis vinifera.*

Physcomitrella *PPOs are monophyletic*

The *PPO* gene family tree (Fig. 1) suggests that the extant PPO sequences in the non-seed plants *P. patens* (13 genes) and *Selaginella moellendorffii* (12 genes) to be monophyletic, but subject to significant lineage specific expansion. The relationships between seed plant PPO clades are unclear and do not allow to reconstruct the detailed history of gene loss and gain. However, lineage specific expansion of *PPO* genes is evident in several seed plant taxa as well, for example within the Rosales, Fabales, and Poales. Within the seed plants, the number of *PPO* genes per species ranges from zero (e.g. *A. thaliana*) to 16 (*Malus domestica*). While most genomes harbour more than one *PPO* gene, several seem to encode only a single PPO, e.g. the two *Citrus* species, *Manihot esculenta*, and *Cucumis sativus*. Interestingly, the *C. papaya* (Brassicales) genome encodes PPOs, evidencing loss of *PPO* genes probably within the Brassicaceae or in the genus *Arabidopsis*.

Physcomitrella *PPO6 is a functional* o*-diphenol oxidase*

To test the functionality of one member of the *P. patens PPO* gene family, this study randomly chose *PPO6* for heterologous expression in *E. coli* under the control of the IPTG-inducible *trc*-promoter. The expression vector pTrc_PpPPO6-his was generated inserting a PCR product encoding for the putative coding sequence of *PPO6*. Expression of PPO6:his was induced and protein extracts were purified using Ni-NTA-columns. Western blot analysis using an anti-His-tag antibody revealed that a major portion of the expressed PPO6:his protein was apparently insoluble and accumulated in inclusion bodies, but a small part of soluble PPO6:his protein could be enriched by purification via Ni-NTA-column. Subsequently, PPO functionality of the recombinant His-tagged PPO6 protein was confirmed in a polarographic enzyme assay using 4-methylcatechol (4-MC) as a substrate. In contrast, control protein extracts for lacZ:his in the same expression system had no measurable *in vitro* PPO activity (Fig. 2). PPO enzyme activities of PPO6:his protein extracts could be inhibited by the addition of KCN to the reaction mixture, which is a necessary prerequisite for a PPO activity assay. Although it was extremely difficult to obtain active recombinant PPO protein from *P. patens*, strong evidence for the functionality of one exemplary *PPO* gene product was found using PPO6.

Searching for phenolic compounds from Physcomitrella *as putative PPO substrates*

To investigate which phenolic compounds could be potential substrates, *P. patens* tissue was analysed for the presence and composition of polyphenolic compounds using UPLC-MS/MS. The compounds protocatechuic acid, 4-hydroxybenzoic acid, salicylic acid, CA, and 4-coumaric acid were detected at concentrations ranging from 1 to 6.5 nmol (g dry weight)$^{-1}$ (Supplementary Fig. S1). In addition, an unknown ester of CA was detected in tissue samples. Based on the spectral data, the ester was tentatively identified as 2-*O*-caffeoylthreonic acid (Supplementary Fig. S2).

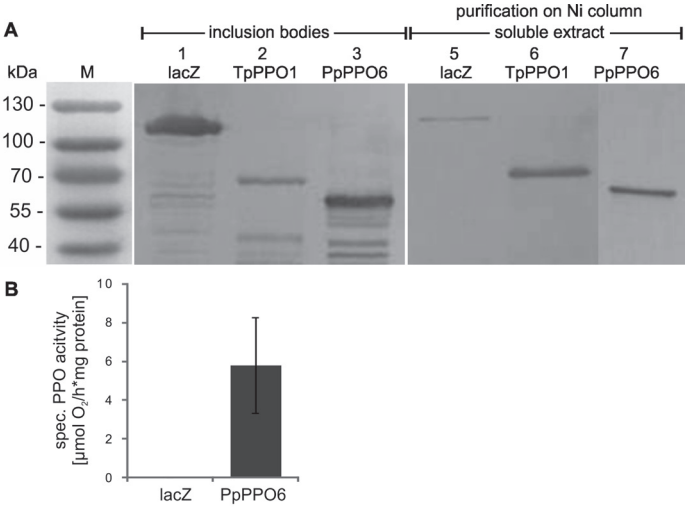

Fig. 2. Recombinant PPO proteins and enzyme activity. (A) Western blot analysis of insoluble (left panel), and soluble protein extracts (right panel) enriched and purified on a Ni-column. Protein extracts of 6 h IPTG-induced *E. coli* cultures expressing His-tagged proteins were separated on a 12.5% SDS gel and detected by an anti-His-tag antibody: lacZ:his (120 kDa), TpPPO1:his (59 kDa, migrates at 65 kDa according to Sullivan *et al.*, 2004), PPO6:his (62 kDa). (B) Specific PPO activity of enriched and purified soluble protein extracts determined polarographically using 4-methylcatechol as a substrate. Bar, standard deviation (n = 3).

Caffeic acid causes growth reduction

As caffeic acid was shown to be an endogenous polyphenolic compound, it was chosen as an example to examine the effects of phenolics on *P. patens* growth and PPO activity. Protonema was cultured on agar medium supplemented with concentrations of CA ranging from 50 to 1000 µM. As indicated in Fig. 3A, plant diameters were lower with increasing CA concentrations. In Fig. 3B, examples of *P. patens* cultivated with 500 µM CA are displayed along with the control showing growth reduction caused by CA. Optical online growth monitoring displaying the relative increase of plant area (Fig. 3C) revealed that substantial growth reduction was apparent 2 days after the exposure to CA.

Additionally, PPO activity, determined polarographically, had a tendency for decreased values in tissue cultivated with CA [227.95 ± 28.91 nmol O_2 h^{-1} (mg protein)$^{-1}$] compared to tissue cultivated in standard culture medium [287.60 ± 13.93 nmol O_2 h^{-1} (mg protein)$^{-1}$] (n = 6).

Generation and molecular characterization of PPO1 knockout plants

To obtain information on the biological functions of PPO in *P. patens*, targeted knockout lines (Schaefer and Zryd, 1997) were generated. For this experiment, *PPO1* was chosen as it had been previously characterized (Richter *et al.*, 2005) and because it is well expressed in protonemata. A *PPO1*-knockout vector was constructed by placing a 730-bp 5′- and a 853-bp 3′-genomic fragment flanking a *35S-NPTII* resistance cassette. From this,

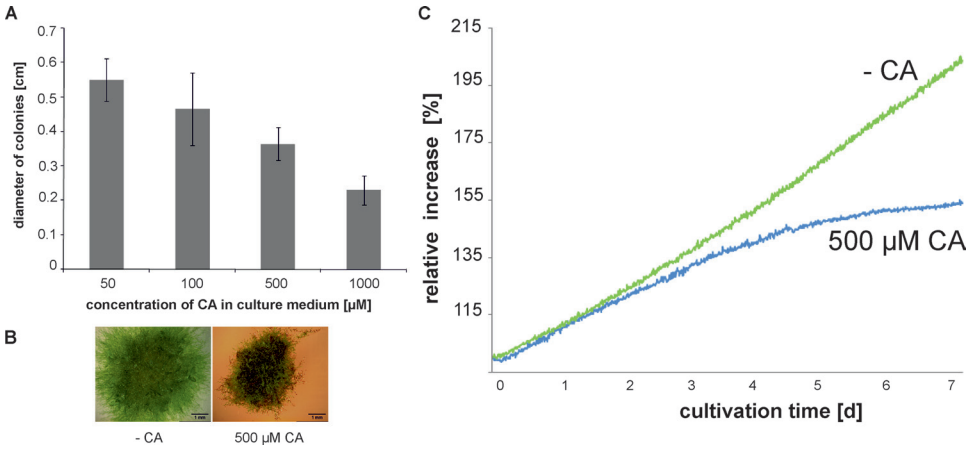

Fig. 3. Growth inhibition of *P. patens* wild type by caffeic acid (CA). (A) Diameter of plants grown on solid culture medium supplemented with different concentrations of CA measured after 4 weeks of cultivation (n = 10, bars represent standard deviation). (B) Growth with 500 µM CA (right) and without CA (left) measured after 4 weeks. (C) Optical online monitoring of arial plant growth displaying the relative area increase of a moss plant cultivated with 500 µM CA and without CA (this figure is available in colour at *JXB* online).

the knockout cassette was amplified by PCR and then used for PEG-based transfection. The expected gene disruption caused by integration of the resistance cassette into the genomic *PPO1* locus was demonstrated by PCR (Supplementary Fig. S3A, B) and subsequent sequencing (not shown). All five selected PPO1 knockout strains (d|ppo1) had undergone a recombination event at the 5′ end of *PPO1*. Plants of d|ppo1 lines 1, 3, and 5 showed additionally recombination at the 3′ end, indicating that a gene replacement had occurred. d|ppo1 lines 5 and 6 had undergone targeted knockout but no gene replacement occurred as the recombination did not take place in the 3′ region of *PPO1*. The expected absence of the *PPO1* transcript in the stable and haploid d|ppo1 lines 1, 3, 5, 6, and 8 was demonstrated by reverse-transcription PCR (Supplementary Fig. S3C). d|ppo1 lines 1 and 5 were arbitrarily chosen for physiological experiments.

PPO1 knockout plants possess a reduced extracellular PPO activity and exhibit a decreased tolerance towards 4-methylcatechol

The only slight decrease in PPO enzymatic activity between tissue extracts of wild type and d|ppo1 lines 1 and 5 revealed that PPO1, if at all, accounts only for a small portion of the overall intracellular PPO activity (Table 1). However, the strong difference in PPO activity between wild type and knockout strains in the extracellular compartment demonstrated that PPO1 is a major extracellular isoform, providing 68% of the overall PPO activity released into the medium. Additionally, this result provides indirect evidence that PPO1 is, besides PPO6, also a functional PPO accepting 4-MC as a substrate.

In order to obtain indications about possible functions of PPO1, the wild type and d|ppo1 lines 1 and 5 were examined for tolerance towards externally applied 4-MC, being a substrate for PPO1. Concentrations as low as 50 µM 4-MC induced a severe die back of mutant tissue and mutant cells lacked fluorescence when FDA staining for vitality was performed. Wild-type protonema, however, was able to resist the 4-MC treatment, and FDA staining indicated that cells were alive and metabolically active (Fig. 4).

Growth assays in which d|ppo1 strains were exposed to various concentrations of CA did not result in significant differences in plant size when compared to wild type (not shown).

PPO1 knockout lines exhibit altered differentiation

Phenotypical observations revealed that the d|ppo1 lines exhibited abnormal protonema growth with shorter and more rounded chloronema cells (Supplementary Fig. S4). Moreover, these transgenic plants were significantly smaller with more gametophores cm^{-2} than wild type (Fig. 5, Table 2). Reduced areal size with enhanced gametophore formation could be partially mimicked with wild-type tissue grown in the presences of the cytokinin isopentenyladenine (100 nM) (Fig. 5 G, H), thus suggesting that the altered phenotype might be related to changes in the homeostasis of cytokinins.

Table 1. *In vitro* PPO activity measured in culture medium and tissue of wild type and d|ppo1 lines. Protein extracts were prepared from tissue and culture medium from cultures grown under standard conditions for 7 days and PPO activity was determined polarographically using 4-methylcatechol as a substrate (n = 3). Values are µmol O_2 h^{-1} (mg protein)$^{-1}$. Different letters within rows indicate significant differences according to ANOVA with post-hoc test after Tukey using $P < 0.01$.

	Wild type	d|ppo1 line 1	d|ppo1 line 5
Culture medium	12.17 ± 3.26[a]	3.93 ± 0.57[b]	3.78 ± 1.42[b]
Tissue	0.19 ± 0.03[a]	0.15 ± 0.01[a]	0.15 ± 0.04[a]

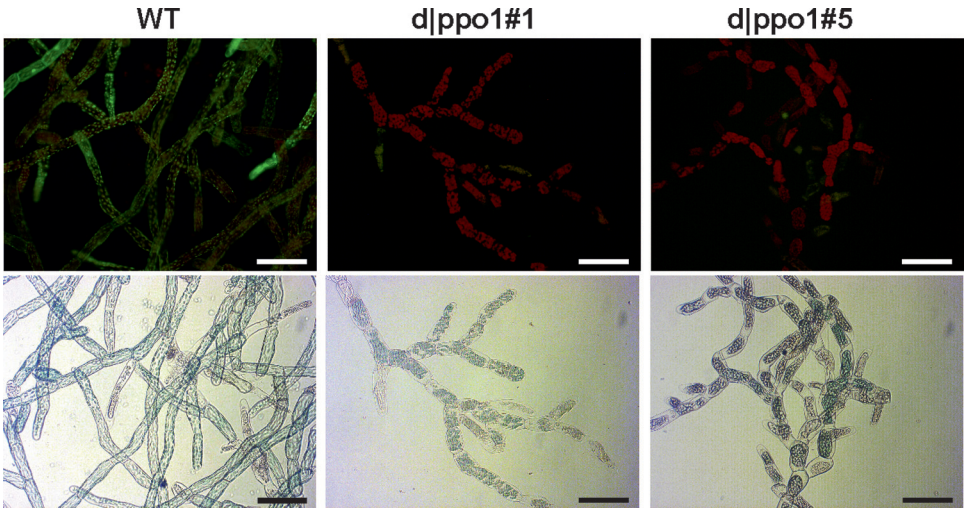

Fig. 4. Effect of 4-methylcatechol (4-MC) application on protonemata of *PPO1* knockout mutants and wild type. Tissue was cultivated under standard conditions in liquid medium supplemented with 50 µM 4-MC and cell vitality was displayed by FDA staining and microscopical observation after 48 h of incubation (upper panel) and bright light microscopy (lower panel). Bars, 100 µm.

PPO1- knockout causes alterations in cytokinin profile

It is well known that gametophore formation in mosses is induced by cytokinins (reviewed in von Schwartzenberg, 2009). To examine whether changes of the cytokinin profile could be a cause for the enhanced differentiation in PPO1-deficient plants, this study carried out a LC-MS based cytokinin analysis in tissue and culture medium and compared d|ppo1 line 5 with the wild type. d|ppo1 line 5 contained increased intracellular amounts of the cytokinin forms *trans*-zeatin-*O*-glucoside, *trans*-zeatin riboside-*O*-glucoside, *cis*-zeatin-*O*-glucoside, *cis*-zeatin riboside-*O*-glucoside, dehydrozeatin riboside-*O*-glucoside and isopentenyladenine (Fig. 6A, 6C, 6E, 6G). In each cytokinin family, at least one compound was significantly increased (t-test, $P < 0.05$).

Moreover, analysis of the culture media also revealed increased extracellular concentrations of isopentenyladenine, isopentenyladenosine-5′-monophosphate, *cis*-zeatin-riboside-5′-monophosphate, and dihydrozeatin riboside-*O*-glucoside in knockout cultures compared to the wild type (Fig. 6B, 6D, 6F, 6H). Also for culture media, at least one compound per family of cytokinin was significantly increased.

PPO1 knockout plants show slower degradation of extracellular radiolabelled isopentenyladenine

To determine if the increase of cytokinin levels found in d|ppo1 line 5 tissue and medium could be a consequence of a reduction in cytokinin breakdown, *in vivo* metabolism studies were carried out by adding [2-^{3}H]-iP to liquid cultures of d|ppo1 lines 1 and 5 and the wild type. From previous studies, it was known that short-term breakdown of [2-^{3}H]-iP in *P. patens* cultures can be easily monitored by counting the radioactivity in the culture medium, which contains 90% of the applied isopentenyladenine (Schwartzenberg *et al*., 1993, 2007).

The amount of the applied [2-^{3}H]-iP (5 pmol ml^{-1}) was comparable to the usual amount of extracellular isopentenyladenine occuring in a *P. patens* wild-type culture (~10 pmol ml^{-1} in 10-day-old liquid cultures, according to Schwartzenberg *et al.*, 2007). Liquid scintillation counting in cultures of d|ppo1 lines 1 and 5 revealed a reduced loss of the applied [2-^{3}H]-iP (Fig. 7), which was found to be significant at 8 h incubation time. HPLC analysis coupled to online liquid scintillation counting (not shown) confirmed the reduction of [2-^{3}H]-iP depletion in the knockout lines, which is likely to be causally connected to the increase of cytokinins and the enhanced differentiation in the PPO1 knockout lines.

Discussion

Phylogenetic analysis suggests emergence of PPOs during the conquest of land by plants

Phylogenetic analysis revealed that *P. patens* possesses 13 paralogous *PPO* genes. Twelve *PPO* genes (1–12) form a monophyletic clade and this *PPO* gene family seems to have established independently from the vascular plant lineage.

PPO13 represents an additional gene located on a separate branch together with *PPOs* from the lycophyte *Selaginella moellendorffii*. It appears likely that the last common ancestor of *P. patens* and the vascular plant lineage possessed at least two *PPO* genes, one giving rise to the multigene family with *PPO1–12* and one which resulted in *PPO13*. Regarding the *PPO* gene families of seed plants, it can be assumed that their last common ancestor harboured two *PPO* genes as well, the descendents of which evolved independently in different plant lineages.

PPO gene duplication within the *P. patens* genome occurred most likely several times. The pairs of *PPO* genes evident in the phylogenetic tree (*PPO1/PPO2*, *3/4*, *5/6*, *8/9*, and *11/12*; Fig. 1) probably were retained after the whole-genome duplication, approximately 45 million years ago (Rensing *et al*., 2007). As indicated by the branch length within the phylogenetic tree, the formation of *PPO* gene families by gene and genome duplications occurred independently in *Selaginella moellendorffii* and seed plants.

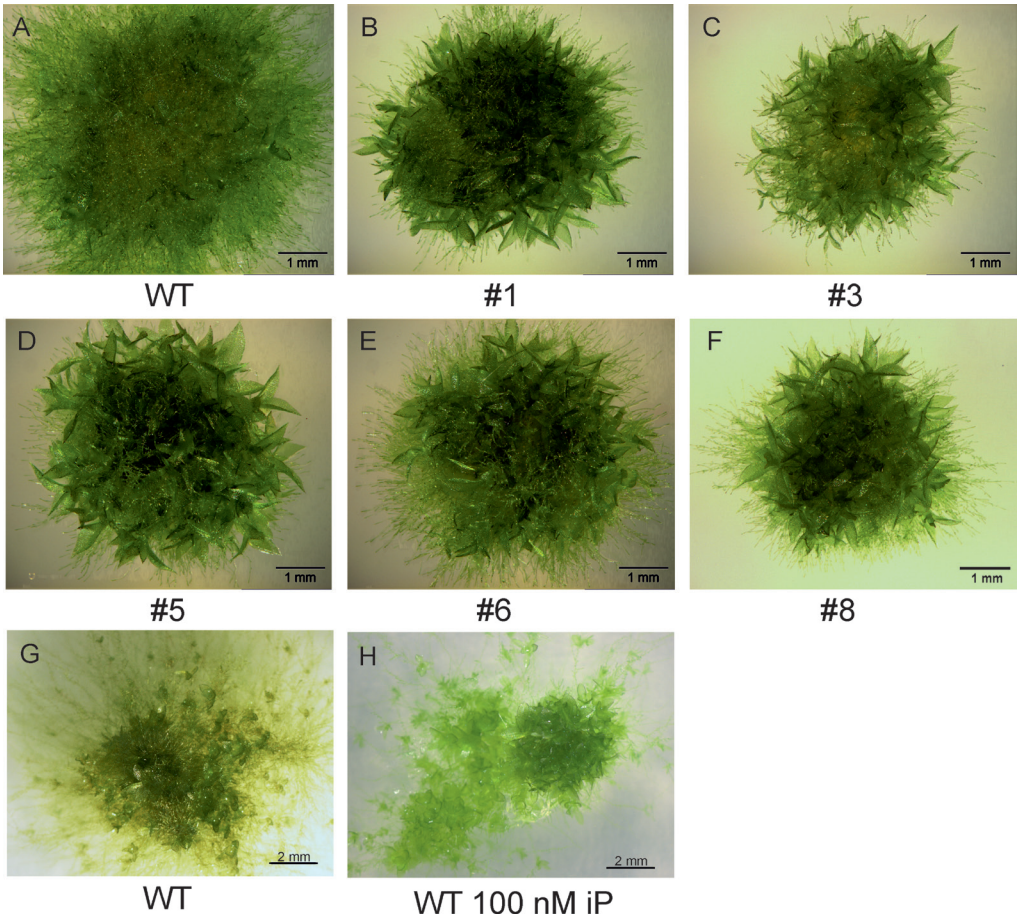

Fig. 5. Phenotypes of *PPO1* knockout plants and wild type. (A–F) Wild type and d|ppo1 lines 1, 3, 5, 6, and 8 cultivated under standard growth conditions for 17 days on the same agar plate. Bars, 1 mm. (G, H) Wild type cultivated for 14 days without and with isopentenyladenine (100 nM). Bars, 2 mm.

When screening the sequenced genomes of the green algae *Chlamydomonas reinhardtii* and *Ostreococcus tauri*, neither *o*-diphenol oxidases and laccases nor tyrosinases were found. Hence, it is most likely that PPO-encoding genes newly occurred during the evolution of the earliest land plants. However, there is a lack of sequenced genomes on the level of basal streptophytes like charopycean and zygnematophycean algae. Organisms of these clades need to be screened for PPO activity and corresponding genes. Analysis of algae adapted to non-aquatic habitats (Lewis, 2007) for PPO would also enable a better estimation of the exact evolutionary stage when PPOs first occurred. Sherman *et al.* (1991) analysed the distribution of PPO activity in a broad variety of aquatic and terrestrial plant species and found indication for PPO activity in the streptophytes *Coleochaete* and *Nitella*. The occurrence of PPOs in streptophytes other than seed plants might point towards an early function of PPOs that has developed further in higher organization forms and differentially evolved to perform diverse functions in different plant species.

Table 2. Gametophore formation in wild-type and d|ppo1 plants
Freshly disintegrated protonema tissue was transferred to agar plates (day 0) and cultivated for 7 days under standard conditions. d|ppo1 lines showed an enhanced production of gametophores compared to wild type. Values are means with standard deviations ($n = 8$). Different letters indicate significant differences according to ANOVA with post-hoc test after Tukey using $P < 0.01$.

Line	Gametophores after 7 days (cm^{-2})
Wild type	0.57 ± 0.18^{a}
d|ppo1 line 1	4.17 ± 0.83^{b}
d|ppo1 line 5	4.52 ± 1.27^{b}
d|ppo1 line 6	3.25 ± 0.75^{b}
d|ppo1 line 8	3.80 ± 0.91^{b}

Lang *et al*. (2008) pointed out that the adaptation of the first land plants to harsh conditions with high solar (UV) radiation and cycles of flooding and desiccation must have caused substantial changes in morphology and regulatory processes leading to the development of newly generated pathways in *P. patens*. Thus, characterization of the *PPO* gene family of *P. patens*, which, as a model system, occupies an important position in the land plant phylogeny, opens up new possibilities to obtain information on potential and probably more original functions of PPOs.

Although *o*-diphenol oxidases are likely to have evolved within the streptophyte clade, many tyrosinase and laccase genes have been characterized from several fungi, such as *Trichoderma reesei* (Selinheimo *et al.*, 2006) and *Pycnoporus sanguineus* (Halaouli *et al.*, 2006). Genome analysis has revealed that *P.*

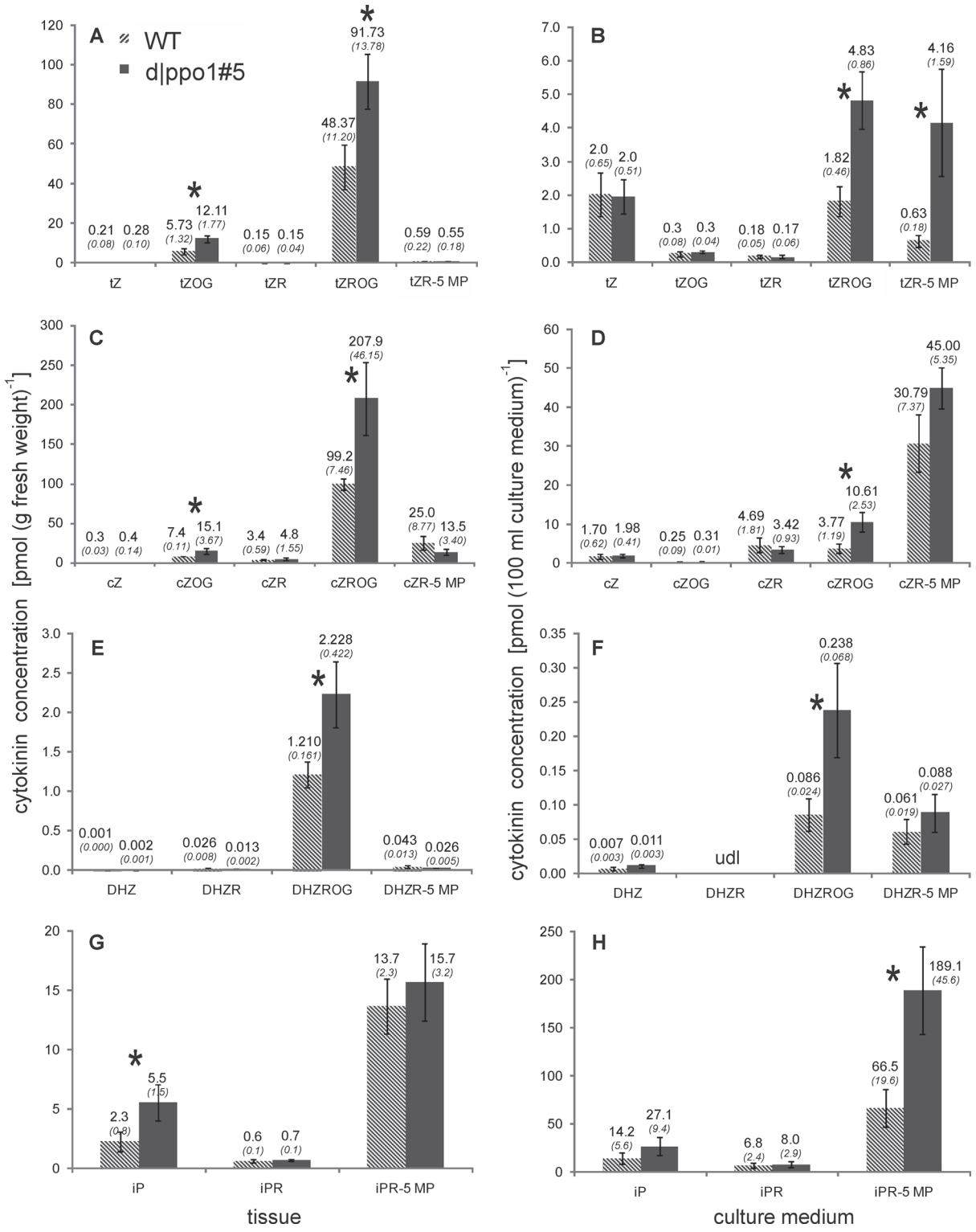

Fig. 6. Cytokinins in wild type and *PPO1* knockout strain d|ppo1 line 5 in tissue (A, C, E, G) and culture medium (B, D, F, H): (A, B) *trans*-zeatins, (C, D) *cis*-zeatins, (E, F) dihydroxyzeatins, (G, H) isopentenyladenine-type cytokinins. Cytokinins were determined from tissue and the corresponding culture medium of wild type and d|ppo1 line 5 after 15 days of culture under standard conditions. Cytokinins were determined from three biological replicates for each genotype (n = 3) by UPLC-MS displayed here as pmol (g fresh weight)$^{-1}$ or pmol (100 ml culture medium)$^{-1}$. Values are means ± standard deviations; numbers above each column are the numeric values. * indicate significant differences in t-test ($P < 0.05$). tZ, *trans*-zeatin; tZOG, *trans*-zeatin-*O*-glucoside; tZR, *trans*-zeatin riboside; tZROG, *trans*-zeatin riboside-*O*-glucoside; tZR-5 MP, *trans*-zeatin-riboside-5'-monophosphate; cZ, *cis*-zeatin; cZOG, *cis*-zeatin-*O*-glucoside; cZR, *cis*-zeatin riboside; cZROG, *cis*-zeatin-riboside-*O*-glucoside; cZR-5 MP, *cis*-zeatin-riboside-5'-monophosphate; DHZ, dihydrozeatin; DHZROG, dihydrozeatin riboside-*O*-glucoside; DHZR-5 MP, dihydrozeatin riboside-5'-monophosphate; iP, isopentenyladenine; iPR, isopentenyladenosine; iPR-5 MP, isopentenyladenosine-5'-monophosphate.

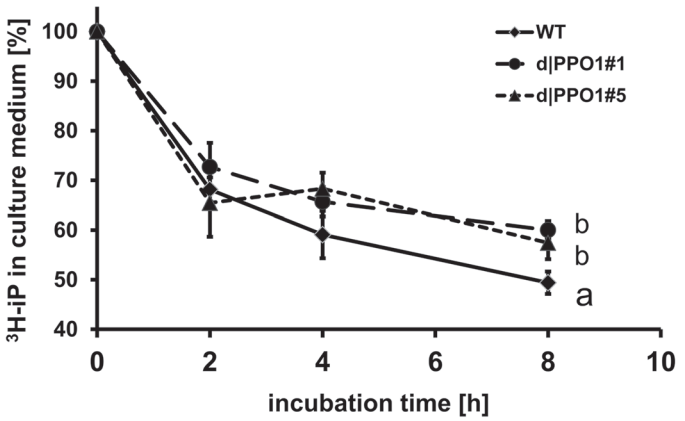

Fig. 7. Time-course of changes in the amount of applied [2-^{3}H]-isopentenyladenine in cultures of d|ppo1 plants compared to wild type (n = 3). Total radioactivity in the culture medium was determined by liquid scintillation counting and is displayed here as percentage radiolabelled isopentenyladenine (t0 = 100%). Different letters indicate significant difference at measurement after 8 h according to ANOVA with post-hoc test after Tukey using $P < 0.05$.

patens also possesses three putative laccase-encoding genes but in the present study's database searches, no tyrosinase-encoding genes have been found in the *P. patens* genome. Consequently, *P. patens* possesses two types of enzymes from the extended group of polyphenol oxidases, three *p*-diphenol oxidases (laccases), and 13 *o*-diphenol oxidases.

The functional gene knockout of *PPO1* with the concomitant strong decrease of extracellular PPO activity (Table 1) provides indirect evidence that PPO1 is secreted to the culture medium and contributes as much as 68% of the total extracellular PPO activity (in 8-day-old protonema cultures). For unknown reasons, this study was unable to express sufficiently high amounts of a PPO1:GFP fusion protein in *P. patens* in order to directly confirm the extracellular targeting of PPO1 (not shown).

However, the indirect evidence of PPO1 being an extracellular enzyme is in clear contrast to PPO localization in seed plants, which is mostly plastidic (Steffens *et al.*, 1994; Yoruk and Marshall, 2003). Ono *et al.* (2006) reported on a vacuolar flavonoid biosynthetic PPO and recently Tran and Constabel (2011) describe PtPPO13 from *Populus trichocarpa* as targeted to the vacuole thus underlining that other than plastidic localizations have to be taken into account. Extracellular localization of PpPPO1 is also supported by prediction of a putative cleavage site (between position 23 and 24, SignalP 4.0, http://www.cbs.dtu.dk/services/SignalP/) and of localization in the secretory pathway (YLoc+, http://abi.inf.uni-tuebingen.de/Services/YLoc/webloc.cgi).

Preliminary quantitative real-time PCR-based analysis of *PPO* gene expression have revealed that different isoforms of the PPO family are differentially expressed and that expression is in addition differentially influenced by factors like culture age, stress by strong-light irradiation or treatment by CA (Richter, 2009). The *PpPPO5* gene has been shown to be induced upon irradiation with UV-B (Wolf *et al.*, 2010). These results suggest that different PPO family members have different functions in *P. patens*.

Heterologous expression of PPO6

From sequence comparison and phylogenetic analysis, it seemed likely that the identified putative *PPO* genes from *P. patens* encode for *o*-diphenol oxidases. Repeated experiments to express recombinant *PPO1* in *E. coli* failed for unknown reasons. However, for PPO6, small amounts of the soluble protein could be purified after heterologous expression in *E. coli* and the *o*-diphenol oxidase activity was confirmed in polarographic assays using 4-MC as a substrate (Fig. 2). The extracts exhibited only low PPO activities not sufficient to allow substrate specificity tests.

In earlier work, Sullivan *et al.* (2004) also could obtain only relatively small amounts of soluble *Trifolium pratense* PPO (TpPPO1) expressed in *E. coli*. Bacterial clones expressing *TpPPO1* were also used as a positive control monitoring proper expression conditions in this study, and protein extracts exhibited *in vitro* PPO activity under identical experimental conditions to those used for *PpPPO6* expression. Nevertheless, as already observed by Sullivan *et al.* (2004), TpPPO1, like PpPPO6, exhibited only low PPO activities relative to the amount of protein used for the assay. Therefore, it seems that PPOs from *P. patens*, like those from seed plants, require appropriate post-translational modification to generate a highly active PPO enzyme, although PPO with limited levels of enzyme activity can be produced in a prokaryotic expression system. In spite of this, functional evidence that one exemplary member of the *P. patens PPO* gene family encodes for an *o*-diphenol oxidase was clearly given by the heterologous expression of *PPO6*.

Phenols from Physcomitrella

P. patens possesses chalcone synthases, catalysing the first step in flavonoid biosynthesis and also phenylalanine ammonia-lyase (PAL) homologues are present in the *P. patens* genome (Wolf *et al.*, 2010). Consequently, as PAL is a key enzyme in the polyphenol synthesis (Boudet, 2007), *P. patens* possesses the general ability and a set of enzymes to synthesize simple phenolic compounds and flavonoids, underlined by the detection of quercetin by Wolf *et al.* (2010).

Using UPLC-MS, this study detected and quantified the phenolic compounds protocatechuic acid, 4-hydroxybenzoic acid, salicylic acid, CA, *p*-coumaric acid, and an ester of CA (Supplementary Figs. S1 and S2). So far, nothing is known about possible functions of these compounds in the bryophyte *P. patens* and a comparison of the amounts of phenolic compounds found in the wild type with those in d|ppo1 plants (not shown) did not allow conclusions on the substrate preference of PPO1. Attempts to determine substrate specificity of recombinant protein of PPO1 and PPO6 were not successful because of limitations in protein quantity.

Effects of caffeic acid application on Physcomitrella *growth*

The application of CA (50 μM) to the culture medium of wild type inhibited protonemal growth and caused browning of the culture medium. Although a much higher than the endogenous

CA concentration was used in these experiments, a considerable extracellular CA consumption in protonemal cultures was monitored spectrophotometrically within 3 days of cultivation (data not shown). These observations suggest that CA applied to the culture medium can be metabolized by certain PPOs produced (and presumably secreted) by *P. patens*.

Bollag *et al.* (1988) reported that the exogenously applied growth-inhibiting phenols 2,6-xylenol and *p*-cresol can be detoxified by an extracellular laccase of the fungus *Rhizoctonia praticola*. Moreover, transgenic *Arabidopsis* seedlings expressing a secreted laccase from *Gossypium arboreum* exhibited an enhanced resistance to certain growth-inhibiting phenolic compounds. This pointed to a role for laccase in transforming phenolics *ex planta* without uptake of the substance by the plant (Wang *et al.*, 2004). The possibility arises that PPOs may play a similar role in *P. patens*.

Effects of PPO1 knockout and tolerance of 4-methylcatechol

From measurements of extracellular PPO activity in concentrated medium extracts of PPO1 knockout plants and wild type (Table 1), this study concluded that PPO1 is a major extracellular PPO with a potential role in detoxification of growth-inhibiting phenolic compounds. The fact that *P. patens* plants lacking PPO1 exhibited a reduced extracellular (*in vitro*) PPO activity using the substrate 4-MC provided indirect functional evidence that *PPO1* encodes a functional *o*-diphenol oxidase. It seems therefore likely that 4-MC, already identified as a substrate for PPO6, is also a substrate for PPO1. Consequently, applied phenolic compounds, potentially toxic for *P. patens* tissue, would be metabolized and removed by an extracellular PPO1-mediated oxidation.

Interesting and significant effects on d|ppo1 protonema were observed after incubation with the PPO substrate 4-MC, which caused an earlier die back in comparison to the wild type, suggesting that this compound is (more) toxic for d|ppo1 plants (Fig 4). Although catechol and its methyl derivatives are widespread phenolic componds in seed plants (Quideau *et al.*, 2011), it was not found in the current LC-MS based analyses of *P. patens* tissue and medium.

As already mentioned above, Wang *et al.* (2004) observed that transgenic *Arabidopsis* seedlings expressing a secreted laccase from *G. arboreum* exhibited an enhanced resistance to certain phenolic compounds. HPLC analysis indicated that the growth-inhibiting phenols were detoxified *ex planta*. The importance of phenolics and their oxidation products in plant–plant interactions and the role of plant PPOs to detoxify exuded phenolics, and thereby decrease their allelopathic action, has been reviewed by Li *et al.* (2010). The increased sensitivity of d|ppo1 plants towards 4-MC supports the view that also PPO1 has a function in detoxifying extracellular phenolic compounds in *P. patens*.

Knockout experiments suggest influence of PPO1 on cytokinin mediated differentiation

All analysed d|ppo1 plants exhibited a reduced areal growth of protonemata when grown on agar connected to a significantly higher production of gametophores cm^{-2} (Fig. 5, Table 2). Growth experiments using hormonal application showed that this increased differentiation to gametophores can be caused by external application of isopentenyladenine to the wild type (Fig. 5G, 5H).

In the work reported in Newman *et al.* (2011), the responsiveness of tomato PPO B to ethylene is well documented and also partial homologies of the *PPO* B promotor with responsive elements for gibberellic acid, abscisic acid, and jasmonic acid suggest that PPO B might be under control of various plant hormones. The current study demonstrated that a *PPO1* knockout causes a significant increase of endogenously produced cytokinins, including intra- and extracellular isopentenyladenine (Fig. 6), which is known to be a key regulator of budding and gametophore production in *P. patens* (Schwartzenberg *et al.*, 2007). The current study also demonstrated that PPO1 knockout plants show a reduced depletion of externally applied [2-^{3}H]-iP (Fig. 7). At this point, the mechanisms that lead from PPO1 deficiency to alterations in cytokinin homeostasis and the resulting developmental changes can only be speculated upon. Lowered cytokinin breakdown, which is likely to be the cause of the reduced isopentenyladenine depletion in the culture medium of the PPO1-deficient plants, is consistent with the proposal that PPOs could be involved in reoxidation of cytokinin oxidase/dehydrogenase, responsible for the cleavage of the isopentenyl side chain (Galuszka *et al.*, 2005). These authors collected evidence that the products of the PPO action on phenolics, the quinones, act as electron acceptors for the reoxidation of cytokinin oxidase/dehydrogenase, thus promoting cytokinin breakdown. This implies that, in the case of *P. patens* plants lacking *PPO1*, lower PPO activities would lead to less quinones and therefore to lower rates of cytokinin oxidase/dehydrogenase reoxidation. As a result, less cytokinin degradation would occur in plants that have a reduced PPO activity, leading to an excess of active cytokinins and consequently causing an increased bud and gametophore production.

Alternatively, the increase of cytokinins in PPO1-deficient plants could also be more distantly related to polyphenol metabolism, so the observed phenotype could be interpreted as a general and rather unspecific response to stress. Wolf *et al.* (2010) observed similarly reduced sizes of colonies and higher gametophore production as photomorphogenetic changes after UV-B irradiation of *P. patens* wild type and discussed this phenomenon as an avoidance reaction, in which increased gametophore occurrence reduces the exposure to UV-B by shadowing basal parts of the plant.

Lower amounts of protonemata with increased gametophore production also minimize the contact surface to the substrate and could be an answer to chemically induced stress. It cannot be excluded that PPO1 deficiency in *P. patens* leads to a type of internal metabolic stress, e.g. change in the general redox status, being at the origin of observed morphogenetic changes. In terms of reproduction and formation of persisting spores, it makes sense that a plant sensing stress accelerates the transition to generative reproduction, the first step of which would be cytokinin-induced gametophore initiation.

Although the phylogenetic analysis suggests that the occurrence of PPOs is connected to the evolution of the land plant lineage, a single and specific function cannot be attributed to these enzymes.

The importance of PPOs is underlined by the high number of *PPO* gene family members, which most probably take over different functions. A characteristic phenotype of d|ppo1 plants, besides the developmental changes, is the strongly increased susceptibility towards 4-MC, suggesting that extracellular PPO1 is involved in the detoxification of external phenolics. Ongoing work on other *PPO* gene family members will elucidate in how far their function and properties differ from the ones of PPO1 and which role PPOs take over in stress adaptation of *P. patens* and other bryophytes.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary information. Primers for *PpPPO1* and *PpPPO6* and deduced and edited amino acid sequence for PPO9

Supplementary Table S1.Properties of *PPO* gene family members

Supplementary Fig. S1. Quantification of phenolic compounds in *P. patens* wild type tissue

Supplementary Fig. S2. ESI(-)-MS spectrum of the unknown ester of caffeic acid

Supplementary Fig. S3. Characterization of *PPO1* knockout (d|ppo1) plants

Supplementary Fig. S4. Protonema growth of *PPO1* knock-out plants and wild type

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