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Research article

Isolation and functional characterization of two Caffeoyl Coenzyme A 3-*O*-methyltransferases from the fern species *Polypodiodes amoena*

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ABSTRACT

Caffeoyl Coenzyme A 3-*O*-methyltransferases (CCoAOMTs) catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to a hydroxyl moiety. CCoAOMTs are important for the synthesis of lignin, which provides much of the rigidity required by tracheophytes to enable the long distance transport of water. So far, no CCoAOMTs has been characterized from the ancient tracheophytes ferns. Here, two genes, each encoding a CCoAOMT (and hence denoted *PaCCoAOMT1* and *PaCCoAOMT2*), were isolated from the fern species *Polypodiodes amoena*. Sequence comparisons confirmed that the product of each gene resembled enzymes known to be associated with lignin synthesis in higher plants. When either of the genes was heterologously expressed in *E. coli*, the resulting recombinant protein was able to methylate caffeoyl CoA, along with a number of phenylpropanoids, flavones and flavonols containing two vicinal hydroxyl groups. Their *in vitro* conversion rate when presented with either caffeoyl CoA or certain flavonoids as substrate was comparable with that of the *Medicago sativa* MsCCoAOMT. Their constitutive expression in *Arabidopsis thaliana* boosted the plants' lignin content, but did not affect that of methylated flavonols, indicating that both PaCCoAOMTs contributed to lignin synthesis and that neither was able to methylate flavonols *in planta*. The transient expression of a *PaCCoAOMT-GFP* fusion gene in tobacco demonstrated that *in planta*, PaCCoAOMTs are likely directed to the cytoplasm.

1. Introduction

Lignin, a phenolic polymer derived mainly from hydroxycinnamyl alcohols, is an important component of the plant cell wall, providing hydrophobicity to the xylem and mechanical support to the stem (Weng et al., 2011). The building blocks of lignin are *p*-coumaryl, coniferyl and sinapyl alcohols, which give rise to, respectively, the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of the lignin polymer. These three units differ with respect to their degree of methoxylation at positions 3 and 5 of the aromatic ring, with the result that both the hydroxylation and methylation steps are important in lignin synthesis (Boerjan et al., 2003). Two S-adenosyl-L-Met (SAM)-dependent *O*-methyltransferases (OMTs) are responsible for the methylation of hydroxyl groups introduced at the 3- and 5- positions of the phenolic ring of monolignol precursors: the caffeic acid 3-OMTs (COMTs; Zubieta, 2002) and the caffeoyl co-enzyme A OMTs (CCoAOMTs). The COMTs range in size from 38 kDa to 42 kDa and do not require Mg^{2+} to methylate caffeoyl and 5-hydroxyferuloyl free acids, aldehydes and alcohols. The CCoAOMTs are rather smaller proteins (23–29 kDa), which do require Mg^{2+} to methylate the aromatic 3-hydroxyl of caffeoyl-CoA and 5-hydroxyferuloyl-CoA in the lignin biosynthetic pathway (Fig. 1). CCoAOMT homologs have been isolated and characterized from a number of plant species, including alfalfa (Inoue et al., 1998), poplar (Meyermans et al., 2000), pine (Li et al., 1999), sorghum (Walker et al., 2016) and tobacco (Martz et al., 1998). The crystal structure of each of MsCCoAOMT (from *Medicago sativa*: Ferrer et al., 2005), McPFOMT (from *Mesembryanthemum crystallinum*: Kopycki et al., 2008) and SbCCoAOMT (from sorghum: Walker et al., 2016) has been determined.

In addition to their established role in lignin formation, the COMTs and CCoAOMTs can also methylate other soluble phenolic metabolites. In several plant species, such as *M. crystallinum* (Ibdah et al., 2003), *Arabidopsis thaliana* (Raes et al., 2003), rice (Lee et al., 2008), poplar (Tsai et al., 2006) and *Vanilla planifolia* (Widiez et al., 2011), CCoAOMT-like enzymes have been shown to successfully methylate various flavonoids, anthocyanins, coumarins and aromatic esters (Hamberger et al., 2007). The *A. thaliana* enzyme CCoAOMT1 contributes to the formation of soluble sinapoyl conjugates in the leaf (Do et al., 2007) and is essential for the synthesis of the coumarin scopoletin

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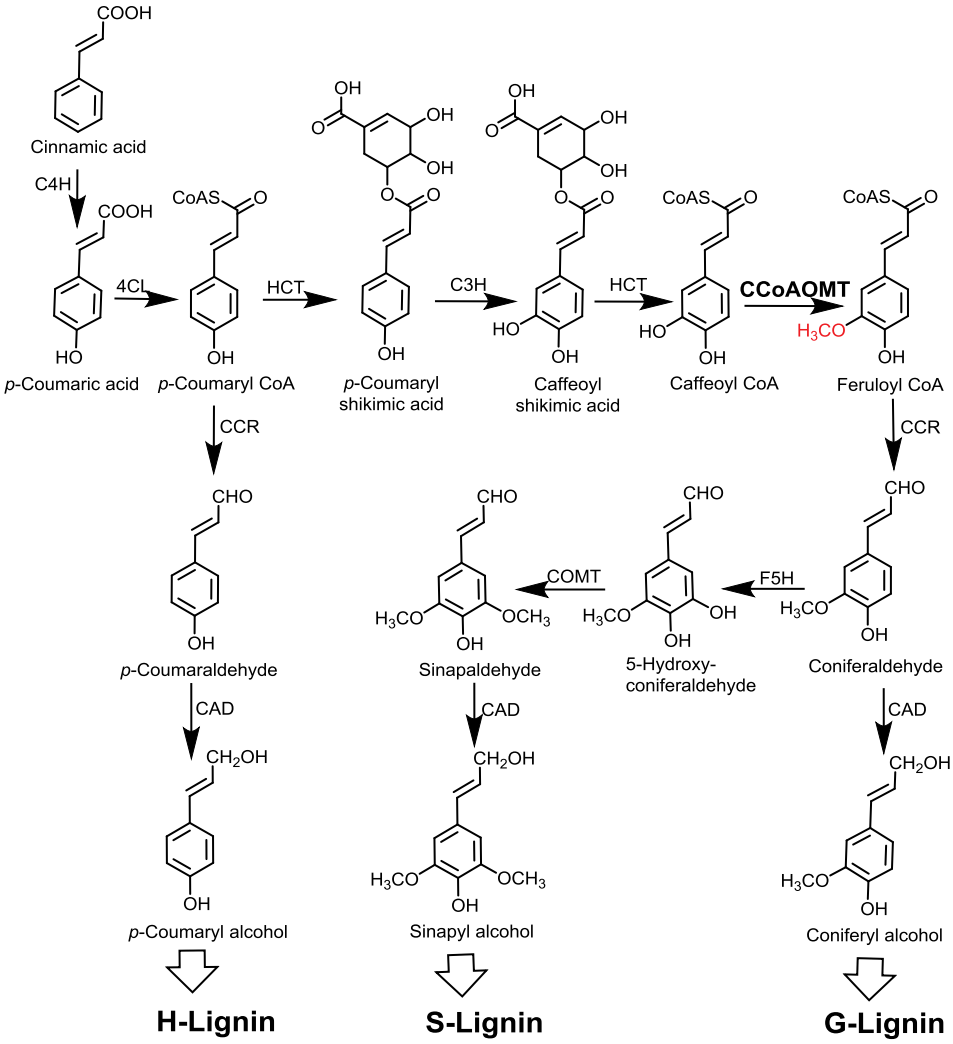

Fig. 1. Enzymes involved in the lignin synthesis pathway. C4H: cinnamic acid 4-hydroxylase, 4CL: 4-coumarate: co-enzyme A ligase, HCT: Hydroxycinnamoyltransferase, C3H: *p*-coumaroyl CoA 3-hydroxylase, CCoAOMT: caffeoyl CoA *O*-methyltransferase, CCR: cinnamoyl CoA reductase, F5H: ferulic acid 5-hydroxylase, COMT: caffeic acid *O*-methyltransferase, CAD: cinnamyl alcohol dehydrogenase.

in the root (Kai et al., 2008). COMTs participate in the methylation of flavonols with orthodihydroxy groups (such as quercetin), resulting in the accumulation of isorhamnetin glycosides in the *A. thaliana* inflorescence (Muzac et al., 2000; Thomas et al., 2003). A functional characterization of CCoAOMT produced by the liverwort species *Plagiochasma appendiculatum* (denoted PaOMT1) implied that it was able to methylate caffeoyl CoA, along with various flavonoids, coumarins and phenylpropanoids (Xu et al., 2015). Its constitutive expression in *A. thaliana*, however, had no effect on the plants' lignin content (unpublished data).

The development of phenylpropanoid metabolism in early land plants facilitated their move out of the aquatic environment, but these plants remained small as they were mechanically weak. The appearance of lignin coincided with the emergence of the first vascular land plants (the tracheophytes) during the Devonian era (Weng et al., 2010). Lignin bestowed the early tracheophytes with sufficient rigidity to stand upright and strengthened their ability to transport water over a long distance, thereby allowing a significant expansion in their size compared with their sister group, the bryophytes. Here, the focus is on the ferns, which represent an early diverging group of euphyllophytes. Given a better understanding of their lignin biosynthesis may provide novel insight into key developmental processes. As yet neither CCoAOMT-like nor true CCoAOMT enzymes have been characterized from any ferns species. Here we report the functional characterization of two distinct caffeoyl-CoA OMT enzymes from ferns *Polypodiodes amoena*. The enzymes metabolized not only caffoyl-CoA, but also flavonoids *in vitro* and increased lignin content when overexpressed in Arabidopsis.

2. Materials and methods

2.1. Plant materials, RNA extraction and reagents

Plants of the fern species *P. amoena* were grown in pots in a growth chamber delivering a 12 h photoperiod at 25 °C. Young leaves were harvested, rinsed in water, snap-frozen in liquid nitrogen, and ground to powder to allow for the extraction of RNA, using a CTAB-based procedure (Gambino et al., 2008). *Nicotiana benthamiana* and *Medicago sativa* plants were grown at 24–26 °C under a 12 h photoperiod. Phenylpropanoid and flavonoid compounds and their methylated forms were obtained from Chengdu Must Bio-technology (Chengdu, China), while all other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. Caffeoyl aldehyde, caffeoyl alcohol, 5-hydroxyconiferyl aldehyde and 5-hydroxyconiferyl alcohol were synthesized using published methods (Kim et al., 2004; Sun et al., 2013). Caffeoyl CoA was generated from caffeic acid with the aid of liverwort 4-coumarate CoA ligase (Gao et al., 2015) and was subsequently purified using a LC-18 SPE column (Supelco, Bellefonte,

PA, USA) (Beuerle and Pichersky, 2002).

2.2. Sequence alignment, phylogenetic analysis and homology modeling

The two putative fern *CCoAOMT* sequences identified from transcriptome sequencing database of the leaves of *P. amoena* (GeneBank: SRR8185331) were designated *PaCCoAOMT1* (MK164417) and *PaCCoAOMT2* (MK164418), while *MsCCoAOMT* (AAC28973.1) of *Medicago sativa* were found from online NCBI database. An alignment of their deduced polypeptide sequences with homologs from maize (AJ242980) and *M. sativa* was carried out using DNAMAN v7.0.2 software (Lynnon Biosoft, Quebec, Canada) (Brenner et al., 2010; Inoue et al., 1998). A plant OMT phylogeny was elaborated by implementing the MEGA v.4.0 program, based on the neighbor-joining method and applying 1000 bootstrap replicates (Tamura et al., 2007). Homology models for *PaCCoAOMT1* and *PaCCoAOMT2* were inferred using the SWISS-MODEL server (swissmodel.expasy.org) (Arnold et al., 2006) and the crystal structure of the *M. sativa* homolog MsCCoAOMT (Protein Data Bank entry 1SUI) as the template (Ferrer et al., 2005). The models were visualized using PyMOL software (www.pymol.org/citing).

2.3. Gene isolation, heterologous expression and purification of recombinant protein

Both *P. amoena* and *M. sativa* cDNA were obtained from the corresponding RNA using a Rever Tra Ace® qPCR RT kit (ToYoBo, Osaka, Japan) in conjunction with an oligo $(dT)_{18}$ primer, following the manufacturer's protocol. The full length *PaCCoAOMT1*, *PaCCoAOMT2* and *MsCCoAOMT* open reading frames (ORFs) were amplified from the corresponding cDNAs using PrimeSTAR® Max DNA Polymerase (Takara, Kusatsu, Japan) and the relevant OMT-F/R primer pair (sequences given in Supplemental Table 1). The resulting amplicons were double-digested with *Bam*HI and *Xho*I (Takara, Kusatsu, Japan) and the restricted products inserted into pET-32a (Novagen, Darmstadt, Germany). After their sequencing-based validation, the recombinant plasmids (along with an empty pET-32a plasmid as a negative control) were each transformed separately into *E. coli* strain BL21 (DE3). To generate recombinant OMT, the transgenic cells were inoculated into 200 mL LB medium containing 100 μg mL^{-1} ampicillin and incubated at 37 °C with shaking (200 rpm) until the OD_{600} reached 0.6, whereupon the expression of the transgene was induced by the addition of 0.5 mM isopropyl-thio-β-D-galactoside for 16 h at 16 °C. Following their harvesting by centrifugation (5000 × *g*, 5 min), the cells were resuspended in binding buffer (20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 7% (v/v) β-mercaptoethanol), lysed by sonication on ice, and the cell debris removed by centrifugation (13000 × *g* for 20 min). His-tagged CCoAOMTs were purified by passing the supernatant containing recombinant protein through a Ni-NTA Sefinose His-bind column (Bio Basic, Toronto, Canada) equilibrated with binding buffer. After rinsing in binding buffer, the purified proteins were eluted with 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 0.25 M imidazole, 7% (v/v) β-mercaptoethanol. SDS-PAGE was used to visualize the degree of homogeneity within each elution fraction. Estimates of protein concentration were obtained by using the Bradford reagent (Beyotime, Shanghai, China), using bovine serum albumin as the standard (Sun et al., 2013).

2.4. Enzymatic assay and determination of reaction kinetics

To demonstrate CCoAOMT activity and identify preferred substrates, the recombinant PaCCoAOMTs and MsCCoAOMT were presented with a range of flavonoid and phenylpropanoid compounds. Optimum reaction temperatures and pHs were obtained as described in Xu et al. (2015); Zhang et al. (2016). The 50 μL reactions used to test for the activity of purified recombinant enzyme each comprised 0.4 M Tris-HCl (pH 7.5), 4 mM DTT, 2 mM $MgCl_2$, 0.5 mM SAM, 0.2 mM substrate and 2 μg recombinant protein. After incubating for 30 min at 37 °C, the reactions were stopped by the addition of an equal volume of acetonitrile. When caffeoyl CoA was provided as the substrate, 12 μL 5 M NaOH was added to hydrolyze its thioester bond and the reaction was incubated at 40 °C for 20 min, then neutralized by the addition of 14 μL 6 N HCl. After extraction in an equal volume of ethyl acetate (EtOAc), the EtOAc fraction was evaporated and the residue dissolved in 0.1 mL methanol. The products were identified by reverse phase HPLC, using a C18 column (XDB-C18, 5 μm; Agilent, California, USA) and a flow rate of 1 mL/min. When flavonoids were used as the substrate, the gradient mobile phase comprised 35% C (methanol)/65% D (0.1% aqueous glacial acetic acid) increasing linearly to 65% C/35% D over 20 min, then 100% C (20–25 min), followed by 35% C/65% D (25–30 min). When phenylpropanoids were used as the substrate, the initial 20 min used 20% C/80% D increasing linearly to 80% C/20% D, then 100% C (20–25 min), followed by 20% C/80% D (25–30 min). The injection volume was 20 μL. Caffeoyl CoA, quercetin and eriodictyol were provided at concentrations varying from 10 μM to 0.5 M in a series of assays performed in triplicate and carried out at the optimal pH and temperature for 10 min to establish the enzyme kinetics of the PaCCoAOMTs. Their catalytic efficiencies were estimated using a standard calibration curve. V_{max} and K_m values were calculated using the Michaelis-Menten equation, as implemented in PRISM 5 software (GraphPad, La Jolla, CA, USA).

2.5. Sub-cellular localization of PaCCoAOMTs

The *PaCCoAOMT1* and *PaCCoAOMT2* sequences were amplified from the protein expression vectors using the gene-specific primer pairs specified in Supplemental Table 1. The resulting amplicons were subcloned into the pGWB5 vector (Nakagawa et al., 2007); after sequence verification, the constructs were transferred into *Agrobacterium tumefaciens* strain GV3101. The transgenic bacteria were infiltrated into *N. benthamiana* leaf epidermal cells following a procedure described elsewhere (Sparkes et al., 2006). Two to three days after infiltration, fluorescence was monitored in the epidermis of the abaxial side of a leaf disc, using a laser scanning microscope equipped with either a 495–570 nm (GFP) or a 650–760 nm (chlorophyll) band pass filter.

2.6. Heterologous expression of PaCCoAOMTs in A. thaliana

Both *PaCCoAOMT* open reading frames were PCR-amplified, using the PaCCoAOMTs-OE-F/-R primer pairs shown in Supplemental Table 1, and the amplicons cloned into the Gateway Entry vector pDONR207 (Invitrogen, Carlsbad, USA), as confirmed by sequencing. An LR recombination reaction (Invitrogen, Carlsbad, USA) was then used to transfer the amplicons into pGWB5 in order to generate the entry vector pDONR207-OMT. The resulting construct was transformed into *Agrobacterium tumefaciens* strain GV3101 as described above, and from there into *A. thaliana* ecotype Col-0 using the floral dip method (Clough and Bent, 1998). About 40 individual transgenic events were generated for each of the *PaCCoAOMT*s, from which twelve lines per construct were retained. Transgenic seed was germinated on 1.0% agar plates containing half strength Murashige and Skoog medium and 2% w/v sucrose; the plates were held under a 16 h photoperiod at a constant temperature of 22 °C. The successful transcription of each *PaCCoAOMT* transgene was monitored in three transgenic lines by reverse transcriptase polymerase chain reaction (RT-PCR), using the AtActin gene (AT3G53750) as the reference. For this purpose, RNA was extracted from two week old seedlings, and converted to cDNA using a Rever Tra Ace® qPCR RT kit (ToYoBo, Osaka, Japan). Each 25 μL RT-PCR consisted of 12.5 μL PrimeSTAR® Max DNA polymerase (Takara, Kusatsu, Japan), 10.5 μL distilled water, 0.5 μL of each 10 μM primer and 1 μL of 0.5 ng/μL cDNA. The reactions were initially denatured at

95 °C for 3 min, then subjected to 25 cycles of 95 °C/10 s, 50 °C/15 s, 72 °C/20 s. The primer pairs (*PaCCoAOMT1*-RTF/R, *PaCCoAOMT2*-RTF/R and Actin-F/R) are detailed in Supplemental Table 1.

2.7. Histochemical and biochemical analysis of PaCCoAOMT transgenic A. thaliana

10 μm thick sections were cut from a fixed position on the stems of six week old wild type and transgenic plants, and stained following a standard phloroglucinol-based protocol (Robinson and Mansfield, 2009). To quantify the lignin content of the stem, a 5 cm section from the base of the main stem of five or more six-week-old plants of each line was ground into powder under liquid nitrogen (Boyes et al., 2001). The powder was extracted by suspension in 70% ethanol held at 65 °C for 3 h, a procedure repeated three times. The residual particulate matter was then extracted overnight in acetone at room temperature. The extractive free residuals were dried at 55 °C, ball-milled into a fine powder and their lignin content quantified using an acetyl bromide-based method (Chang et al., 2008). To estimate the seedling capacity to accumulate flavonol, seeds were surface-sterilized by immersion in 75% ethyl alcohol and raised on agar plates containing 1/2 strength Murashige and Skoog medium containing 1% w/v sucrose. The plates were held in the dark at 4 °C for three days, then transferred for 14 days under a constant temperature of 22 °C and a 16 h/8 h photoperiod. Shoot material was snap-frozen in liquid nitrogen, lyophilized and weighed. Flavonols were extracted by exposing a sample of 30 ± 0.5 mg lyophilized stem to 50% methanol for 1 h in the presence of ultrasound, and the resulting extract hydrolyzed by adding an equal volume of 2 N HCl and holding at 70 °C for 40 min. The resulting products were extracted twice in 0.5 mL ethyl acetate and centrifuged (5000 × *g*, 10 min). After evaporation *in vacuo,* the residue was dissolved in 0.1 mL methanol and a 20 μL aliquot injected into an HPLC device, as described above for the analysis of the reaction products generated from a flavonoid substrate. Standard samples of flavonols were purchased from the Chengdu Must Bio-technology.

3. Results and discussion

3.1. Isolation and sequence analysis of two CCoAOMT genes from P. amoena

The SwissProt annotation of the transcriptome sequencing dataset of *P. amoena* (GeneBank: SRR8185331) suggested the presence of two genes likely encoding a SAM-dependent methyltransferase: these were designated *PaCCoAOMT1* and *PaCCoAOMT2*. One of the two sequences comprised a 765 bp open reading frame and the other a 864 bp open reading frame, and the respective length of their predicted translation products was 254 and 287 residues, representing polypeptides of molecular mass, respectively, 28.75 kDa and 32.28 kDa. A sequence alignment indicated that the two proteins shared a sequence high identity of 60.4%; their homology with the products of *At1g67990* (ABH04519.1) was, respectively, 47.3% and 41.9% (Clark et al., 2001); with *P. appendiculatum* OMT1 (ALS88170.1) 49.3% and 53.0% (Xu et al., 2015), with maize CCoAOMT (AJ242980) 68.2% and 60.6% (Brenner et al., 2010) and with alfalfa MsCCoAOMT (AAC28973.1) 69.3% and 61.3% (Inoue et al., 1998) (Fig. 2). MsCCoAOMT is a *bona*

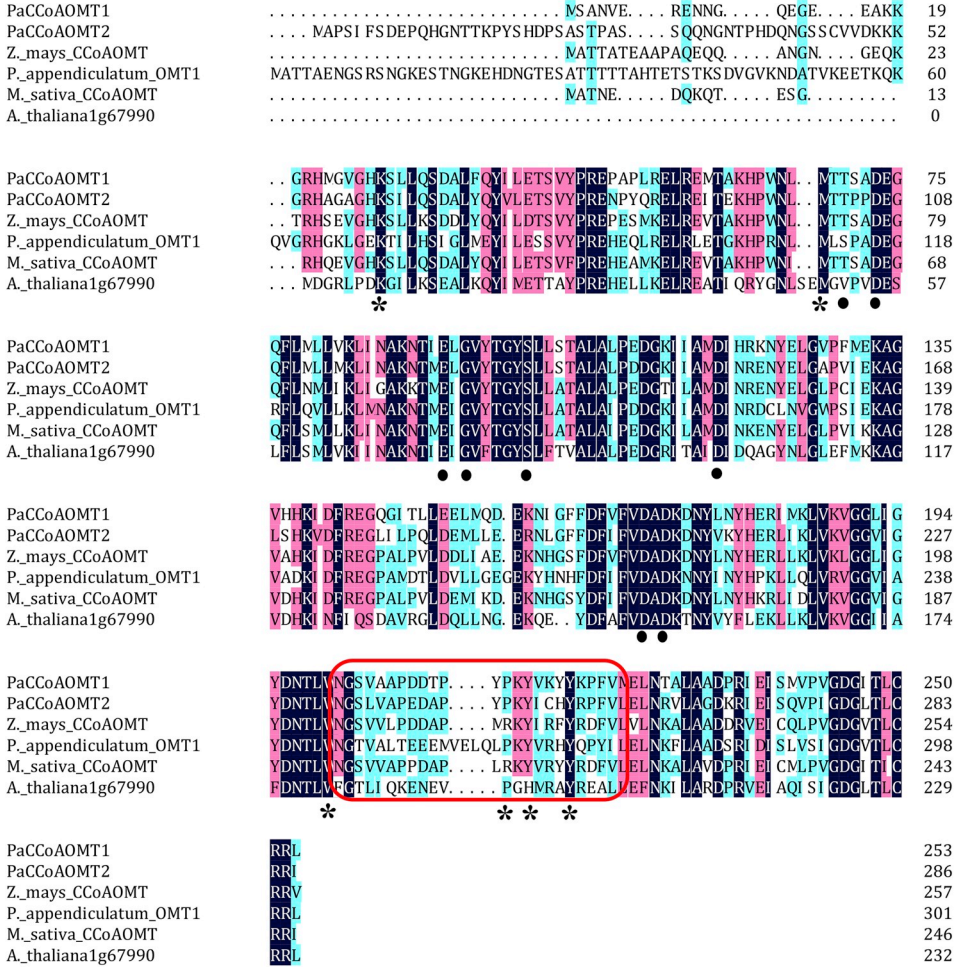

Fig. 2. Alignment of the PaCCoAOMT polypeptide sequences with those of *Z. mays* CCoAOMT (AJ242980), *P. appendiculatum* OMT1 (ALS88170.1), *A. thaliana At1g67990* (ABH04519.1) and *M. sativa* CCoAOMT (AAC28973.1). The substrate binding residues are indicated by asterisks below the sequence. Dots below the sequence indicate metal and SAM binding residues. The insertion loop is shown boxed in red. Fully conserved residues are highlighted in dark blue, strongly conserved (score = 0.75) ones in pink and weakly conserved (score = 0.5) ones in light blue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

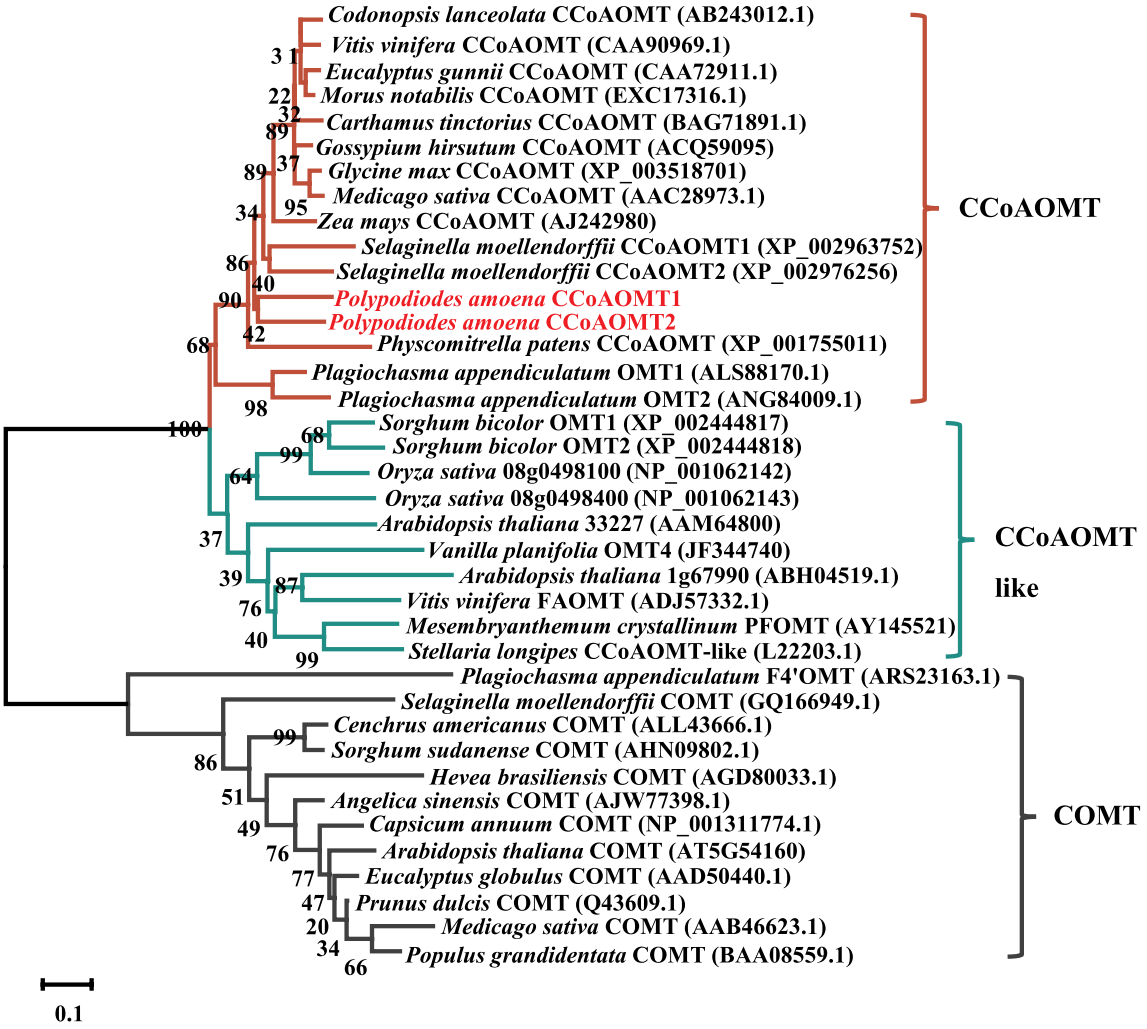

Fig. 3. The phylogeny of the PaCCoAOMTs. The tree was constructed using the neighbor-joining method, applying 1000 bootstrap replicates. Bootstrap values shown as percentages next to branch points. The length of each branch reflects the distance between nodes.

fide caffeoyl-CoA OMT sequence, and its crystalline structure has been elucidated (Ferrer et al., 2005). Most of the MsCCoAOMT sites associated with substrate, metal ion and SAM binding were conserved in both *PaCCoAOMT1* and *PaCCoAOMT2*: the exception was that residue 206R was represented by 213P in *PaCCoAOMT1* and by 246P in *PaCCoAOMT2*. Protein homology modeling further suggested that *PaCCoAOMT1* and *PaCCoAOMT2* both possess an overall tertiary structure and a substrate binding pocket largely identical to that of MsCCoAOMT. The crystal structure of alfalfa CCoAOMT revealed that the metal ion has a critical role in deprotonating the caffeoyl 3-hydroxyl group and maintaining the resultant oxyanion in close proximity to the reactive methyl group of AdoMet (Ferrer et al., 2005). In the crystal structure of MsCCoAOMT, the N-terminal region and the insertion loop domain near the C-terminus have an important relationship with the selectivity and positional specificity of the substrate. Such mechanism could be applied to *PaCCoAOMT1* and *PaCCoAOMT2* (Supplemental Fig. 1). A phylogenetic analysis (Fig. 3) revealed two evolutionary clades: one represents the CCoAOMTs, while the other clusters the COMTs. In the first group, true CCoAOMTs form a distinct clade, whereas the CCoAOMT-like form another clade. Both *PaCCoAOMT1* and *PaCCoAOMT2* were belonged to the former clade and were closely related to the OMTs encoded by the *P. patens* genome. A *P. appendiculatum* sequence was at the base of this clade and is probably the progenitor of the higher plant CCoAOMTs.

3.2. Purification of the PaCCoAOMTs and their catalytic activity

Recombinant MsCCoAOMT, PaCCoAOMT1 and PaCCoAOMT2 proteins were generated by expressing the relevant coding sequences in *E. coli* BL21 (Supplemental Fig. 2). Their *in vitro* enzyme activity was then characterized by offering a range of possible substrates, using SAM as the methyl group donor. The products of these reactions were identified by HPLC separation, using the corresponding *meta-O*-methylethers as standards (Table 1). When provided with either caffeoyl CoA, caffeoyl aldehyde or 5-OH coniferaldehyde, PaCCoAOMT1 exhibited a conversion rate comparable to that of MsCCoAOMT, while that of PaCCoAOMT2 was slightly lower in the presence of either caffeoyl CoA or caffeoyl aldehyde, but higher in the presence of caffeoyl alcohol or 5-OH coniferaldehyde. Overall, in spite of the slight differences in the conversion rate against different phenylpropanoids substrates, however, the enzyme activity displayed by the two fern OMTs was comparable to that of *bona fide* higher plant CCoAOMTs. All three recombinant enzymes were also able to catalyze flavonoids possessing aromatic vicinal dihydroxyl groups, thereby forming one or two

Table 1
Relative activity of purified PaCCoAOMT1, PaCCoAOMT2 and MsCCoAOMT recombinant proteins with selected substrates.

Substrates	MsCCoAOMT	PaCCoAOMT1	PaCCoAOMT2
Caffeoyl CoA	36.28 ± 2.07	34.84 ± 1.38[a]	27.19 ± 2.29
Caffeic acid	ND	ND	ND[b]
Caffeoyl aldehyde	14.79 ± 0.32	13.32 ± 0.47	6.64 ± 0.57
Caffeoyl alcohol	ND	ND	8.04 ± 0.34
5-OH Ferulic acid	ND	ND	ND
5-OH Coniferaldehyde	3.92 ± 0.61	3.79 ± 0.33	11.07 ± 0.13
5-OH Coniferyl alcohol	ND	ND	ND
Quercetin	59.10 ± 8.36	56.77 ± 4.32	43.16 ± 5.61
Luteolin	61.31 ± 3.06	55.71 ± 1.52	37.42 ± 2.58
Eriodictyol	13.85 ± 5.03	18.00 ± 6.56	48.48 ± 5.06
Baicalein	27.80 ± 0.62	26.43 ± 0.59	11.95 ± 2.06
Scutellarein	28.62 ± 4.21	23.13 ± 3.91	14.76 ± 2.45
Kaempferol	ND	ND	ND
Naringenin	ND	ND	ND

[a] Activities presented are nmol (mg min) $^{-1}$ ± STDEV of three replicates.
[b] No product detected.

methylated products, but were unable to catalyze either kaempferol or naringenin (which lack a 3′—OH group). HPLC profiles of the various reaction products are illustrated in Fig. 4. On the basis of retention time and UV spectrum, quercetin was catalyzed to isorhamnetin (Fig. 4 a-d) and caffeoyl CoA to feruloyl CoA (Fig. 4 j-m). Providing eriodictyol generated two products (Fig. 4 e-i), the major one being homoeriodictyol (eriodictyol-3′—*O*-methylether), while the minor one shared the retention time and UV spectrum of hesperetin (eriodictyol-4′—*O*-methylether); the formation of two products rather than one likely reflects the existence of a chiral C on the eriodictyol C-ring.

MsCCoAOMT, PaCCoAOMT1 and PaCCoAOMT2 showed a somewhat different substrate specificity: MsCCoAOMT has the highest conversion rate of luteolin, while quercetin was the favorite substrate for PaCCoAOMT1 and eriodictyol for PaCCoAOMT2. Although PaCCoAOMT1 and PaCCoAOMT2 share higher sequence similarity to each other than to MsCCoAOMT, PaCCoAOMT1 is biochemically more similar to MsCCoAOMT than to PaCCoAOMT2. Both PaCCoAOMTs and MsCCoAOMT can methoxylation of catalyzing flavonoids with aromatic vicinal dihydroxyl groups as substrates *in vitro*, and showed comparable

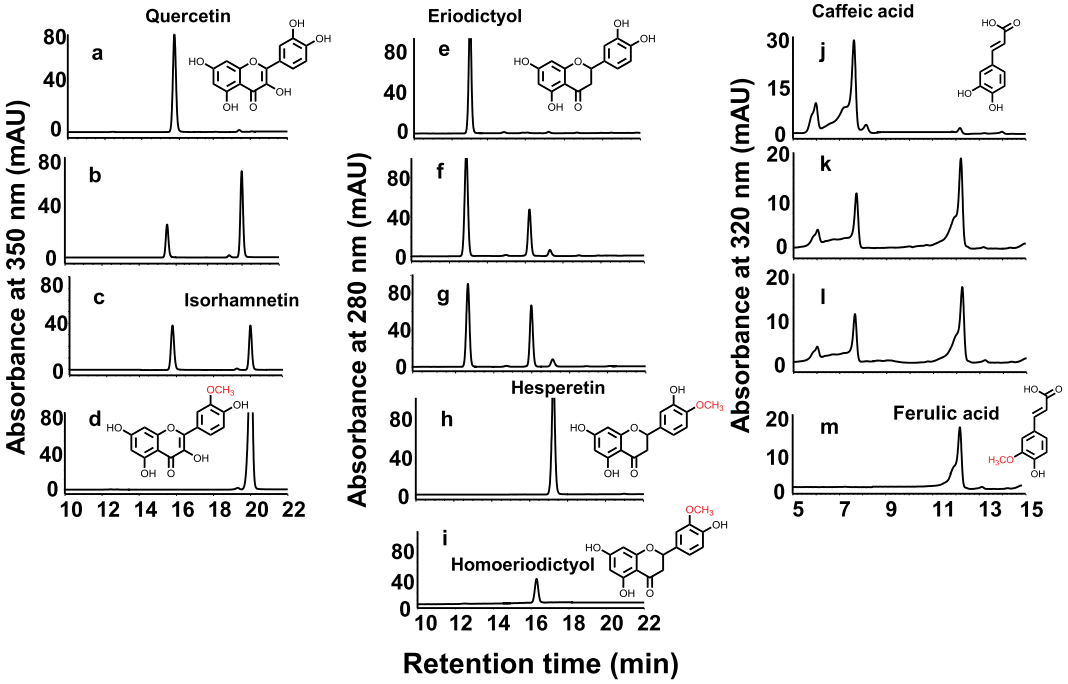

Fig. 4. In vitro assays of recombinant PaCCoAOMT1 and PaCCoAOMT2. HPLC profiles of the products generated by the empty vector control (a) and PaCCoAOMT1 (b), PaCCoAOMT2 (c) using quercetin as substrates. Isorhamnetin standard (d). HPLC profiles of the products generated by the empty vector control (e) and PaCCoAOMT1 (f), PaCCoAOMT2 (g) using eriodictyol as substrates. Hesperetin (h) and homoeriodictyol (i) standard. HPLC profiles of the products generated by the empty vector control (j) and PaCCoAOMT1 (k), PaCCoAOMT2 (l) using caffeoyl CoA as substrates. Ferulic acid (m) standard. Note: when caffeoyl CoA was provided as the substrate, NaOH was added to hydrolyze its thioester bond, then neutralized by the addition of HCl. So caffeic acid and ferulic acid were detected instead of the CoA-esters.

Table 2
The kinetic parameters of recombinant PaCCoAOMT1 and PaCCoAOMT2 using caffeoyl CoA, quercetin and eriodictyol as substrates.

Substrate	Enzyme	K_m (μM)	V_{max} (nmol $mg^{-1}min^{-1}$)	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$)
Caffeoyl CoA	PaCCoAOMT1	47.74 ± 11.88	85.08 ± 4.88	0.04 ± 0.002	878.30
	PaCCoAOMT2	51.14 ± 13.60	68.47 ± 4.27	0.04 ± 0.002	738.84
Quercetin	PaCCoAOMT1	48.95 ± 10.11	274.50 ± 14.81	0.14 ± 0.007	2763.69
	PaCCoAOMT2	91.59 ± 18.06	88.02 ± 5.63	0.05 ± 0.003	530.32
Eriodictyol	PaCCoAOMT1	96.36 ± 22.17	103.8 ± 7.89	0.05 ± 0.003	530.88
	PaCCoAOMT2	54.65 ± 7.48	241.5 ± 8.97	0.13 ± 0.004	2438.57

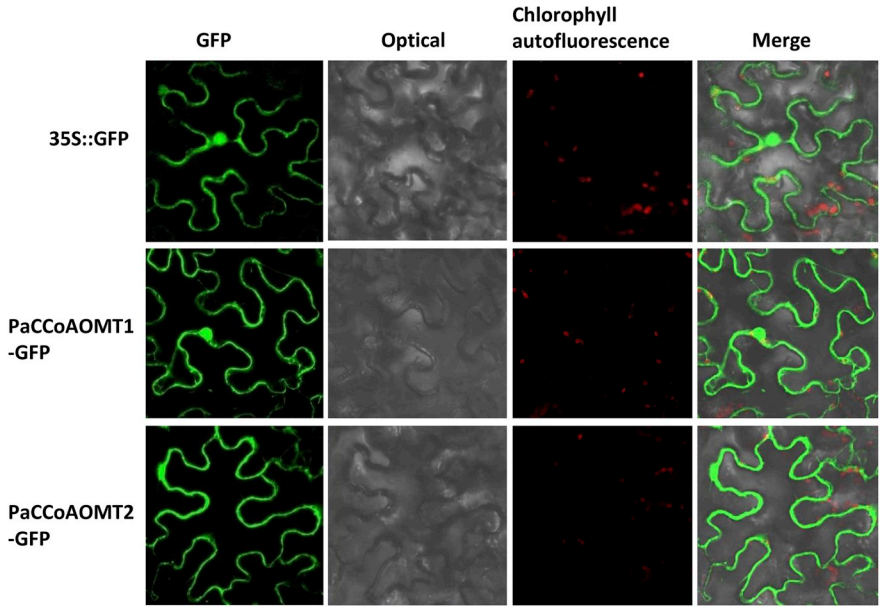

Fig. 5. Sub-cellular localization of the products of the 35S::PaCCoAOMT1-GFP and 35S::PaCCoAOMT2-GFP transgenes in transiently transformed *N. benthamiana* leaf discs. The GFP signal appears green, while chlorophyll fluoresces red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

conversion rate (Table 1). It has been generally accepted that CCoAOMT-like enzymes are able to methylate flavonoids, anthocyanins, coumarins and aromatic esters, while true CCoAOMTs specifically methylate caffeoyl-CoA and 5-hydroxyferuloyl-CoA (Maury et al., 1999). The indication from the present data was that this criterion applied to distinguish between the CCoAOMTs and CCoAOMT-like enzymes is insufficiently rigorous.

The enzyme kinetics measured in reactions performed under optimal pH and temperature conditions, with quercetin, eriodictyol and caffeoyl CoA supplied as substrate, are given in Table 2. The K_m value of PaCCoAOMT1 with quercetin, eriodictyol and caffeoyl CoA were 48.95, 96.36 and 47.74 μM, with K_{cat}/K_m values of 2763.69, 530.88 and 878.3 $M^{-1} s^{-1}$, respectively. For PaCCoAOMT2, the K_m and K_{cat}/K_m values for quercetin, eriodictyol and caffeoyl CoA were 91.59, 54.65, and 51.14 μM and 530.32, 2438.57, and 738.84 $M^{-1} s^{-1}$, respectively. These results show that PaCCoAOMT1 has much higher specificity for quercetin than for eriodictyol, while PaCCoAOMT2's substrate specificity is the opposite. With the caffeoyl CoA, both PaCCoAOMT1 and PaCCoAOMT2 displayed a similar K_m value and catalytic efficiency.

3.3. Sub-cellular localization of PaCCoAOMT1 and PaCCoAOMT2

The sub-cellular localization of the two PaCCoAOMTs was examined by transiently expressing GFP fusion constructs in *N. benthamiana* leaf epidermal cells. This experiment showed that the transgene product in both cases was directed to the cytoplasm (Fig. 5), consistent with the behavior of PaF6OMT (Zhang et al., 2016).

3.4. Expressing the PaCCoAOMTs in A. thaliana increases cell wall lignin content

An RT-PCR assay detected transcript from both *35S::PaCCoAOMT* transgenes in T_3 plants, while this was not the case for control wild type plants (Fig. 6A). Application of the Wiesner (phloroglucinol-HCl, wherein a violet-red color is indicative of lignins) histochemical stains (Robinson and Mansfield, 2009) showed that the vasculature of each of the transgenics showed stronger phloroglycinol staining compared to the wild type (Fig. 6C–E). Measurement of the total lignin content of stems of six week old T_3 plants using the acetyl bromide method (Moreira-Vilar et al., 2014). The result confirmed that the presence of the two transgenes increased the deposition of lignin by, respectively, 26.8% and 32.2%. In line *PaCCoAOMT2*-OE-2, the increase reached as high as 35.6% (Fig. 6B). As a result, the total lignin content of the PaCCoAOMT1 and PaCCoAOMT2 transgenic plants was significantly raised comparing with wild type plants. The implication is that *PaCCoAOMT1* and *PaCCoAOMT2* act primarily as a caffeoyl co-enzyme A *O*-methyltransferase, involved in synthesis of lignin monomers. A previous study has reported that bryophytes do not synthesize lignin, although they do have lignin-like phenolics in their cell walls (Ligrone et al., 2007). The polypeptide sequence and predicted structure of the two PaCCoAOMTs resembled those of typical true CCoAOMTs, so the suggestion is that gain of *bona fide* CCoAOMTs activity from precursors acting on flavonoid happened prior to the fern/seed plant split and participating in lignin biosynthesis pathways.

3.5. The flavonol content of PaCCoAOMT transgenic A. thaliana plants

Both PaCCoAOMT1 and PaCCoAOMT2 exhibited a high conversion efficiency with respect to the *in vitro* conversion of quercetin to isorhamnetin. A comparison drawn between plants derived from three independent transgenic events for each of the PaCCoAOMT genes showed that no difference could be detected between the transgenics and wild type with respect to their isorhamnetin accumulation (Supplemental Fig. 3). The implication was that expressing the PaCCoAOMTs could enhance the accumulation of lignin, but did not increase the content of flavonols. The conclusion was that both PaCCoAOMTs participate in lignin formation in plants.

4. Conclusions

The present investigation has focused on two CCoAOMTs encoded by the genome of the fern *P. amoena*. It confirmed that these CCoAOMTs possess a comparable level of enzyme activity as the model plant MsCCoAOMT. Both the PaCCoAOMTs and MsCCoAOMT were able to methylate flavonoids *in vitro*, with a preference for the flavonoid quercetin, although they are involved in lignin synthesis *in planta*. The research has shed some light on the origin and evolution of lignin synthesis in a species of fern, a group of plants considered to be the

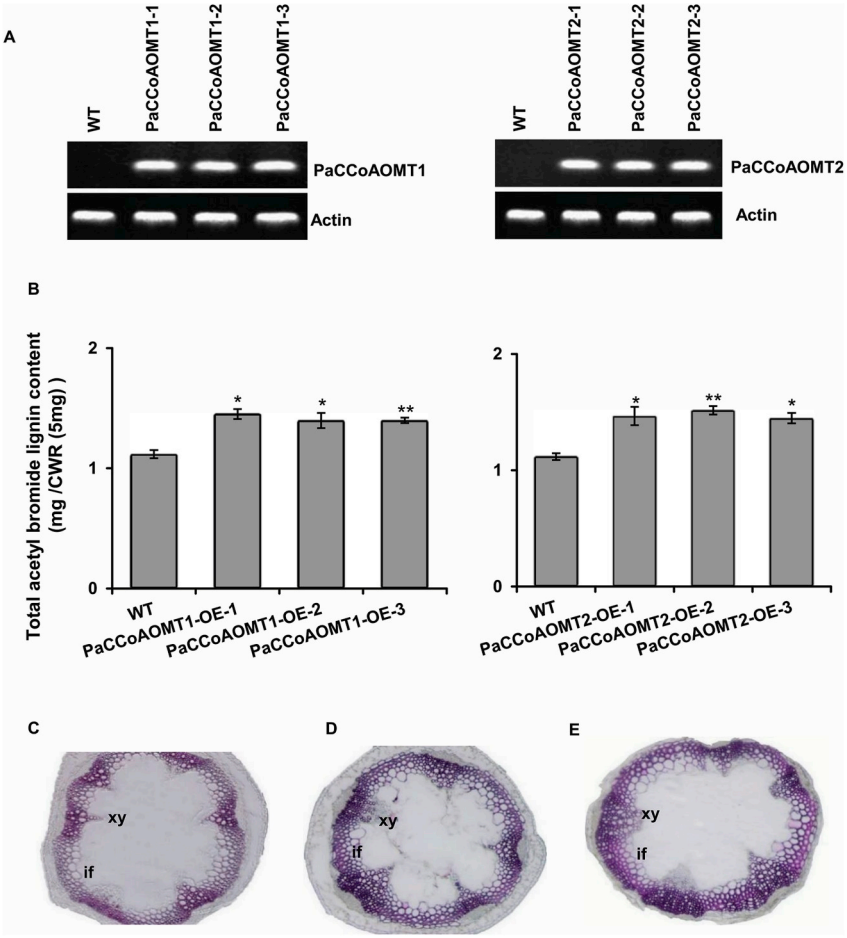

Fig. 6. The heterologous expression of PaCCoAOMT genes in *A. thaliana* and its effect on lignin content. (A) RT-PCR profiling shows that both PaCCoAOMT1 and PaCCoAOMT2 were transcribed in the selected transgenic lines; *AtActin* was used as the reference sequence. (B) UV analysis of total acetyl lignin content in per five milligram of extract-free cell wall residues (CWR) in transgenic and wild type plants. Data shown in the form mean ± SD of three replicated. *, **: means differ significantly from the wild type level at, respectively, P < 0.05 and < 0.01. Phloroglucinol-HCl stained stem sections sampled from (C) wild type *A. thaliana* plant, (D) transgenic plant expressing 35S::PaCCoAOMT1 and (E) transgenic plant expressing 35S::PaCCoAOMT2. Lignin is stained violet-red. if: interfascicular fiber, xy: xylem. Bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

progenitors of modern vascular plants. It has also identified candidate genes for the enzymatic synthesis of methylated flavonoids.

Author contributions

A.-X.C. and H.-X.L. conceived the research plan and designed the experiments; X-S. Z, R.N, P.-Y.W, T.-T, Z and C.-J.S performed the experiments and analyzed data; X-S.Z. and A-X.C. wrote the paper. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest in the present investigation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.01.021.

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