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Enzymatic production of oroxylin A and hispidulin using a liverwort flavone 6-*O*-methyltransferase

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Oroxylin A and hispidulin, compounds which are abundant in both *Scutellaria* and liverwort species, are important lead compounds for the treatment of ischemic cerebrovascular disease. Their enzymatic synthesis requires an *O*-methyltransferase able to interact with the related flavonoid's 6-OH group, but such an enzyme has yet to be identified in plants. Here, the gene encoding an *O*-methyltransferase (designated PaF6OMT) was isolated from the liverwort species *Plagiochasma appendiculatum*. A test of alternative substrates revealed that its strongest preferences were baicalein and scutellarein, which were converted into, respectively, oroxylin A and hispidulin. Allowed a sufficient reaction time, the conversion rate of these two substrates was, respectively, 90% and 100%. PaF6OMT offers an enzymatic route to the synthesis of oroxylin A and hispidulin.

Keywords: baicalein; liverworts; *O*-methyltransferase; *Plagiochasma appendiculatum*; scutellarein

Liverworts, which evolved during the transition of marine to terrestrial plants, harbor an abundance of secondary metabolites, including terpenoids, lignin, and the phenolic bibenzyls and flavonoids [1–4]. The flavonoids, represented by, among others, luteolin, apigenin, oroxylin A, and hispidulin, are a particularly common class of compound in liverwort tissues [5,6]. Their diversity in structure is mirrored by their range of biological activity [7]. The flavonoids arise from the general phenylpropanoid pathway. Their precursor chalcone is formed from the condensation of *p*-coumaroyl-CoA with three units of malonyl-CoA [8]. The enzyme chalcone isomerase converts the chalcone core into the basic flavanone structure [9], which in turn is modified by various reductases, isomerases, and hydroxylases to form flavanone, flavone, dihydroflavonol, isoflavones, and anthocyanidins [10]. The functional diversity of the flavonoids results in part from further modification to the hydroxyl groups via acetylation, glycosylation, or methylation [11]. The latter, which is particularly prominent, induces the physiological properties diversity and alters the chemical reactivity of flavonoids more hydrophobic [12]. The *O*-methyltransferases (OMTs), which are commonplace in plants, animals, and microorganisms, are a very diverse group of enzymes: some hundreds of plant members of the family are known [13]. Class I OMTs are lower molecular weight (26–29 kDa) and Mg^{2+}-dependent, while Class II OMTs are typically 38–40 kDa and do not require a metal cation for catalytic activity [14]. Although Class I OMTs are characterized by caffeoyl-coenzyme A OMTs, there are various enzymes that possess distinct regiospecific to polyhydroxy flavonoids. Some Class I OMTs are able to methylate the OH group present at position 7 of the A-ring, position 3 of the C-ring, positions 3′, 4′, or 5′

Abbreviations
CatOMT, catechol OMT; GFP, green fluorescent protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; LB, Luria–Bertani; OMTs, *O*-methyltransferases; ORF, open reading frame; SAM, *S*-adenosyl-L-methionine.

of the B-ring or other dimethylation [8,15–18]. However, the methylation of the scutellarein 6-OH group is a rare example that involves the species *Ocimum basilicum* (sweet basil) [19]. Moreover, the methylation of the scutellarein 6-OH group is less favorable than that of the 7-OH group. A cation-dependent OMT from *Mesembryanthemum crystallinum* can accept a broad spectrum of substrates having a vicinal dihydroxyl structure, and is able to methylate the 6-OH group of scutellarein [20]. However, the methylation is not specific to 6-OH and the enzyme catalytic efficiency is low. Neither the *O. basilicum* nor the *M. crystallinum* enzymes can efficiently and specifically catalyze 6-OH groups in flavonoids.

Here, a Mg^{2+}-dependent flavone 6-OMT has been identified in the liverwort species *Plagiochasma appendiculatum*. When the enzyme was subsequently purified, it had a strong substrate preference for baicalein and scutellarein, and its catalytic product was solely the respective 6-mono-*O*-methylether (respectively, oroxylin A and hispidulin) (Fig. 1). Thus, this enzyme represents the first documented plant OMT to show a substrate preference for the flavone 6-OH group. Manipulation of the reaction conditions allowed baicalein and scutellarein to be highly efficiently converted by the enzyme, thereby providing a viable enzymatic means of synthesizing the valuable molecules oroxylin A and hispidulin.

Materials and methods

Plant materials and reagents

Two-month-old *P. appendiculatum* thallus, grown in a greenhouse delivering a daytime temperature of 25°C and a 12-h photoperiod, was cleaned and snap-frozen in liquid nitrogen. The frozen tissue was ground to powder in the presence of liquid nitrogen, and the powder extracted via the CTAB method [21]. Caffeoyl CoA, caffeoyl alcohol, caffeoyl aldehyde, and 5-OH coniferyl alcohol were all synthesized in the laboratory using established methods [22,23]. Baicalein, scutellarein, oroxylin A, hispidulin, quercetin, luteolin, eriodictyol, and esculetin were purchased from Chengdu Must Bio-technology (Chengdu, China). Catechol was purchased from Shanghai aladdin Biochemical Technology Co. Ltd (Shanghai, China). Solvents and other reagents required were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

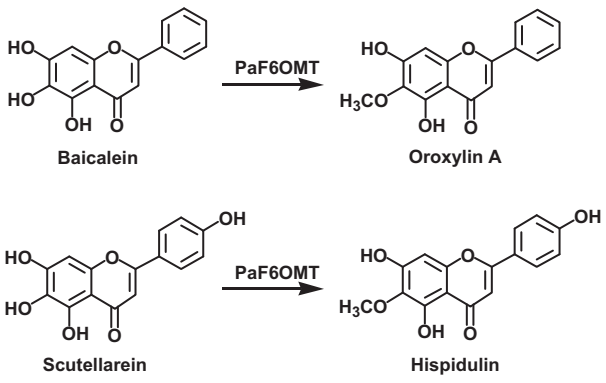


Fig. 1. Reactions catalyzed by PaF6OMT.

Sequence analysis

The full-length sequence of the target gene (designated *PaF6OMT*) was derived from the *P. appendiculatum* transcriptome sequencing database. Using the PaF6OMT open reading frame (ORF) sequence as a Blast query, a number of homologs were identified in the GenBank database (http://www.ncbi.nlm.nih.gov/). A peptide alignment between the predicted products of *PaF6OMT* and *Medicago sativa* CCoAOMT (AAC28973.1), *P. appendiculatum* OMT1 (KP729179), *Rattus norvegicus* CatOMT (1JR4-A), and *Nocardia concave* CatOMT (WP-051178395.1) was conducted using DNAMAN v5.2.2 software (Lynnon, Biosoft, Quebec, Canada). The subsequent phylogenetic analysis was based on the maximum likelihood method, as implemented in the software package MEGA v4.0 [24].

Heterologous expression and protein purification

The extracted RNA was converted to cDNA using a RevertAid™ First Strand cDNA Synthesis kit (Takara, Otsu, Japan) with an oligo $(dT)_{18}$ primer, in accordance with the manufacturer's protocol. The PaF6OMT sequence was amplified from *P. appendiculatum* cDNA using the primer pair PaF6OMT-F/R (sequences given in Table S1). The resulting amplicon was double-digested by *Bam*H I and *Hin*d III (Takara) and then inserted into the corresponding cloning site of the pET32a vector (Novagen, Darmstadt, Germany). After validation by sequencing, the recombinant plasmid was introduced into *Escherichia coli* BL21 (DE3), and the recombinant cells were cultured at 37 °C, with constant rotation (200 rpm), in 200 mL Luria–Bertani (LB) liquid medium containing 100 $\mu g \cdot mL^{-1}$ ampicillin. Once the OD_{600} had reached 0.4–0.6, 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added, and culture was held at 18 °C for 18 h with shaking at 110 rpm. The cells were then harvested by centrifugation (5000 ***g***, 5 min) and resuspended in binding buffer (20 mM Tris/HCl (pH 8.0), 500 mM NaCl, 5 mM imidazole). The cells were repelleted (5000 g, 5 min), rinsed twice in the same buffer, lyzed by ultrasound and recentrifuged (12 000 ***g***, 10 min, 4 °C). The final supernatant was passed through a Ni-NTA Sefinose His-bind column (Bio Basic,

Ontario, Canada), following the supplier's protocol. Recombinant protein was eluted from the column by flushing with 2-mL binding buffer, followed by 2-mL 20 mM Tris/HCl (pH 8.0), 500 mM NaCl, and 500 mM imidazole. An empty vector transferred into *E. coli* served as the negative control. The soluble fraction and the purified protein produced by both cells harboring either the recombinant plasmid or the empty vector were denatured by boiling, then separated by SDS/PAGE (12% acrylamide) [25]. Proteins were visualized in the gel by Coomassie blue R250 staining. Protein concentrations were determined using Bradford's reagent (Beyotime, Shanghai, China), using known concentrations of BSA for calibration.

Enzyme assays

To demonstrate PaF6OMT activity and identify its preferred substrates, the enzyme was presented with various flavonoids, phenylpropanoids, and coumarins. The 50-μL reactions used to test the activity of the purified enzyme each comprised 200 mM Tris/HCl (pH 7.5), 4 mM DTT, 2 mM $MgCl_2$, 0.5 mM SAM, and 0.2 mM substrate and 2 μg recombinant proteins. After incubating for 30 min at 37 °C, the reactions were stopped by the addition of an equal volume of acetonitrile. HPLC was performed using a reverse phase 4.6 × 150 mm XDS-C18 column (Agilent, Palo Alto, CA, USA). The methanol (in water plus 0.1% (v/v) glacial acetic acid) gradient was 45–75% (v/v) for baicalein and scutellarein, 35–65% (v/v) for the other flavonoids, and 20–50% (v/v) for the phenylpropanoids and coumarins. The flow rate was 1.0 $mL{\cdot}min^{-1}$. The reaction products from baicalein and scutellarein were identified on the basis of their NMR spectrum, as captured on an AV 600 spectrometer (Bruker, Karlsruhe, Germany) in DMSO-d_6 with TMS as internal standard.

To ascertain the effect of varying the reaction temperature on enzyme activity, the reactions were run at 25, 30, 35, 37, 40, 45, and 50 °C, all at pH 7.5. The test for pH sensitivity involved running the reactions at 37 °C using as buffer 400 mM of either MES (pH 5.0–6.0), potassium phosphate (pH 7.0), or Tris/HCl (pH 7.5–9.0). In study of kinetic parameters, the substrates baicalein and scutellarein concentrations were constructed from 10 to 200 μM. The assays which were done in triplicate were carried out at the optimal pH and temperature for 5 min by PCR. The catalytic efficiency of PaF6OMT protein was estimated by a standard calibration curve of reaction products. Subsequently, the V_{max} and K_m values were calculated by using the Michaelis–Menten equation implemented of GRAPHPAD PRISM 5 software (La Jolla, CA, USA).

The dependence of PaF6OMT on Mg^{2+} was tested by including 1 mM EDTA as a metal chelator. The influence of protein amount on the conversion rate was tested by fixing the incubation time to 1 h and the reaction temperature to 37 °C, then varying the amount of PaF6OMT included in a 50-μL reaction from 1 μg to 14 μg, with SAM and Mg^{2+} raised in proportion. The effect of incubation time was explored by allowing it to vary from 5 min to 4 h in the presence of 6 μg PaF6OMT. The catalytic efficiency of PaF6OMT was calculated according to the result revealed by HPLC.

Subcellular localization of PaF6OMT activity

The subcellular site of PaF6OMT activity was deduced from the behavior of a GFP fusion plasmid in transiently transformed *Nicotiana benthamiana* leaves. The *PaF6OMT* sequence was amplified from the sequenced clone, using as the primer pair PaF6OMT-GFP-F/R (sequences given in Table S1), and the amplicon was then introduced into the pCAMBIA1301 binary vector (CAMBIA, Canberra, ACT, Australia). The engineered vector was validated by sequencing. Validated recombinant plasmids were transferred into *Agrobacterium tumefaciens* strain EHA105 using the freeze/thaw method [26]. A single *A. tumefaciens* colony was seeded into 2-mL yeast extract peptone medium supplemented with 100 $\mu g{\cdot}mL^{-1}$ kanamycin and 50 $\mu g{\cdot}mL^{-1}$ rifamycin, and the culture was held overnight at 30 °C with shaking. A 5-μL aliquot of the medium was seeded into 10-mL fresh medium, and once the OD_{600} had reached about 0.5, the cells were harvested by centrifugation (2000 ***g***, 20 min), rinsed, and resuspended in 10-mL 50 mM MES/KOH (pH 5.6), 2 mM Na_3PO_4, 0.5% (w/v) glucose, 100 μM acetosyringone until OD_{600} reached to 1.0. The *A. turnefaciens* harboring the gene encoding silencing inhibitor protein p19 or the empty vector control was cultured with the same way above [27]. The 1 : 1 mixture of the target gene (or the empty vector) and p19 gene was infiltrated into the epidermal leaf cells of *N. benthamiana* after being activated by incubation at 25 °C for 2–3 h with shaking. About 2 or 3 days post infiltration, GFP activity and chlorophyll were visualized by confocal microscopy, following excitation with a 488 nm Ar laser. The band pass filters used were 495–570 nm (GFP) and 650–760 nm (chlorophyll).

Results and Discussion

cDNA isolation and PaF6OMT sequence analysis

By searching a transcriptome sequencing dataset for *P. appendiculatum* thallus, a putative SAM-dependent methyltransferase isoform was identified. The full-length cDNA, designated *PaF6OMT*, was amplified from the cDNA of the thallus of *P. appendiculatum*. The open reading frame, of which length is 729 bp, was predicted to encode 242 amino acids with a calculated molecular mass of 27.4 kDa and a theoretical isoelectric point of 6.26. The outcome of a BlastX search suggested that PaF6OMT would be a

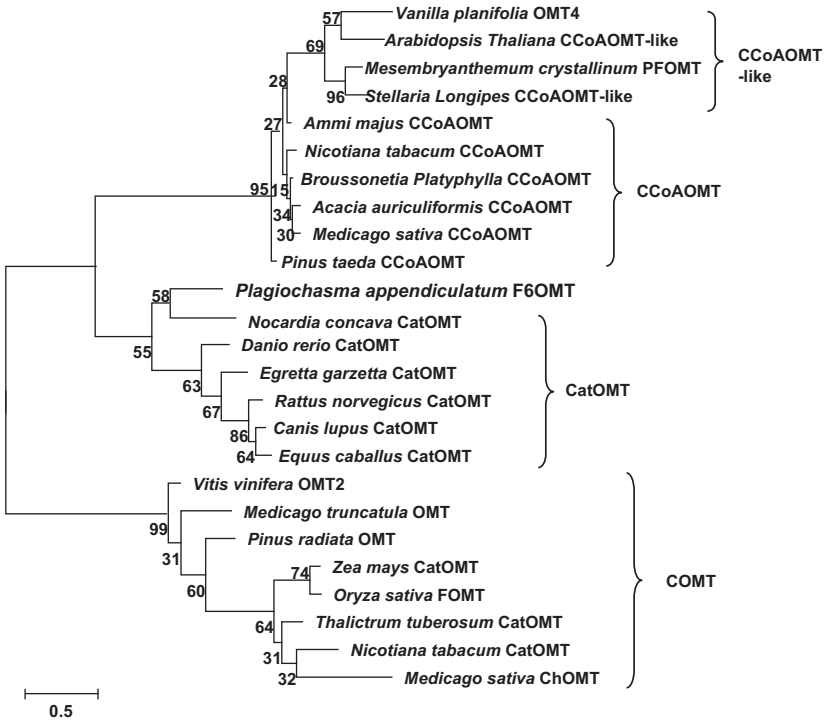


Fig. 2. Phylogenetic tree of PaF6OMT and other OMTs. The tree was constructed by the MEGA v4.0 program using maximum likelihood method. Except PaF6OMT from *Plagiochasma appendiculatum*, accession numbers of the other OMTs sequences used are listed in Table S2.

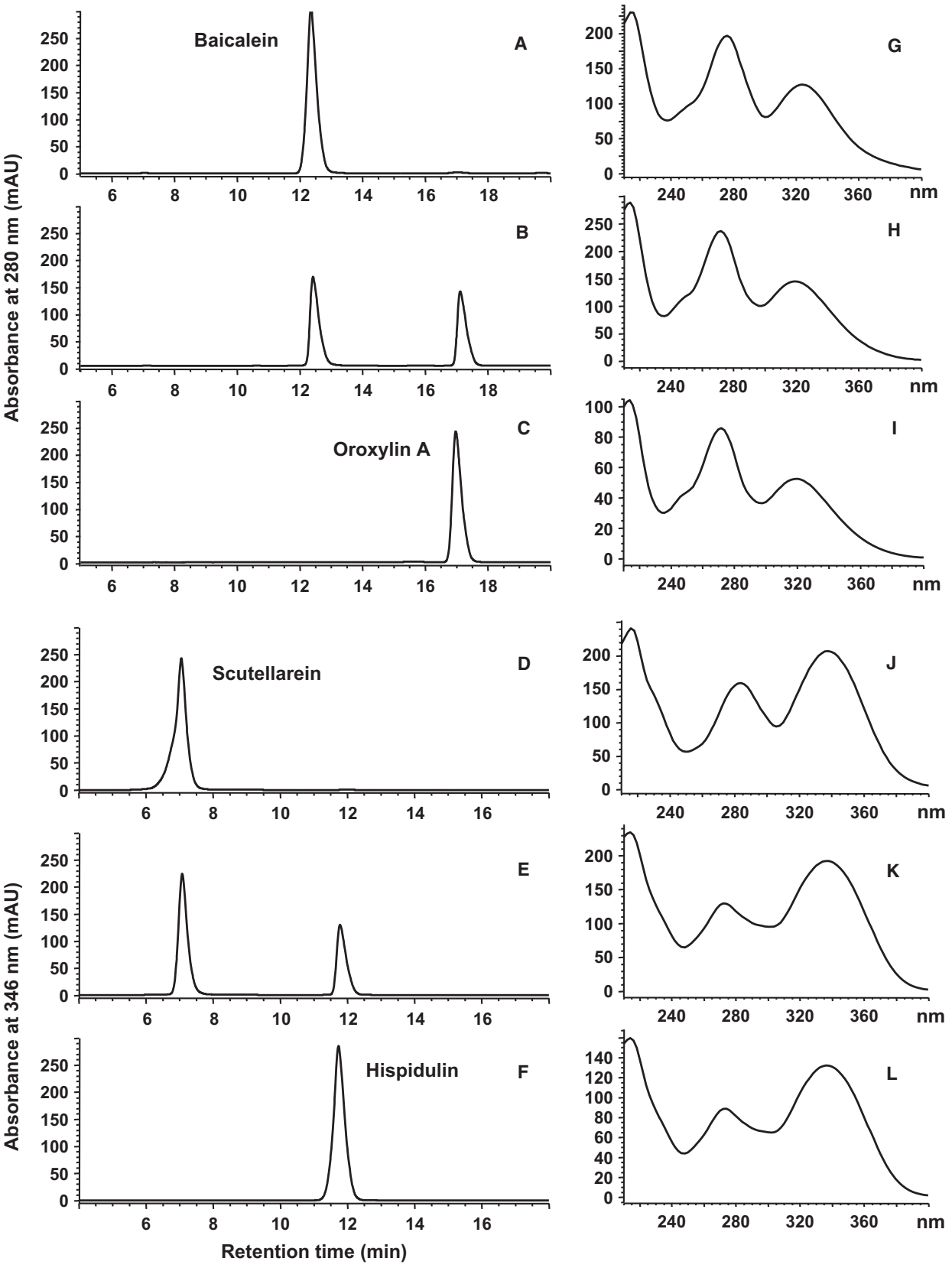
SAM-dependent methyltransferase. However, its sequence shared only 19.2% identity with the alfalfa enzyme CCoAOMT (Fig. S1), used as the classical model for this class of enzyme. Sequence alignment also revealed differences with respect to both the metal- and SAM-binding sites: only two of the six substrate-binding sites present in *M. sativa* CCoAOMT [28] were retained in PaF6OMT. The level of similarity between PaF6OMT and PaOMT1 (KP729179), the first *O*-methyltransferase characterized in a liverwort [29], was also low (16.2%). However, the sequence matched more closely with both *R. norvegicus* CatOMT (1JR4-A) (33.5%) and *N. concave* CatOMT (WP-051178395.1) (45.2%). The phylogenetic analysis showed that PaF6OMT was far from the branch of CCoAOMT and gathered to the cluster of animal catechol methyltransferase (Fig. 2). Not surprisingly, most of the residues shown to interact with SAM and Mg^{2+} cofactors in *R. norvegicus* CatOMT crystals [30] are well conserved in plant CCoAOMTs that require the same cofactors. *R. norvegicus* and *N. concava* CatOMTs belong to animal catechol methyltransferases which was the closest non-CCoAOMT to this CCoAOMT family [28]. Class I OMTs from plants and animals have similar crystallographic structure and relatively high sequence conservation, and the liverwort *P. appendiculatum* belonged to the most

Table 1. Relative activity of purified PaF6OMT recombinant proteins with selected substrates.

Substrate	Product	PaF6OMT
Scutellarein	Hispidulin	50.67 ± 1.979 (100)[a]
Baicalein	Oroxylin A	35.72 ± 1.08 (70)
Quercetin	Isorhamnetin	13.39 ± 1.22 (26)
Luteolin	Chrysoeriol	12.85 ± 0.77 (25)
Eriodictyol	Hesperetin	10.70 ± 0.64 (21)
Esculetin	Scopoletin	5.18 ± 0.11 (10)
Caffeoyl CoA	Feruloyl CoA	3.38 ± 0.41 (7)
Caffeoyl alcohol	Coniferyl alcohol	4.84 ± 0.16 (10)
Caffeoyl aldehyde	ND[b]	0
Caffeic acid	ND	0
5-OH Ferulic acid	ND	0
5-OH Coniferyl alcohol	ND	0
Catechol	ND	0

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

Fig. 3. HPLC profiles of the products generated by (A, D) the empty vector control and (B, E) PaF6OMT using baicalein and scutellarein as substrates, respectively. (C) oroxylin A standard; (F) hispidulin standard; UV spectra of (G) baicalein, (H) oroxylin A, (J) scutellarein, and (K) hispidulin standard; UV spectra of (I) enzymatic product of baicalein and (L) enzymatic product of scutellarein.


Baicalein
A
B
Oroxylin A
C
Scutellarein
D
E
Hispidulin
F
G
H
I
J
K
L
Absorbance at 280 nm (mAU)
Absorbance at 346 nm (mAU)
Retention time (min)
nm

original higher plants. So it is consistent with that high similarity between PaF6OMT and animal CatOMTs. It is also important to note that PaF6OMT has a longer N-terminal region than MsCCoAOMT and RnCatOMT. And its polar insertion loop domain is less than MsCCoAOMT. These may result the unique function of the enzyme which were different from the traditional CCoAOMT or animal CatOMT.

Purification and functional identification of the recombinant PaF6OMT

To investigate the extraordinary activity of the recombinant PaF6OMT speculated from the sequence analysis, the gene was heterologously expressed in *E. coli* and the recombinant protein was subsequently purified. Based on SDS/PAGE separation, its N-terminal hexahistidine-tagged and metal affinity-purified form proved to have an apparent molecular mass of ~ 47.8 kDa, consistent with its predicted mass (Fig. S2).

PaF6OMT was provided *in vitro* with a range of potential substrates: the flavonoids baicalein, scutellarein, quercetin, luteolin, and eriodictyol; the phenylpropanoids caffeoyl CoA, caffeoyl alcohol, caffeoyl aldehyde, caffeic acid, 5-OH ferulic acid, and 5-OH coniferyl alcohol; the coumarin esculetin; catechol. The reaction products were identified firstly by HPLC separation using their corresponding putative methylethers as standards. The enzyme showed a stronger preference for baicalein and scutellarein than for any of the other substrates (Table 1, Fig. 3). The provision of baicalein and scutellarein as a substrate generated a product which shared the same HPLC retention time, UV spectrum as oroxylin A and hispidulin, respectively. When the reaction products of baicalein and scutellarein were examined using ^{1}H NMR, it was concluded that a hydroxyl signal (δ_H 8.82 s) present in baicalein (Fig. S3A, Table 2) was replaced by a methoxyl (δ_H 3.76 s) in its product (oroxylin A) (Fig. S4A, Table 2), which was located at C-6 supported by HMBC and HMQC of baicalein (Fig. S3C,D). The similar methoxyl signal (δ_H 3.75) was clearly visible in the ^{1}H NMR profile of product hispidulin (Fig. S5A, Table 2) and was assigned to C-6 by consulting the literature [31–33]. The conclusion was that products of baicalein and scutellarein were, respectively, oroxylin A and hispidulin. The reaction products of quercetin and luteolin were identified by HPLC separation using the corresponding 3′-*O*-methylether (isorhamnetin and chrysoeriol) as the product standards. Eriodictyol was

Table 2. Spectroscopic data for baicalein, oroxylin A and hispidulin.

	Baicalein[a]		Oroxylin A		Hispidulin	
Position	δ_C	δ_H(J)	δ_C	δ_H(J)	δ_C	δ_H(J)
2	162.9		163.6		129.0	
3	104.5	6.94 s	104.8	6.98 s	102.3	3.75 s
4	182.1		182.1		182.0	
5	147.0	12.66 s	153.3	12.94 s	152.7	13.08 s
6	129.3	8.82 s	132.0	3.76 s 3H	131.9	3.75 s 3H
7	153.6	10.58 s	153.3	10.82 s	157.1	10.72 s
8	94.0	6.64 s	94.0	6.64 s	94.8	5.77 s
9	149.8		152.8		152.8	
10	104.5		104.8		104.2	
1′	131.0		132.3		121.6	
2′	126.3	8.08 d (7.5)	126.7	8.09 d (7.9)	129.0	7.94 d (8.8)
3′	129.1	7.61 m	129.1	7.63 m	102.5	6.93 d (8.8)
4′	131.9	7.61 m	130.4	7.63 m	161.6	10.36 s
5′	129.1	7.61 m	129.1	7.63 m	102.5	6.93 d (8.8)
6′	126.3	8.08 d (7.5)	126.7	8.08 d (7.9)	129.0	7.94 d (8.8)

[a]Recorded in DMSO-d_6 at 600 MHz (^{1}H NMR) or 150 MHz (^{13}C NMR). δ in ppm. J in Hz. ^{13}C multiplicities were determined by HSQC experiment. The ^{1}H and ^{13}C spectroscopic data was assigned by consulting the previously reports.

Table 3. Kinetic parameters for purified recombinant PaF6OMT using baicalein, and scutellarein as substrates.

Substrate	K_m (μM)	V_{max} (nmol·mg^{-1}·min^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (M^{-1}·s^{-1})
Baicalein	43.21 ± 6.239	86.21 ± 4.264	0.039 ± 0.002	911.8
Scutellarein	37.00 ± 5.184	85.18 ± 3.823	0.039 ± 0.002	1052.1

accepted as substrate and generated an unusual para-methylated product due to the existence of chiral carbon on its C-ring [29]. PaF6OMT can also accept esculetin, caffeoyl CoA, and caffeoyl alcohol as substrates, converting them to scopoletin, feruloyl CoA, coniferyl alcohol, respectively, as identified by chromatography with the corresponding authentic standard. While catechol OMT (CatOMT) can efficiently convert catechol into guaiacol *in vitro*, PaF6OMT was unable to do this, even though the two sequences of CatOMTs are phylogenetically closely related to one another. To determine the conditions for optimal enzyme activity, the pH dependence of PaF6OMT was first investigated in reactions where scutellarein was the substrate and *S*-adenosyl-L-methionine (SAM) was the methyl donor; a second set of reactions tested the temperature dependence of the enzyme at pH 7.5. The influence of pH was slight, and enzyme activity was greatest in the pH range 7–9 (data not shown). With respect to temperature, the optimal range was 35–45 °C, with activity declining sharply when the temperature rose above 50 °C. When a metal chelator (EDTA) was included in the reaction, methyltransferase activity was abolished (date not shown). The enzyme's kinetic parameters were determined at 37 °C and a pH of 7.5, using baicalein and scutellarein as alternative substrates (Table 3). Its affinity for the two substrates was comparable, although the catalytic efficiency for scutellarein was higher than that for baicalein. Experiments in which the amount of protein or the incubation time were varied under optimal reaction conditions showed that the catalytic rate fell as both the quantity of PaF6OMT and the incubation time were increased. Product (oroxylin A or hispidulin) yield rose as the quantity of PaF6OMT was raised, reaching 100% in the presence of 14 μg (oroxylin A) or 9 μg (hispidulin) of enzyme after 1 h incubation. In the presence of 6 μg of enzyme, the proportion of converted baicalein and scutellarein after 1 h at 37 °C was estimated to have been, respectively, 75% and 95% (Fig. 4A), while the product yields after 30 min were 63% (oroxylin A) and 87% (hispidulin), and after 90 min, respectively, 92% and 97% (Fig. 4B). After 4 h, an almost complete conversion of both substrates was achieved. Experiments in the synthetic production hispidulin must ensure the protection of other OH groups in scutellarein, while methylating the 6-OH group. A recently published protocol for generating hispidulin from scutellarein comprises a six-step reaction and requires at least 30 h to complete [34]. In contrast, the use of PaF6OMT required just a single step and the reaction was completed within 45 min. The enzyme is readily to be purified as recombinant protein from *E. coli*. It provides a safe, clean, and rapid means of converting baicalein and scutellarein into, respectively, the valuable products oroxylin A and hispidulin.

Subcellular localization of PaF6OMT

To investigate the subcellular localization of PaF6OMT, the fusions of PaF6OMT-GFP was constructed with the green fluorescent protein (GFP) and expressed under the control of the 35S promoter by transient assay in tobacco. When 35S::PaF6OMT-GFP transgene was transiently expressed in *N. benthamiana*, it was observed that gene was expressed largely in the cytoplasm (Fig. 5). However, PaOMT1 from *P. appendiculatum* has been reported directing to the chloroplast [29]. It may attribute to the differences in their enzyme features. The implication is that *in vivo*, the

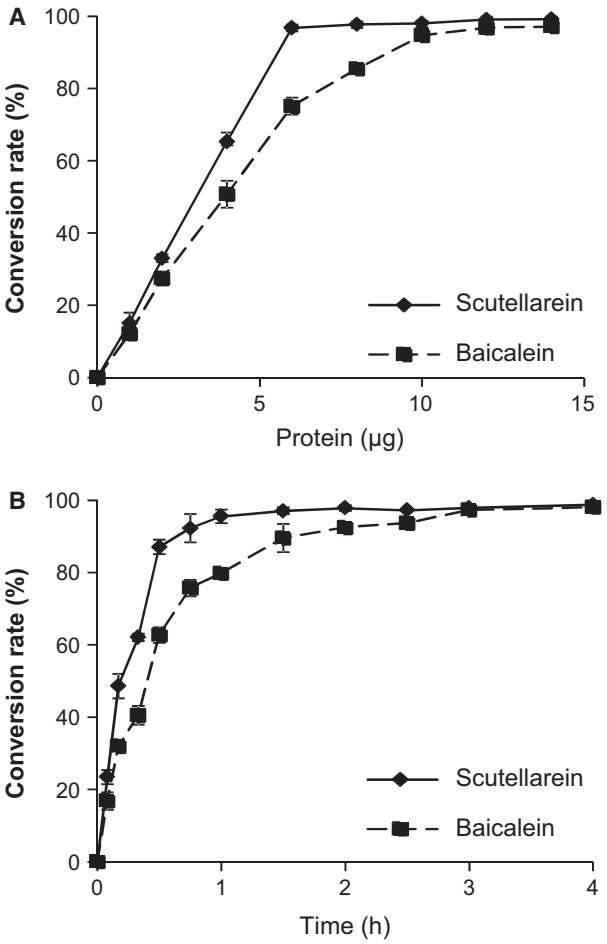


Fig. 4. Effect of protein amount or the incubation time on the conversion rates of baicalein and scutellarein at pH 7.5 and 37 °C. (A) The amount of PaF6OMT was varied from 1 μg to 14 μg; (B) The incubation time was varied from 5 min to 4 h.

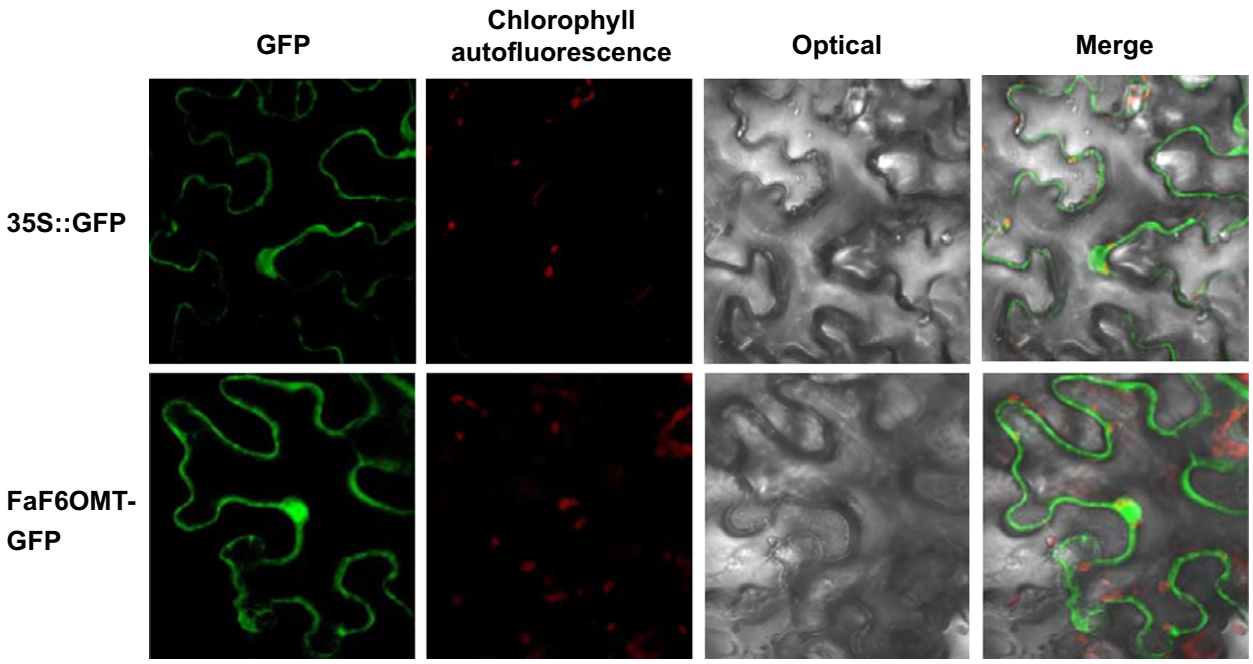


Fig. 5. Subcellular localization of the fusion gene *p35S::GFP* (empty vector control) and *p35S::PaF6OMT-GFP* when transiently expressed in *N. benthamiana* leaves. GFP protein is shown in green and chlorophyll in red.

methylation of 6-OH flavonoids probably occurs in the cytosol. Given that flavonoid synthesis is carried out by enzyme complexes [35], the likelihood is that PaF6OMT is a member of an endoplasmic reticulum multi-enzyme complex.

Conclusion

In conclusion, it has been possible to isolate a gene encoding an OMT from the liverwort species *P. appendiculatum* and to show that its product PaF6OMT catalyzes the 6-*O*-methylation of baicalein and scutellarein. The enzyme belongs to a novel subbranch of the class I OMTs, and to represent the first Mg^{2+}-dependent *O*-methyltransferase which interacts preferentially with 6-OH flavones. In contrast to either the complex extraction/separation steps needed to produce oroxylin A and hispidulin from plant material, or the multi-step synthetic reactions required to derive these compounds from baicalein and scutellarein, the enzymatic catalysis driven by PaF6OMT requires a simple, single-step reaction under mild, aqueous conditions, and produces a high yield.

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Author contributions

A-XC designed the experiments. Y-YZ, R-XX and SG performed the experiments. Y-YZ and A-XC analyzed the data and wrote the paper. All the authors analyzed the results and edited and approved the final version of the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Sequence alignment of PaF6OMT with *M. sativa* CCoAOMT (AAC28973.1), *P. appendiculatum* OMT1 (KP729179), *N. concava* CatOMT (WP-051178395.1), and *R. norvegicus* CatOMT (1JR4-A).
Fig. S2. SDS/PAGE analysis of recombinant PaF6OMT proteins from *E. coli*.
Fig. S3. NMR spectroscopic data for baicalein in DMSO-d_6: (A) ^{1}H NMR spectrum (600 MHz) of baicalein in DMSO-d_6; (B) ^{13}C NMR spectrum (150 MHz) of baicalein in DMSO-d_6; (C) HSQC spectrum (600 MHz) of baicalein in DMSO-d_6; (D) HMBC spectrum (600 MHz) of baicalein in DMSO-d_6.
Fig. S4. NMR spectroscopic data for oroxylin A in DMSO-d_6: (A) ^{1}H NMR spectrum (600 MHz) of oroxylin A in DMSO-d_6; (B) HMBC spectrum (600 MHz) of oroxylin A in DMSO-d_6.
Fig. S5. NMR spectroscopic data for compound hispidulin in DMSO-d_6: (A) ^{1}H NMR spectrum (600 MHz) of hispidulin in DMSO-d_6; (B) HMBC spectrum (600 MHz) of hispidulin in DMSO-d_6.
Table S1. Sequences of oligonucleotide primers used in this study.
Table S2. Accession numbers of amino acid sequences used for phylogenetic construction.