RESEARCH PAPER

Methylation mediated by an anthocyanin, O-methyltransferase, is involved in purple flower coloration in Paeonia

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Abstract

Anthocyanins are major pigments in plants. Methylation plays a role in the diversity and stability of anthocyanins. However, the contribution of anthocyanin methylation to flower coloration is still unclear. We identified two homologous anthocyanin O-methyltransferase (AOMT) genes from purple-flowered (PsAOMT) and red-flowered (PtAOMT) Paeonia plants, and we performed functional analyses of the two genes in vitro and in vivo. The critical amino acids for AOMT catalytic activity were studied by site-directed mutagenesis. We showed that the recombinant proteins, PsAOMT and PtAOMT, had identical substrate preferences towards anthocyanins. The methylation activity of PsAOMT was 60 times higher than that of PtAOMT in vitro. Interestingly, this vast difference in catalytic activity appeared to result from a single amino acid residue substitution at position 87 (arginine to leucine). There were significant differences between the 35S::PsAOMT transgenic tobacco and control flowers in relation to their chromatic parameters, which further confirmed the function of PsAOMT in vivo. The expression levels of the two homologous AOMT genes were consistent with anthocyanin accumulation in petals. We conclude that AOMTs are responsible for the methylation of cyanidin glycosides in Paeonia plants and play an important role in purple coloration in Paeonia spp.

Key words: Anthocyanin O-methyltransferase, catalytic activity, flavonoids, flower coloration, Paeonia, single amino acid substitution.

Introduction

The colours of flowers, fruits, and leaves are critically important traits in plants for UV protection and attracting pollinators and seed-dispersing organisms (Grotewold, 2006). The major classes of pigments that cause plant coloration are flavonoids, carotenoids, and betalains. The flavonoids are the best-studied class of pigments and are known to be widely

Abbreviations: 3D, three-dimensional; AOMT, anthocyanin O-methyltransferase; Ch7Neo, chrysoeriol 7-O-neohesperidose; Cy3G, cyanidin 3-O-glucoside; Cy3G5G, cyanidin 3,5-di-O-glucoside; DAD, diode-array detection; Dp3G, delphinidin 3-O-glucoside; DW, dry weight; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; Is3G7G, isorhamnetin 3,7-di-O-glucoside; Km3G, kaempferol 3-O-glucoside; Lu7G, luteolin 7-O-glucoside; Lu7Neo, luteolin 7-O-neohesperidose; MS, mass spectrometry; OMT, O-methyltransferase; ORF, open reading frame; Pg3G, pelargonidin 3-O-glucoside; Pn3G, peonidin 3-O-glucoside; Pn3G5G, peonidin 3,5-di-O-glucoside; qPCR, quantitative PCR; Qu3G7G, quercetin 3,7-di-O-glucoside; Qu3R, quercetin 3-O-rutinoside; SAM, S-adenosylmethionine.

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distributed among the angiosperms (Tanaka et al., 2008). Flavonoids play important roles in various ecological and physiological processes in plants, including pigmentation, UV absorption, antioxidation, defence responses, and signal transduction, among others (Tahara, 2007).

Anthocyanins are the largest group of flavonoids and the greatest contributors to floral coloration, providing the basis for orange, pink, red, magenta, scarlet, purple, blue, and blue/black flower colours (Tanaka et al., 2009). The basic biosynthetic pathways of three major anthocyanidins, namely pelargonidin, cyanidin, and delphinidin, have been well characterized (Fig. 1A). Further modifications to anthocyanidins including methylation, glycosylation, and acylation in these three aglycones produce a wide variety of anthocyanin compounds that strengthen the flower colour phenotypes (Rausher, 2008; Wessinger and Rausher, 2012). Previous studies that investigated the methylation of flavonoids have focused primarily on flavones and isoflavones (Ibrahim et al., 1998; Noel et al., 2003; Lam et al., 2007; Wang, 2011). Methylation of anthocyanins was first reported in petunia at the 3′ and 5′ positions of aglycones (Wiering and Devlaming, 1977; Jonsson et al., 1983; Bruglieri et al., 2003; Provenzano et al., 2014). Anthocyanin O-methyltransferase (AOMTs) were isolated from different grape cultivars, named VvAOMT and FAOMT, and characterized in vitro and in vivo, and they were found to be anthocyanin 3′- and 3′,5′-O-methyltransferase (Hugueney et al., 2009; Lucker et al., 2010). Akita et al. (2011) revealed a gene (CkmOMT2) from purple-flowered fragrant cyclamen, and an enzyme assay with heterologously expressed CkmOMT2 in vitro demonstrated that CkmOMT2 exhibited methylation activity with anthocyanins. A red/purple flower colour in a fragrant cyclamen mutant was bred by ion-beam irradiation, which was caused by the loss of the CkmOMT2 region (Kondo et al., 2009). Previous studies on the chromatic properties of anthocyanins from red grapes (Heredia et al., 1998) and tree peony petals (Sakata et al., 1995) demonstrated the effects of methylation on the hue; specifically, the higher the number of methoxyl groups,
the more pronounced the shift towards purple. In addition, it has been proposed that the methylation of B-ring hydroxyl groups causes a small shift towards red (Tanaka et al., 2008). Although there has been some progress on anthocyanin methylation, a large portion of methyltransferases of plant origin needs to be further characterized, as several questions remain to be answered, such as the molecular mechanism of anthocyanin methylation, the way in which methyltransferase takes part in the methylation of anthocyanins in vivo, and the relationship between anthocyanin methylation and floral coloration.

Methylation is an alkylation reaction that transfers an activated methyl group from S-adenosylmethionine (SAM) to the N-, C-, O-, or S-nucleophiles of acceptor molecules (Klimasauskas and Weinhold, 2007). Most methyltransferases methylate hydroxyl and carboxyl moieties, and are referred to as O-methyltransferases (OMTs). A large family of OMTs in plants contributes to the vast structural and functional diversity of plant natural products (Wang, 2011), which can be classified into two types according to their sequence homology and substrate variance (Joshi and Chiang, 1998; Noel et al., 2003; Lam et al., 2007). Type I OMTs include a group of homodimeric OMTs with molecular weights of 38–43 kDa and are cation independent, which methylate both flavonoids and isoflavonoids. Type II OMTs have lower molecular weights (23–27 kDa) and are cation dependent. Most type II OMTs have been shown to be specific for caffeoyl coenzyme A esters of phenylpropanoids (CCoAOMTs), which are thought to be key enzymes in the biosynthesis of lignin (Ye et al., 1994). However, Ibdah et al. (2003) reported that a novel Mg\(^{2+}\)-dependent PFOMT from Mesembryanthemum crystallinum (ice plant) with a molecular weight of 26.6 kDa and high similarity to type II OMTs was specific for the methylation of flavonols and caffeoyl-CoA. Thus, there is a novel subclass within type II OMTs with diverse substrates that are not restricted to lignin synthesis. The identification of cation-dependent OMTs such as ROMT15/17 from Oryza sativa (Lee et al., 2008), VvAOMT from Vitis vinifera (Hugueney et al., 2009), FAOMT from V. vinifera (Lucker et al., 2010), and ObF8OMT (Berim and Gang, 2013) with preferences for flavonoid substrates confirmed the appropriateness of the definition of the new subclass of type II OMTs. This progress highlights the occurrence of novel flavonoid methylation enzymes that differ from type I OMTs and underscores the need to further investigate their catalytic mechanisms.

Plants in the genus Paeonia are important ornamentals throughout the world. These beautiful plants have large flowers in a variety of colours and shapes. China has a long history of cultivating and breeding Paeonia cultivars and has rich collections of germplasm resources (Ji et al., 2012). However, a large proportion of the available cultivars have a purple or ‘purplish’ flower, but rarely a red flower. Our previous studies on flavonoids from the flowers of various Paeonia spp. demonstrated that peonidin derivatives were the major anthocyanidins that accumulated in most cultivars (Wang et al., 2001; Jia et al., 2008; Li, 2010), indicating that methylation modifications of anthocyanins are prevalent in Paeonia spp. As such, these plants provide a good model system for the investigation of methylation mechanisms and their influence on flower coloration.

In this study, we characterized an AOMT (PsAOMT) from a purple-flowered plant from the genus Paeonia and characterized its homologue PtAOMT from another plant in the genus Paeonia with a vivid red flower using both in vitro and in vivo methods. The catalytic activity PTAOMT was 60-fold less than that of PsAOMT. By using site-directed mutagenesis, we demonstrated that the vast difference in catalytic activities between these two enzymes was caused by the substitution of one key amino acid. This work characterized the subclass of type II OMTs by integrating biochemical, molecular, and phytochemical analysis, which will support an understanding of the anthocyanin methylating mechanism and shed light on its influence on flower coloration. The efficient enzyme PsAOMT and its key amino acid are responsible for effective activity and could be applied to the specifically targeted molecular breeding of ornamental and crop plants or the development of healthy and beneficial products.

**Materials and methods**

**Chemical sources**

Cyanidin, delphinidin, peonidin, pelargonidin 3-O-glucoside (Pg3G), and delphinidin 3-O-glucoside (Dp3G) were purchased from Extrasynthese (Genay, France). Quercetin, quercetin 3-O-rutinoside (Qu3R), and caffeic acid were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Cyanidin 3,5-di-O-glucoside (Cy3G5G), cyanidin 3-O-glucoside (Cy3G), kaempferol 3-O-glucoside (Km3G), kaempferol, luteolin, apigenin, naringenin, and epi catechin were purchased from Sigma-Aldrich (Shanghai, China). Analytical-grade methanol and acetonitrile were obtained from Promtart (Elk Grove, CA, USA).

**Plant materials and culture conditions**

A tree peony cultivar (Paeonia suffruticosa cv. ‘Gunpohden’) and an herbaceous peony Paeonia tenuifolia were used. The plants were grown at the Beijing Botanical Garden. The tobacco and strawberry plants were cultivated in a greenhouse under a 14 h light/10 h dark photoperiod. The temperature was maintained at 25 °C during the light period and 18 °C during the dark period.

**Cloning candidate AOMT cDNA and phylogenetic analysis**

An open reading frame (ORF) of a segment of expressed sequence tag (EF529149) from a cDNA library of the tree peony (Shu et al., 2009) is highly homologous to genes for the flavonoid OMT (PFOMT) from M. crystallinum (Ibdah et al., 2003) and anthocyanin OMT (VvAOMT) from grapevine (Hugueney et al., 2009) and was used for a reference sequence for cloning AOMTs from Paeonia plants. Total RNA was isolated from the two Paeonia petals with an RNAprep pure kit (Tiangen, Beijing, China). One microgram of total RNA was used as the template for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (Promega, WI, USA). The ORFs of PsAOMT and PtAOMT were cloned with high-fidelity PrimerSTAR HS polymerase (TaKaRa, Ohtsu, Japan) by using the AOMT forward/reverse primers (Supplementary Table S1, available at JXB online) from P. suffruticosa cv. ‘Gunpohden’ and P. tenuifolia. The amino acid sequences of PsAOMT and PtAOMT were aligned with CLUSTAL X (Thompson et al., 1997) and refined manually. MEGA 5.1 software was used to reconstruct a phylogenetic tree by using the maximum-likelihood test method (Tamura et al., 2011), with 1000 bootstrap replicates.
The sequenced cDNA of *AOMT* was inserted into the pMAL-c5X expression vector (NEB, MA, USA), which contains a maltose-binding protein tag. Recombinant AOMTs were purified with an amylose resin column (NEB). Site-directed mutagenesis was performed by using a Fast Mutagenesis System kit (TransGen, Beijing, China). The sequences of the primers used for this protocol are given in Supplementary Table S1.

Characterizing the recombinant AOMTs

The assay reaction conditions were optimized prior to performing quantitative analyses. The influence of pH on AOMT activity was assessed within a pH range of 4.5–8.5 using MES (pH 4.5–6.5) and Tris/HCl (pH 7.5–8.5) buffers. The effect of divalent cations on the enzyme activity was estimated by adding aqueous solutions of MgCl₂, CaCl₂, ZnCl₂, MnCl₂, CoCl₂, or EDTA (all at 10 mM final concentration) to the reaction mixture. The optimal concentrations of metal ions were assessed by testing different concentrations of MgCl₂ (0.1, 0.2, 0.5, 1.0, 5.0, and 10 mM). The optimized conditions were as follows: purified recombinant AOMT (2 μg) was assayed in a final volume of 200 μl containing 200 μM SAM, 1.0 mM MgCl₂, 14 mM β-mercaptoethanol, 100 mM Tris/HCl (pH 7.5), and 20 μM flavonoid substrates (the chemical structures are shown in Supplementary Fig. S1, available at JXB online). Incubation was performed at 35 °C and stopped with 800 μl of methanol containing 2% formic acid, followed by centrifugation at 12 000 rpm for 10 min. The upper liquid was prepared for high-performance liquid chromatography (HPLC) analysis, and 20 μl of reaction sample was loaded. Anthocyanin and flavonol profiles were recorded at 525 and 350 nm, respectively. The substrates and products were identified by comparison with standards and HPLC electrospray ionization mass spectrometry (HPLC-ESI/MS). For the kinetic studies, purified AOMT was incubated under the above optimized conditions with the exception of a range of substrate concentrations from 5 to 200 μM for Km determination (Hugueney et al., 2009). Reaction products were analysed by HPLC, using the method reported by Yang et al. (2009).

Molecular modelling of the PsAOMT and PtAOMT active sites

Three-dimensional models of PtAOMT and PsAOMT were generated by using the I-TASSER Protein Structure and Function Predictions web server (Roy et al., 2010). Homology models were built by using the known three-dimensional (3D) structure of caffeoyl coenzyme, a 3-O-methyltransferase (CCoAOMT, PDB code 1SUI) from alfalfa (Ferrer et al., 2005). The best models of PsAOMT and PtAOMT were evaluated based on their template modelling score (0.90), root mean square deviation (1.75), and sequence identity (54.0%) in relation to the 3D structure of CCoAOMT. Because recombinant AOMTs form dimers in solution, PsAOMT and PtAOMT were modelled as homodimers by using the COOT program (Krissinel and Henrick, 2004). Substrate-binding sites were predicted by docking Cy3G5G with the PsAOMT dimer 3D model in the SWISS-Dock program (Grosdidier et al., 2011).

Transient expression in strawberry fruit

*Fragaria*×*ananassa* cv. ‘Hongyan’ mature plants with fruits that were just turning red were used for these experiments. A single colony of *Agrobacterium* strain EHA105 with PsAOMT or PtAOMT and a single colony with *Arabidopsis* *PRODUCTION OF ANTHOCYANIN PIGMENTI* (PAP; GenBank accession no. AF325123) were cultured and diluted to an OD₆₀₀ of 0.1–0.3, and injected into strawberry fruits according to the method of Hoffmann et al. (2006). The infiltrated fruits were harvested after 4 d, and the extracts were analysed according to Yang et al. (2009).

Tobacco transformation

PsAOMT and PtAOMT were cloned into the pBI121 binary vector (Clontech). Sequence-confirmed constructs were then introduced into *Agrobacterium* strain EHA105 by electroporation. A single positive colony was co-cultured with leaf sections of sterile *Nicotiana tabacum* cv. Nc89 according to a previously reported protocol (Horsch et al., 1985). Finally, kanamycin-resistant plantlets were transferred to soil mix, acclimatized, and grown in a greenhouse. Positive transgenic lines were selected by PCR, and empty-plasmid transgenic plantlets were used as controls. The transgenic tobacco lines with similar expression levels of PsAOMT and PtAOMT were used for further study.

Quantitative PCR (qPCR) analysis of AOMT expression

Flower petals at five different developmental phases were sampled in triplicate. Total RNA was prepared as described above. Quantitative assays of gene expression were performed using an UltraSYBR Mixture (CW BIO, Beijing, China) and analysed with a Stratagene (CA, USA) Mx3000P instrument as described by Lan et al. (2009). The relative quantification of mRNA transcripts was performed in triplicate with normalization to *Actin* (GenBank accession no. JN105299) (Zhao et al., 2012). The primers used in the qPCR analysis were flanked by an intron (Supplementary Table S1). The PCR products were sequenced to confirm that the correct gene was amplified. AOMT expression in the roots, stems, leaves, and sepals was also studied.

HPLC diode-array detection (HPLC-DAD) and HPLC-MS analyses of flavonoids extracts

The flavonoids (anthocyanins, flavones, and flavonols) in petals from *P. suffruticosa* cv. ‘Gunpohden’ and *P. tenuifolia* at five stages, in transgenic tobacco flowers, and in strawberry fruits were extracted using a method described by Yang et al. (2009). The extraction protocol was as follows: transgenic tissues (tobacco and strawberry) were extracted with 2% formic acid/methanol (v/v) assisted by sonication at 20 °C for 20 min. All extracts were filtered through a 0.22 μm membrane before injection. The solvent and gradient method for the separation of transgenic tobacco flower extracts, strawberry fruit extracts, and enzyme assay extracts was as follows: solvent A, 10% aqueous formic acid; solvent B, 0.1% formic acid in acetonitrile; constant gradient from 5 to 40% B within 25 min, maintain 40% B for 5 min, and then return to 5% B in 5 min. The flow rate was 0.8 ml min⁻¹. The column temperature was maintained at 30 °C, and 10 μl of analyte was injected. DAD data were recorded from 200 to 800 nm.

An HPLC-ESI-MS® system was used as described by Zhu et al. (2012). The positive-ion mode was adapted for anthocyanins; both the positive-ion and negative-ion modes were employed for colourless flavonoids. MS spectra were recorded over an m/z range of 100–1000.

The identification of anthocyanins and colourless flavonoids was according to past research on *Paeonia* plants (Wang et al., 2001; Li et al., 2009). The anthocyanins and colourless flavonoids were quantified using Cy3G and Qu3R as standards, respectively, by linear regression. The anthocyanin quantity was expressed as μg of Cy3G equivalents g⁻¹ of dry weight (DW), and by using the calibration curve, the following was obtained: anthocyanin (mAU)=0.474 [Cy3G (μg ml⁻¹)] – 1.275 (r²=0.999). Colourless flavonoids were expressed as μg of Qu3R equivalents g⁻¹ of DW, with the following calibration curve: (mAU)=0.437 [Qu3R (μg ml⁻¹)] – 2.485 (r²=0.997). All samples were analysed in triplicate.

Colour measurements (CIELab system)

Chromatic analyses were performed based on the CIE 1976 (*L*‘*a*’*b*’) system (Gonnet, 1998). The colours were expressed as *L**, *a**, and *b* values. The value of *L* represents lightness, from black (0) to white
An anthocyanin OMT involved in floral coloration

(100); a* describes red (positive) to green (negative); b* describes yellow (positive) to blue (negative); and C* represents the chroma or saturation of the colour. The colour parameters were measured with an NF333 spectrophotometer according to Zhu et al. (2012).

Results

Flavonoid accumulation during the floral development of Paeonia plants

We investigated the flavonoid profiles of purple flower petals from *P. suffruticosa* cv. ‘Gunpohden’ and the vivid red flower petals of *P. tenuifolia* at five different developmental stages (S1–S5, from colourless to full floral expansion) (Fig. 2). Petal anthocyanins were identified as Cy3G, Cy3G5G, peonidin 3-O-glucoside (Pn3G), and peonidin 3,5-di-O-glucoside (Pn3G5G) according to Wang et al. (2001) (Fig. 1B). The anthocyanins accumulated during development and reached a maximum at the bloom stage (S5). The total concentration of anthocyanins was 10.73 ± 2.29 mg g⁻¹ of DW and 13.25 ± 0.76 mg g⁻¹ of DW at S5 for *P. suffruticosa* cv. ‘Gunpohden’ and *P. tenuifolia*, respectively. Interestingly, peonidin-derived anthocyanins (Pn3G and Pn3G5G, 94%) were the primary anthocyanins of purple flowers (*P. suffruticosa* cv. ‘Gunpohden’), and cyanidin-derived anthocyanins (Cy3G and Cy3G5G, 76%) were the primary anthocyanins in vivid red flowers (*P. tenuifolia*) (Fig. 2 and Supplementary Table S2, available at *JXB* online). Other flavonoid compound profiles of *P. suffruticosa* cv. ‘Gunpohden’ and *P. tenuifolia* flowers were analysed at 350 and 280 nm, respectively (Supplementary Fig. S2, available at *JXB* online). Co-pigments (flavonols and flavones) were abundant in the petals of *P. suffruticosa* cv. ‘Gunpohden’, and they were primarily quercetin 3,7-di-O-gluco-side (Qu3G7G), isorhamnetin 3,7-di-O-glucoside (Is3G7G), luteolin 7-O-glucose (Lu7G), chrysoeriol 7-O-glucose (Ch7G), luteolin 7-O-neohesperidose (Lu7Neo) and chrysoeriol 7-O-neohesperidose (Ch7Neo) according to Li et al. (2009) (Supplementary Fig. S2). The amounts at different developmental stages are shown in Supplementary Fig. S3, available at *JXB* online. By contrast, the red flowers of *P. tenuifolia* contained negligible amounts of co-pigments (Supplementary Fig. S2). Given the hypothesis that anthocyanins are responsible for coloration, the anthocyanin methyltransferase might be the reason for the observed colour difference given the anthocyanins that were characterized from these two plants.

Fig. 2. Flowers from five developmental stages and the anthocyanin accumulation at five developmental stages (S1–S5) from *Paeonia suffruticosa* cv. ‘Gunpohden’ (A) and *P. tenuifolia* (B), as well as expression patterns of AOMT transcripts at the corresponding developmental stages and in different tissues, measured by qPCR. The expression values have been normalized against the β-Actin gene and are expressed as relative abundances.
In silico analysis of candidate AOMTs

To confirm our hypothesis, two genes with ORFs of 708 bp that encoded for methyltransferase were obtained from *P. suffruticosa* cv. ‘Gunpohden’ and *P. tenuifolia*. The deduced amino acid sequences of these genes contained a conserved domain similar to those of the SAM or AdoMet MTases superfamily (cl17173) and the Methyltransf_3: O-methyltransferase superfamily (pfam01596), and were designated *PsAOMT* and *PtAOMT*, respectively. The putative peptides of these proteins had 235 aa and a calculated molecular mass of 26.4 kDa, and they each contained a conserved structural fold of seven β-sheets (Fig. 3) (Cheng and Roberts, 2001). Surprisingly, there were a total of four amino acids (positions 13, 85, 87, and 205) that differed between *PsAOMT* and *PtAOMT* (Fig. 3). A BLAST analysis indicated that *PsAOMT* and *PtAOMT* were homologous to several previously characterized type II subclass OMTs. *PsAOMT* shared 73% identity with *VvAOMT* from grapevine (Hugueney et al., 2009), 62% with *GmAOMT* from *Glycine max* (Kovinich et al., 2011), and 58% with *PFOMT* from *M. crystallinum* (Ibdah et al., 2003). Phylogenetic analysis demonstrated that *PsAOMT* belongs to the type II OMTs (with a low molecular weight and Mg\(^{2+}\) dependence) and was similar to the typical example of this class of enzyme, CCoAOMT (Fig. 4). A subclass of the type II OMTs specific for flavonoid OMTs has been proposed (Ibdah et al., 2003), and thus *PsAOMT* is a possibly new member of this subclass together with *VvAOMT*, *McPFOMT*, and several other polypeptides described in a patent (Brugliera et al., 2003) (Fig. 3).

![Fig. 3. Sequence alignments of *PsAOMT* and *PtAOMT* with predicted secondary structural elements. The reference sequences *VvAOMT* (grapevine, BQ796057), *GmAOMT* (black soybean, ADX43927) and *McPFOMT* (ice plant, AY145521) are also included. α-Helices and β-strands are represented as cylinders and arrows, respectively. The residues conserved in all OMTs are shaded. (This figure is available in colour at JXB online.]](http://jxb.oxfordjournals.org/content/6568/1/6568/F3.expansion?cmd=full&imageId=JXB/article/F3)
An anthocyanin OMT involved in floral coloration and quercetin, PsAOMT had a higher affinity for Cy3G and Cy3G5G (Table 1), suggesting that cyanidin-derived anthocyanins are high-affinity substrates for PsAOMT. Unlike anthocyanins, anthocyanidin cyanidin could be used as substrate by PsAOMT with a low activity. In addition, we could not detect the methylated products of the anthocyanidin delphinidin (Supplementary Table S3), which suggested that delphinidin might be unstable in the reaction system, or might be due to the low enzymatic activity of PsAOMT. PsAOMT could also methylate Lu7G and Lu with weak affinity. The following substrates could not be methylated by PsAOMT: Km3G, kaempferol, luteolin, apigenin, naringenin, epicatechin, and caffeic acid (Table 1). Surprisingly, although PtAOMT possesses identical substrate specificity and biochemical properties, the catalytic activity of PtAOMT was much lower than that of PsAOMT (approximately 60-fold lower) (Table 1).

A single amino acid substitution can dramatically alter the catalytic activity of AOMTs

The high amino acid sequence identities of PsAOMT and PtAOMT indicated that their divergent enzymatic activity might result from differences in amino acid residues at four positions. Four separate site-directed mutations were constructed by using the low-activity enzyme PtAOMT as a template, namely PtAOMT-G13E, PtAOMT-T85A, PtAOMT-R87L, and PtAOMT-T205R. When assayed for 5 min under the optimized reaction conditions with Cy3G5G as a substrate, only Pn3G5G was detected from the PtAOMT-R87L reaction product, and the observed in vitro catalytic efficiency of PtAOMT-R87L was equal to that of PsAOMT (Table 2). To further characterize these mutations, the reaction durations were extended from 5 to 30 min, and the recombinant G13E, T85A, and T205R enzymes displayed 1.4, 4.1, and 9.1%, respectively, of the PsAOMT catalytic activity (100%). The PtAOMT activity was 1.5% of that of PsAOMT (100%), and the increased duration of the assays did not lead to a detectable increase in PtAOMT activity (Table 2). However, the single R87L mutation of PtAOMT led to an equally high activity (104.5%) as that of PsAOMT (100%). This result indicated that a leucine residue at position 87 plays an important role in the catalytic activity of these AOMTs. To validate that a leucine at position 87 was indeed a key amino acid residue, two mutations (L87R and L87A) of PsAOMT were constructed. Both these mutant enzymes exhibited decreased catalytic activity (similar to
Table 1. Overview of PsAOMT and PtAOMT activities with various substrates under optimized assay conditions

| Substrate                     | PsAOMT | PtAOMT |
|-------------------------------|--------|--------|
|                               | $K_m$ (μM) | $V_{max}$ (nM$^{-1}$) | $K_{cat} \times 10^{-3}$ (s$^{-1}$) | $K_{cat}/K_m$ (M$^{-1}$s$^{-1}$) | Specific activity (pkat mg$^{-1}$) |
| Pelargonidin 3-O-glucoside    | 0.5 (0) | 0.5 (0) | 0.5 (0) | 0.5 (0) | 0.5 (0) |
| Cyanidin 3,5-di-O-glucoside   | 1.06 (0.01) | 1.06 (0.01) | 1.06 (0.01) | 1.06 (0.01) | 1.06 (0.01) |
| Cyanidin 3-O-glucoside        | 1.76 (0.02) | 1.76 (0.02) | 1.76 (0.02) | 1.76 (0.02) | 1.76 (0.02) |
| Delphinidin 3-O-glucoside     | 4.11 (0.35) | 4.11 (0.35) | 4.11 (0.35) | 4.11 (0.35) | 4.11 (0.35) |
| Quercetin 3-O-rutinoside      | 12.32 (0.32) | 12.32 (0.32) | 12.32 (0.32) | 12.32 (0.32) | 12.32 (0.32) |
| Luteolin 7-O-glucoside        | 146.26 (10.07) | 146.26 (10.07) | 146.26 (10.07) | 146.26 (10.07) | 146.26 (10.07) |
| Kaempferol 3-O-glucoside      | –      | –      | –      | –      | –      |
| Cyanidin                      | –      | –      | –      | –      | –      |
| Delphinidin                   | –      | –      | –      | –      | –      |
| Quercetin                     | 4.33 (0.07) | 4.33 (0.07) | 4.33 (0.07) | 4.33 (0.07) | 4.33 (0.07) |
| Luteolin                      | –      | –      | –      | –      | –      |
| Kaempferol                    | –      | –      | –      | –      | –      |
| Apigenin                      | –      | –      | –      | –      | –      |
| Naringenin                    | –      | –      | –      | –      | –      |
| Epicatechin                   | –      | –      | –      | –      | –      |
| Caffeic acid                  | –      | –      | –      | –      | –      |

Table 2. Comparison of catalytic activities of the recombinant AOMTs generated using site-directed mutagenesis, using Cy3G5G as the substrate

Activity (%): recombinant protein activity as a percentage of the ‘full’ activity measured for PsAOMT; SD, standard deviation with three repetitions.

| Polypeptide | Incubation for 5 min [% (±SD)] | Incubation for 30 min [% (±SD)] |
|-------------|--------------------------------|--------------------------------|
| PsAOMT      | 100(0)                          | 100(0)                          |
| PtAOMT-G13E | 0(0)                           | 1.4(0.1)                        |
| PtAOMT-T85A | 0(0)                           | 4.1(0.3)                        |
| PtAOMT-R87L | 100.7(1.1)                     | 104.5(0.7)                      |
| PtAOMT-T205R| 0(0)                           | 9.4(0.5)                        |
| PsAOMT-L87R | 0(0)                           | 4.0(0.2)                        |
| PsAOMT-L87A | 0(0)                           | 4.8(0.4)                        |
| PtAOMT      | 0(0)                           | 1.5(0.3)                        |

the activity of recombinant PtAOMT) (Table 2). A kinetic study of PtAOMT-R87L showed that its substrate specificity was identical to that of PsAOMT and its specific activity was similar to that of PsAOMT (Supplementary Table S4, available at JXB online). It is worth noting that the $K_m$ of PtAOMT-R87L for Cy3G was slightly lower than its $K_m$ for Cy3G5G; the opposite was true for PsAOMT. These results demonstrated that Leu-87 is a critically important residue for the catalytic activity of these OMT enzymes and that a single amino acid mutation can cause a dramatic decrease in activity without a loss of function.

Structural basis for substrate discrimination in AOMTs

The 3D models of PsAOMT and PtAOMT were computed and analysed to investigate the structural basis for their activity differences (Supplementary Fig. S7, available at JXB online). The SAM-binding residues (Met-49, Glu-73, Ser-81, Asp-99, Asp-151, and Asp-153) were conserved in both OMTs and were very similar to those of CCoAOMT (Fig. 5). After careful analyses of the PsAOMT model and the substrate-binding site of CCoAOMT, the residues Tyr-45, Val-48, Trp-181, Phe-182, Lys-194, Phe-196, and Asp-226 were predicted to be the putative substrate-binding pocket of Cy3G5G (Fig. 5). To our surprise, the single amino acid residue (Leu-87) that contributes the substrate specificity of PsAOMT is far from the putative substrate-binding pocket (Supplementary Fig. S7).

In vivo characterization of AOMTs

We first investigated the catalytic activities of the Paeonia AOMTs in vivo by using transient expression in strawberry fruits. The strawberry fruit mostly contained the pelargonidin type of anthocyanins (da Silva et al., 2007), which are known for not being AOMT substrates. To provide appropriate substrates for the enzymatic reactions, the anthocyanin biosynthesis-related R2R3 MYB transcription factor PAP1 from Arabidopsis (Borevitz et al., 2000) was introduced along with the two Paeonia AOMT sequences by agroinfiltration (Hoffmann et al., 2006). The simultaneous expression of PAPI and AOMT transcripts resulted in the synthesis of Cy3G (m/z 449 [M$^+$]) and Pn3G (m/z 463 [M$^+$]) in strawberry fruits (Fig. 6A). It should be noted that there are several anthocyanin compounds that occur naturally in strawberries (a1–a6 shown in Supplementary Table S5). Pn3G accumulated to higher levels than those of Cy3G in the PAPI and PsAOMT transformants. Conversely, the Cy3G levels were higher than the Pn3G levels in the PAPI and PtAOMT transformants (Fig. 6A).

Next, we used Agrobacterium-mediated transformation of tobacco for in vivo studies of AOMT function. Tobacco has pink flowers that are known to contain cyanidin-3-O-rutinoside...
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(Ac3R). Five and four transgenic tobacco lines were found to accumulate similar mRNA levels of PsAOMT and PtAOMT (Supplementary Fig. S8, available at JXB online), respectively. The anthocyanin profiles of these transgenic and control lines were analysed by HPLC-MS (Fig. 6B). The results demonstrated that five 35S::PsAOMT lines accumulated peonidin-3-O-rutinoside (Pn3R), with a peak of m/z 609 [M]+, accounting for 21.7 ± 1.8% of total anthocyanin, and Pn3R was also detected in four 35S::PtAOMT transgenic lines, accounting for a lower 1.9 ± 0.2% total anthocyanin content. This finding indicated that the proteins encoding PsAOMT and PtAOMT had a contrasting catalytic activity in vivo, which was consistent with the characterization of recombinant proteins in vitro.

To evaluate the influence of the introduced PsAOMT and PtAOMT on flower coloration, chromatic analyses of transgenic tobacco lines and controls were performed. One-way analysis of variance was performed for the colour parameters \( L^* \), \( a^* \), \( b^* \), and \( C^* \), and significant differences were observed between 35S::PsAOMT and control flowers (Fig. 7). The 35S::PsAOMT transgenic lines had higher values of \( a^* \) towards red, and lower values of \( b^* \) towards blue, leading to an \( h \) change towards a purple hue compared with that of the control, which demonstrated that methylated anthocyanins would result in purplish flower colours. The colour parameters of the 35S::PtAOMT lines fell between those of the 35S::PsAOMT lines and the control and showed no significant differences among them (Fig. 7).

This result further validated the OMT activity of AOMTs of Paeonia and underscored the effects that dramatically different enzymatic efficiencies can cause, leading to differences in the biosynthesis and accumulation of these compounds.

AOMT expression patterns are consistent with anthocyanin accumulation

To determine whether there was any correlation between the expression levels of AOMT transcripts and flavonoid accumulation during floral development in Paeonia spp., the corresponding flower petals from S1–S5 were harvested and assayed for the expression of AOMT transcripts. The results indicated that the PsAOMT expression levels in P. suffruticosa cv. ‘Gunpohden’ were consistent with the anthocyanin accumulation levels in petals. As shown in Fig. 2, anthocyanins were barely detected at the beginning of the colourless stage (S1), and PsAOMT transcripts were also detected at low levels (Fig. 2). From the flower coloration stage onward, the PsAOMT expression level increased and reached a maximum when the flower bud became loose and ready to bloom (S3) (Fig. 2). The methylated anthocyanin Pn3G5G accumulated to its maximum amount at S5 because of the continuous expression of PsAOMT. The expression levels of PsAOMT showed no obvious relationship with the accumulation of flavones and flavonols because the methylated colourless flavonoids (Is/Ch glycosides) already existed before the onset of S1 (Supplementary Fig. S3) when PsAOMT transcripts were hardly detected. The concentrations of these compounds did not change with the increasing expression levels of PsAOMT transcripts (Fig. 2 and Supplementary Fig. S3). These results indicated that PsAOMT was related to and specific for methylated anthocyanin accumulation in Paeonia. The expression pattern of PtAOMT in P. tenuifolia was identical to that of PsAOMT in P. suffruticosa cv. ‘Gunpohden’ (Fig. 2). However, there was no noticeable correlation between the PtAOMT expression levels and the accumulation of methylated anthocyanin compounds (Fig. 2 and Supplementary Fig. S3). We inferred that this finding was caused by the relatively weak enzyme activity of PtAOMT in comparison with that of PsAOMT.

In addition, AOMT expression was evaluated in various vegetative organs (Fig. 2). PsAOMT transcripts could be detected at very low levels in roots and stems, and they were detected at slightly higher levels in leaf and sepal tissues, which are known to accumulate low anthocyanin concentrations. PtAOMT transcripts were detected in the roots and leaves at very low levels and were noticeable in the stems and sepals.

AOMT catalyses the biosynthesis of methylated anthocyanins and leads to purple coloration in Paeonia flowers

We sequenced AOMT cDNA from five tree peony cultivars (‘Fengdan’, ‘Hongling’, ‘Liuguangyicai’, ‘Chaoyi’, ‘Songhui’, and ‘Gunpohden’). The cDNAs of PtAOMT were about 1225 bp in length. The deduced amino acid sequences of PtAOMT from ‘Fengdan’, ‘Hongling’, ‘Liuguangyicai’, ‘Chaoyi’, and ‘Songhui’ and ‘Gunpohden’ were identical. The deduced amino acid sequence of PsAOMT was identical to that of PtAOMT.

Fig. 5. Molecular model of the PsAOMT active site. The SAH (S-adenosyl-l-homocysteine), Cy3G5G, and substrate-binding residues are represented as sticks, and labelled in cyan, grey, and white, respectively. The oxygen, nitrogen, and sulfur atoms are labelled in red, blue, and yellow, respectively.

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and ‘Qinglongwomochi’), two herbaceous peony cultivar (‘Dafugui’ and ‘Zixiayingri’), and one intersectional hybrid (‘Hexie’) between tree peony and herbaceous peony, which all have a red/purple or purple flower colour (Supplementary Table S2, Supplementary Fig. S9, available at JXB online). A chemical analysis of anthocyanins from the five cultivars by HPLC-MS showed that methylated anthocyanins (peonidin glycosides) accounted for 87–95% of the total anthocyanins (Supplementary Table S2, Supplementary Fig. S11, available at JXB online) and cyanidin glycosides were detected as minor compounds, indicating that all the cultivars possessed efficient methyltransferases to catalyse the methylation of cyanidin glycosides to peonidin glycosides. As a result, peonidin glycosides accumulated in the petals, leading to the purple

Fig. 6. Characterization of AOMT activity in vivo using transient expression in strawberry and the stable transformation of tobacco. (A) Anthocyanin profiles of strawberry fruits with transient PBI121 or PAP1 expression, and the co-expression of PAP1/PsAOMT, and PAP1/PtAOMT (4 d of expression). Other anthocyanins are shown in Supplementary Table S5, available at JXB online. (B) Anthocyanins from transgenic tobacco flowers (35S::PsAOMT, 35S::PtAOMT and empty vector control, respectively) were analysed by HPLC-MS.
The dimer of type II OMTs lacking a domain for dimerization in the N-terminal region could be formed by hydrophobic interactions. Each monomer could interact with substrate(s) and cofactor(s), unlike type I OMTs, which require a homodimeric structure to perform methylation (Noel et al., 2003). Surprisingly, we found that the leucine at position 87 of PsAOMT was vital for the high activity level, and a mutant with an arginine residue at the same position in PsAOMT led to a remarkable decrease in enzymatic activity.

OMT-catalysed methylation reactions require a methyl donor (SAM), a methyl accepter (substrate), and an appropriately conformed reaction centre. Typically, the SAM-binding residues comprise a highly glycine-rich sequence of E/DXGXGXG known as motif I, which is highly conserved in the N-terminal region of OMTs (Martin and McMillan, 2002). The N terminus and a variable insertion loop near the C terminus played important roles in the substrate specificity (Kopycki et al., 2008). The key amino acid at position 87 was located in the α4 helix proximal to motif I (Fig. 3), suggesting that a mutation in this amino acid may influence the binding of SAM or the methyl transfer. Although the PtAOMT and PsAOMT activities differ greatly in terms of catalytic efficiency, these enzymes shared the same substrate specificities, indicating that the substrate-binding regions of both proteins were probably conserved. The SAM-binding site might be altered by the single amino acid substitution at position 87 in the two proteins, and this mutation may be responsible for the observed differences in the enzyme turnover rates but may not affect substrate regioselectivity. The substitution may also affect the methylation reaction. The currently recognized catalytic mechanisms of OMTs are S_{N}2-like reactions, which are metal dependent, acid/base residues, or mediation by proximity and desolvation (Liscombe et al., 2012). The arginine residue is polar, unlike leucine (a neutral amino acid), which may change the hydrophobic environment around SAM or the catalytic centre. However, Leu-87 of PsAOMT is far from the putative substrate-binding pocket; however, mutations at distant residues can alter the properties of the active site region and change the activity as reported for glutathione S-transferase (Ketterman et al., 2001), alcohol dehydrogenase (Nagel et al., 2013), and regulator proteins (Freeman et al., 2011). Mutations at distant residues might cause dynamic motion at the active site that is involved in the conformational change of the substrate-binding region and then alter the catalytic efficiency and substrate specificity.

To elucidate the mechanism precisely, X-crystal structures of AOMTs must be further investigated.

**Discussion**

*Functional characterization of AOMTs from Paeonia and their active switch*

A phytochemical analysis in *Paeonia* plant petals on methylated anthocyanin compounds accumulation suggested that strong methylation activity occurs in the petals (Wang et al., 2001; Zhang et al., 2007; Jia et al., 2008; Li et al., 2009). Therefore, we identified and characterized genes in this study for anthocyanin methyltransferases that belonged to the new type II OMTs. The characterization of recombinant PsAOMT and PtAOMT with a wide range of flavonoid compounds and caffeic acids as substrates demonstrated that they both methylated flavonoids with a vicinal dihydroxy on the B-ring and a 3-hydroxyl on the C-ring. The kinetic parameters of PsAOMT showed that the specific activities of anthocyanins and flavonols were similar to their activity in VvAOMT, and the \( K_{m} \) values for anthocyanins were smaller than those in VvAOMT (Hugueney et al., 2009), displaying a better affinity with the optimum substrates. In addition, PsAOMT showed much lower catalytic activities with anthocyanidins, quercetin, and luteolin, indicating that it was able to methylate glycosylated flavonoids rather than aglycones. In peony flowers, the major anthocyanins are both methylated and glycosylated, and the order of aglycone modifications \textit{in vivo} remain unknown. The present work suggested that methylation may occur after glycosylation during anthocyanin biosynthesis, which is consistent with reports in grape berries (Ford et al., 1998; Hugueney et al., 2009). To clarify the order of methylation and glycosylation \textit{in vivo}, the further characterization of the flavonoid glycosyltransferase in *Paeonia* will be meaningful.
Supplementary Fig. S11) and the other six colourless flavonoids (Supplementary Fig. S3) as well as AOMT expression were investigated during flower development. The results indicated that PsAOMT expression was notably consistent with the accumulation of monomethylated peonidin glycosides in *P. suffruticosa* cv. ‘Gunpohden’ flowers. In contrast, peonidin glycosides were barely accumulated in *P. tenuifolia* flowers, because of weak PtAOMT activity, while the AOMT activity and accumulation of methylated colourless flavonoids seemed less relevant. Although recombinant PsAOMT could employ flavonols and flavones as substrates *in vitro* (Table 1), the methylated colourless flavonoids in tree peony flowers were minor components, such as Is3G7G, Ch7G, and Ch7Neo (Supplementary Fig. S3). Moreover, these methylated colourless flavonoids were detected at early stages before colouration, and the content increased slowly, displaying no obvious correlation with the *PsAOMT* expression. This finding might be explained by the specificity of the substrate structure and anthocyanin competition, or there may be other flavone and flavonol OMTs in peonies.

**Methylation of anthocyanins is involved in the purplish flower colouration of *Paeonia* spp.**

In ornamental plants, the mechanism of blue flower colouration has been extensively studied, and consists primarily of delphinidin anthocyanins (Yoshida *et al.*, 2009). However, the basis of coloration of purple flowers from cyanidin-based anthocyanins remains unclear at present. Cyanidin-based anthocyanins confer red/purple and purple colours to plants, with some exceptions such as cornflower (a blue colour) (Shiono *et al.*, 2005). The flower colour phenotype is controlled primarily by pigment chemical structures and the concentrations of particular pigments (Nakayama *et al.*, 2012). In the present study, the two plant materials presented purple and vivid red colours, although they contained the same four cyanidin-based derivatives and similar total concentrations of anthocyanins. The vast difference in methylated anthocyanins (peonidin glycosides) was dominant in purple flowers and for cyanins as the major pigment in red flowers. This finding suggested that methylation varied the colour of cyanidin-based anthocyanins from red to purple. To elucidate the influence of methylation on coloration, transgenic tobacco lines with overexpressed *PsAOMT* were generated. The flower colour of transgenic tobacco plants with different methylation profiles corresponding to those in Fig. 6 demonstrated significant changes in chromatic parameters towards a purple hue, compared with that of the control (Fig. 7). A similar phenomenon was reported in fragrant cyclamen (Akita *et al.*, 2011). A purple-flowered fragrant cyclamen lacking an enzyme for anthocyanin OMT (*CkmOMT2*) (ion-beam irradiation deletion) produced red/purple flower phenotypes (Akita *et al.*, 2011).

Sakata *et al.* (1995) studied 38 tree peony cultivars in Japan and concluded that hydroxylation and methylation both contributed considerably to the blueing of flower colours. Chemical and chromatic analyses of hundreds of tree peony cultivars showed that the methylation level of anthocyanins was significantly correlated with the purpleness of the flower colour (Li, 2010). Supplemental information including the flower colour, chromatic parameters, anthocyanin methylation levels and the key amino acid of corresponding AOMTs for 10 typical purple or red-flowered cultivars are summarized in Supplementary Table S2. Taken together, these findings show that there was a noticeable correlation for cultivars that contained a key leucine amino acid and then generated a high level of methylated anthocyanins, resulting in a purple flower.

In comparison with the methylation of anthocyanins in tree peony, the minor differences in glycosylation might play less of a role in flower coloration because it may simply increase the solubility or decrease anthocyanin vacuolar inclusions (Morita *et al.*, 2005). The former study demonstrated that, in tree peony flowers, glycosylation was present only with monoglucosides, and the number of glucose moieties did not contribute to the bluing of the tree peony flower (Sakata *et al.*, 1995; Li, 2010).

Co-pigments (colourless flavonoids) are another difference between the two plant materials. Other flavonoid compound profiles of *P. suffruticosa* cv. ‘Gunpohden’ and *P. tenuifolia* flowers were analysed at 350 and 280 nm (Supplementary Fig. S2). Red flowers contained negligible amounts of co-pigments, and co-pigments were abundant in purple flowers (Supplementary Fig. S2). Co-pigments interacting with anthocyanins at the proper concentrations and ratios can result in a bathochromic shift (Asen *et al.*, 1971). However, a study of Japanese tree peony cultivars showed that there were pink-flowered cultivars with high co-pigment contents and purple-flowered cultivars with low co-pigment contents, suggesting that co-pigmentation might not be a major factor in the blueing of tree peony flowers (Sakata *et al.*, 1995). As the content of flavonols increased, the blue flower of lisianthus changed towards red (Nielsen *et al.*, 2002). Flavone accumulation in transgenic torenia made the flower bluer (Aida *et al.*, 2000). Nevertheless, these studies were based primarily on blue flowers with delphinidin-based anthocyanins. Whether these compounds influence coloration in flowers with cyanidin-based anthocyanins, and the mechanism of any such influence, will require further investigation.

Similar chemical and chromatic analyses of lotus cultivars have also been investigated, and the results suggested that the methylation level of anthocyanins was significantly correlated with the hue towards purpleness (Yang, 2009; Yang *et al.*, 2009). Heredia *et al.* (1998) reported a chromatic characterization of five anthocyanins from red grape skins obtained in a spectroscopic study, and showed that as the degree of methylation increased, a shift towards purple was observed. This finding suggested that anthocyanin methylation might commonly contribute to purple coloration, especially in cyanidin-based pigment systems.

In conclusion, we characterized an AOMT in *Paeonia* plants. Based on *in vitro* and *in vivo* experiments, we grouped this AOMT into a recently defined subclass of type II OMTs and identified the specificity for anthocyanin substrates. In addition, we characterized the biochemical properties of recombinant PsAOMT and found that a mutation in a key amino acid residue could dramatically change the catalytic
efficiency. Moreover, the AOMT is likely to be responsible for the methylation of anthocyanins and to contribute to the purple coloration of flowers in *Paeonia* plants. Thus, research on AOMTs in *Paeonia* is an important step for understanding anthocyanin modifications, and their contribution to flower coloration innovation is relevant for ornamental plants.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** The chemical structure of substrates used for *in vitro* analysis of recombinant PsAOMT and PtAOMT.

**Supplementary Fig. S2.** HPLC profiles of flower extracts from *Paeonia suffruticosa* cv. ‘Gunpohden’ (A) and *Paeonia tenuifolia* (B) at 350 and 280 nm.

**Supplementary Fig. S3.** The accumulation of colourless flavonoids in *Paeonia suffruticosa* cv. ‘Gunpohden’ petals during flower development.

**Supplementary Fig. S4.** SDS-PAGE of recombinant PsAOMT.

**Supplementary Fig. S5.** The catalytic activity of PsAOMT with quercetin 3-O-rutinoside (Qu3R) as a substrate under different reaction conditions, including a pH gradient of buffer solutions, the presence of different diveralent cations, and various Mg" concentrations.

**Supplementary Fig. S6.** Profiles of products methylated by recombinant PsAOMT using a series of substrates in the *in vitro* system by HPLC analysis.

**Supplementary Fig. S7.** Superimposed 3D models of PsAOMT and PtAOMT represented as cartoons.

**Supplementary Fig. S8.** The expression of *AOMT*s in transgenic tobacco lines according to RT-PCR.

**Supplementary Fig. S9.** Flower phenotypes of different *Paeonia* cultivars used in this study.

**Supplementary Fig. S10.** Amino acid sequence alignments of the AOMTs from 10 *Paeonia* plants.

**Supplementary Fig. S11.** HPLC profiles of anthocyanins in flower petals.

**Supplementary Table S1.** The primer sequences used in site-directed mutagenesis and qPCR.

**Supplementary Table S2.** A summary of the 10 specimens, including information regarding the flower colour, chromatic parameters, total anthocyanin content, methylation levels of the anthocyanins, and the key amino acid(s) of the AOMTs.

**Supplementary Table S3.** Identification of products methylated by recombinant PsAOMT using a series of substrates in the *in vitro* system.

**Supplementary Table S4.** PtAOMT-R87L activities with major substrates under optimized assay conditions.

**Supplementary Table S5.** Anthocyanins in strawberry fruits as detected by HPLC-MS (demonstrated in Fig. 6).

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