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

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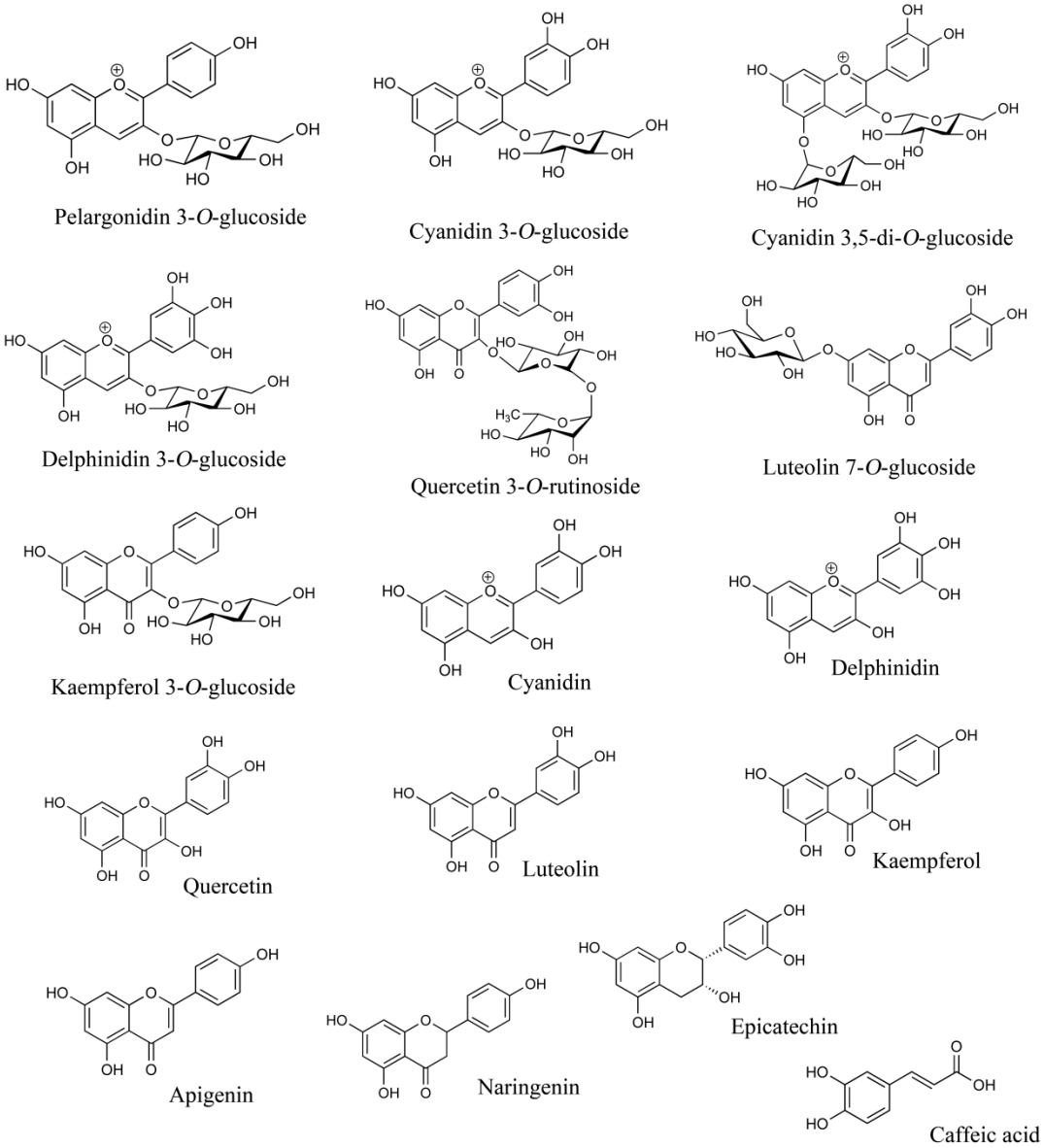

Figure S1. The chmical structure of substrates used for *in vitro* analysis of recombinant PsAOMT and PtAOMT.

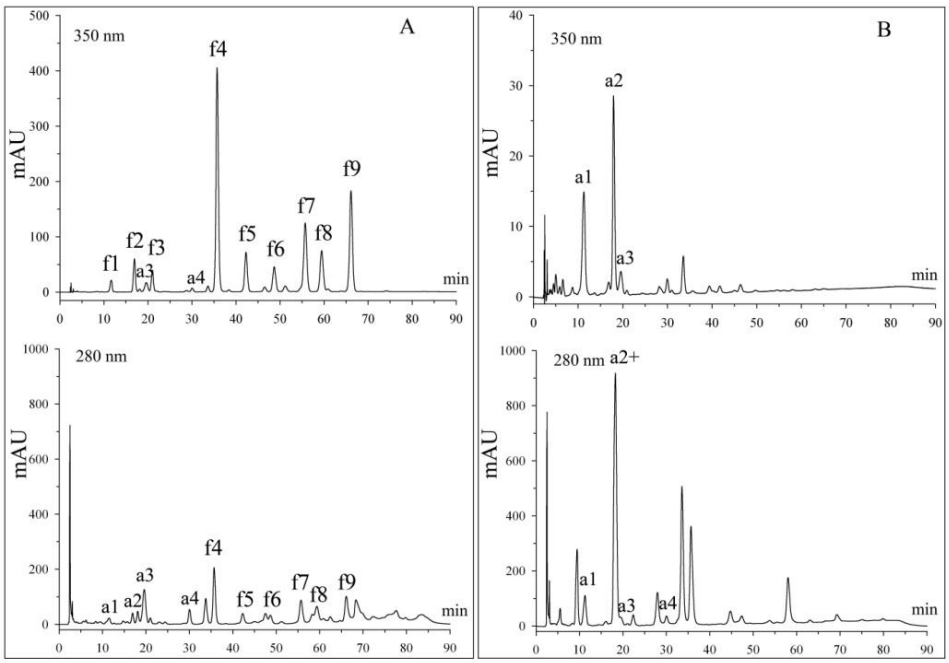

Figure S2. HPLC profiles of flower extracts from *Paeonia suffruticosa* cv. 'Gunpohden' (A) and *Paeonia tenuifolia* (B) at 350 nm and 280 nm. a1, Cy3G5G; a2, Cy3G; a3, Pn3G5G; a4, Pn3G; f1, Qu3G7G; f2, Km3G7G; f3, Is3G7G; f4, Lu7G; f5, Lu7Neo; f6, Ap7G; f7, Ap7Neo; f8, Ch7G; and f9, Ch7Neo. The unlabeled peaks are unknown peaks. Peak a2+ was a mixture that included a2 and an unknown compound.

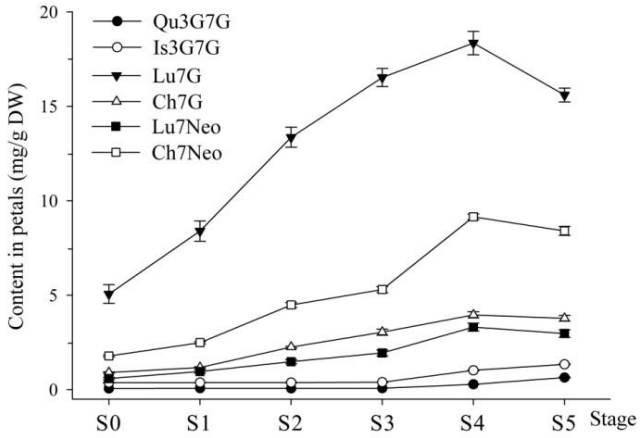

Figure S3. The accumulation of colorless flavonoids in *Paeonia suffruticosa* cv. 'Gunpohden' petals during flower development.

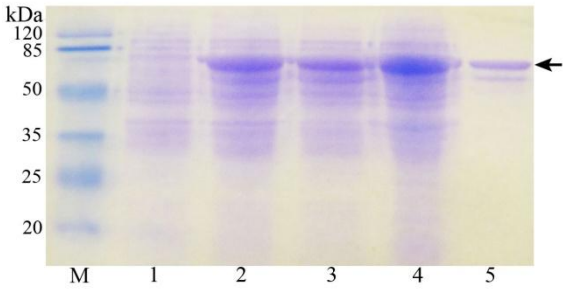

Figure S4. SDS-PAGE of recombinant PsAOMT. Lane M, protein size markers; Lanes 1–2, total *E. coli* protein before or after induction; Lanes 3–4, fragmented *E. coli* supernatant and pellet after induction with fusion protein; and Lane 5, purified MBP-PsAOMT fusion protein.

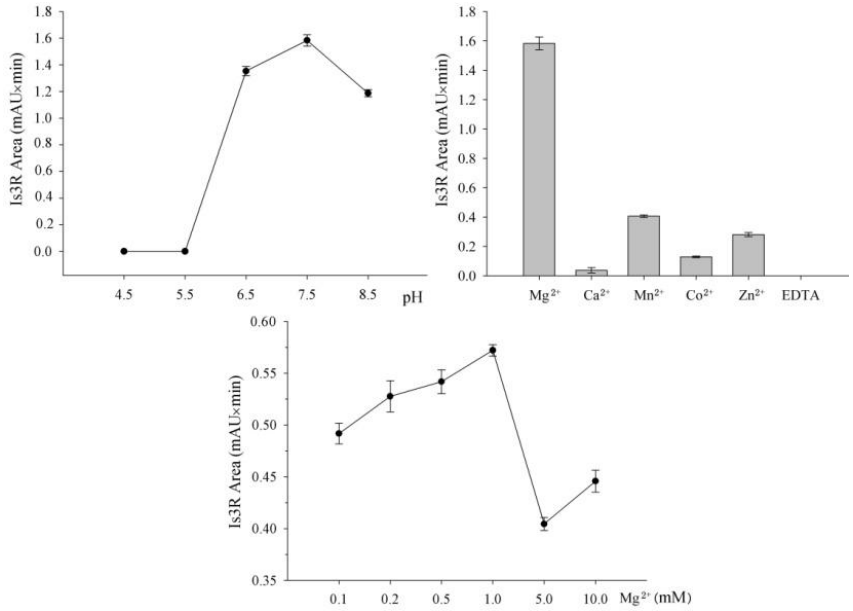

Figure S5. The catalytic activity of PsAOMT with quercetin 3-*O*-rutinoside (Qu3R) as a substrate under different reaction conditions, including a pH gradient of buffer solutions, the presence of different divalent cations, and various Mg^{2+} concentrations. The mAU areas of the methylated product and isorhamnetin 3-*O*-rutinoside (Is3R) are presented as the means of triplicate experiments.

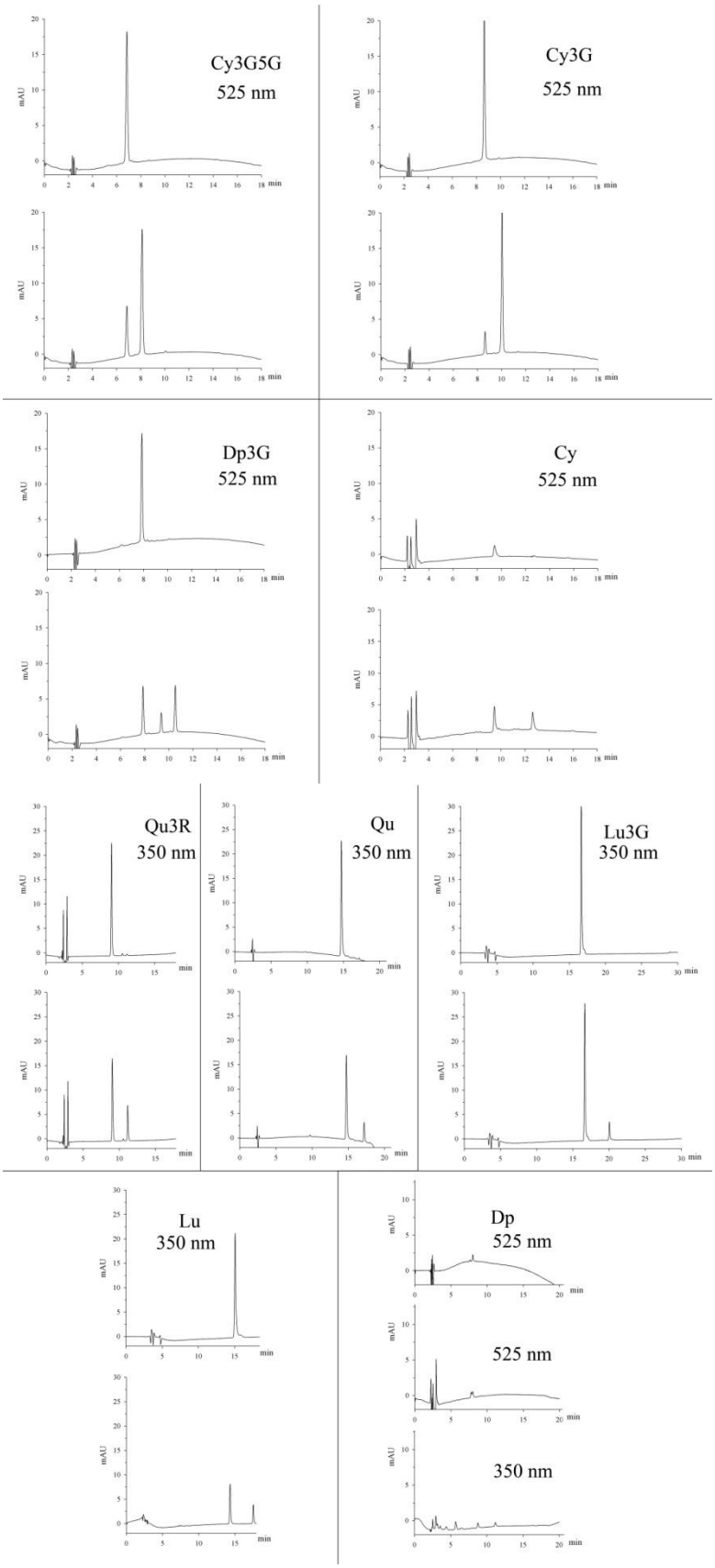

Figure S6. Profiles of products methylated by recombinant PsAOMT using a series of substrates in the *in vitro* system by HPLC analysis.

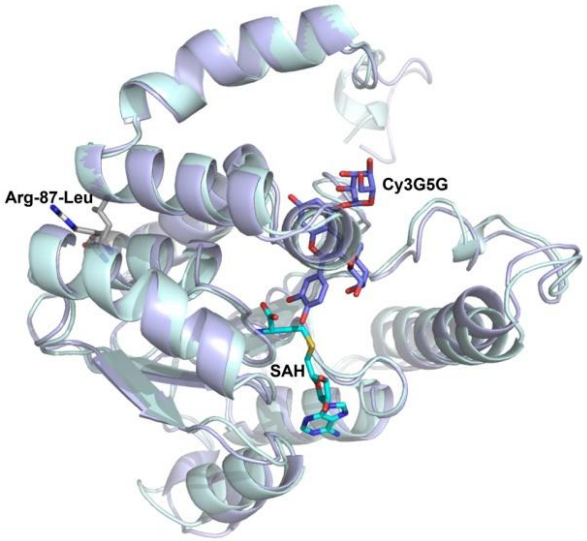

Figure S7. Superimposed 3D-models of PsAOMT and PtAOMT are represented as cartoons; PsAOMT and PtAOMT are labeled in light blue and pale cyan, respectively. SAH, cyanidin 3,5-di-*O*-glucoside (Cy3G5G), and the key amino acid residue Arg-87-Leu are represented as sticks and labeled in cyan, slate and white, respectively. The oxygen, nitrogen, and sulfur atoms are labeled, in red, blue, and yellow, respectively.

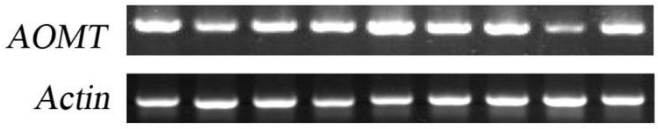

Figure S8. The expression of *AOMT*s in transgenic tobacco lines according to RT-PCR. Lanes 1–5, transgenic lines with 35S::*PsAOMT*; Lanes 6–9, transgenic lines with 35S::*PtAOMT*. The expression of *Actin* (GQ339768) was used as standard.

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

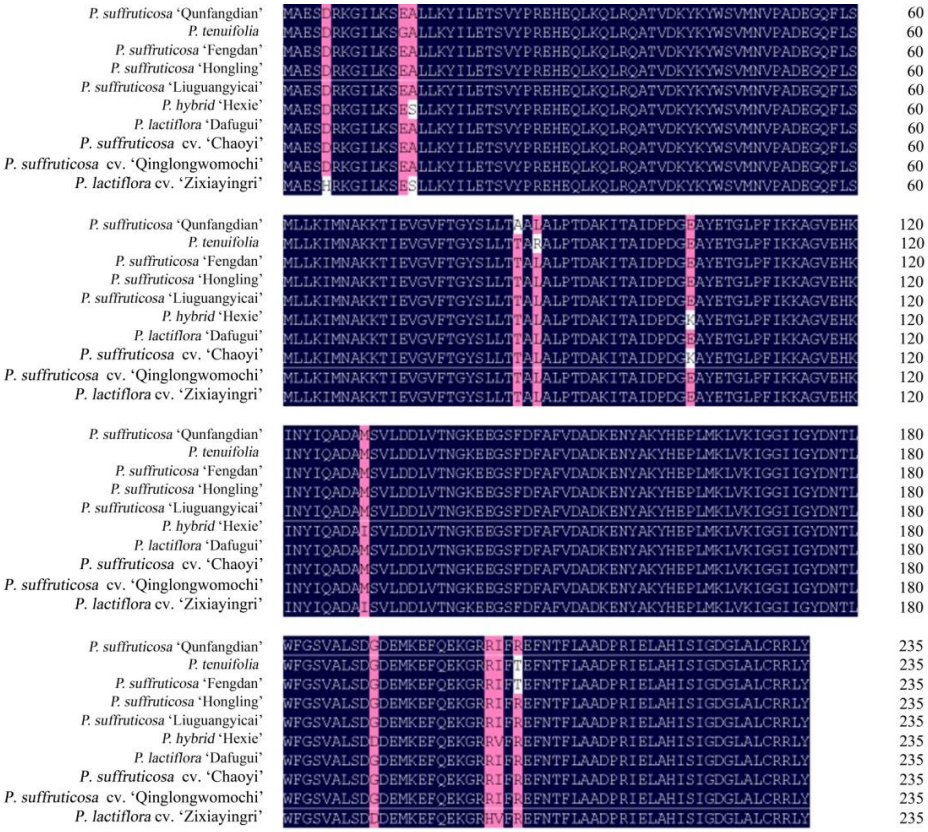

Figure S10. Amino acid sequence alignments of the AOMTs from ten *Paeonia* plants.

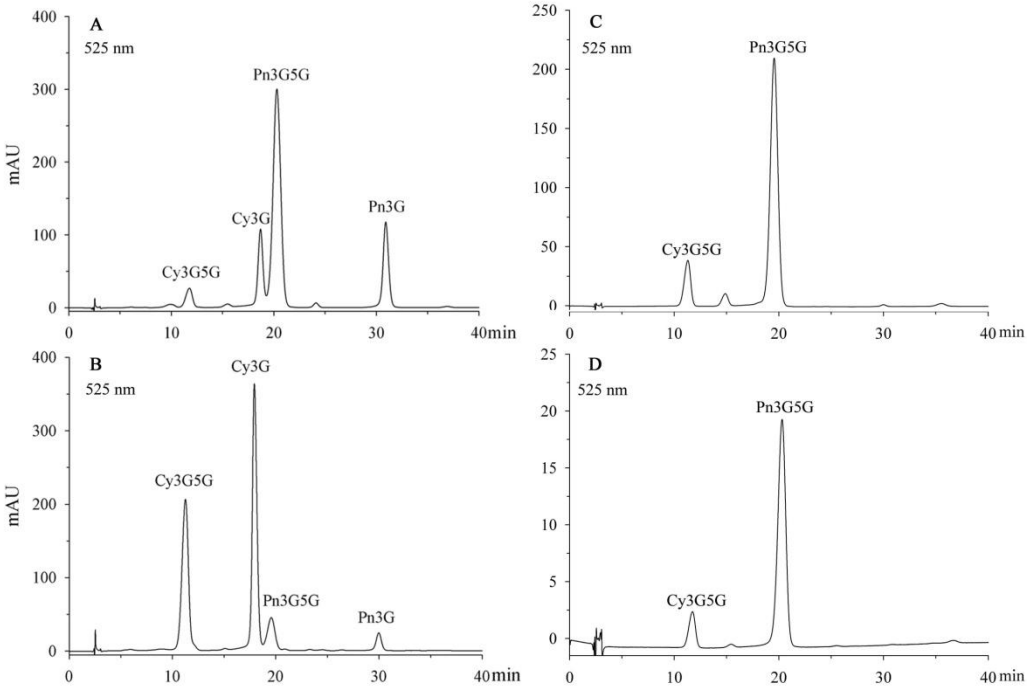

Figure S11. HPLC profiles of anthocyanins in flower petals. A, *Paeonia suffruticosa* cv. 'Gunpohden'; B, *Paeonia tenuifolia*; C, *Paeonia hybrid* ('Hexie'); D, *Paeonia lactiflora* cv. 'Dafugui.'

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

Primer Name	Oligonucleotide sequence
AOMT-forward	ATGGCCGAGTCAGATAGGAAGGGT
AOMT-reverse	TAAAATGATCCTTCTGCCATGCT
PtAOMT–G13E-forward	TATTCTTAAAAGTGAAGCCCTTTTG
PtAOMT–G13E-reverse	TCACTTTTAAGAATACCCTTCCTAT
PtAOMT–T85A-forward	TTATTCTCTTCTCACTGCCGCTCGT
PtAOMT–T85A-reverse	CAGTGAGAAGAGAATAACCAGTAAAG
PtAOMT–R87L-forward	TCACTACCGCTCTTGCTCTGCCTAC
PtAOMT–R87L-reverse	AGAGCGGTAGTGAGAAGAGAATAAC
PtAOMT–T205R-forward	GGCGCATCTTTAGAGAGTTCAACAC
PtAOMT–T205R-reverse	CTAAAGATGCGCCTGCCCTTTTCCTG
PsAOMT–L87R-forward	CTTCTCACTGCCGCTCGTGCTCTGC
PsAOMT–L87R-reverse	CGAGCGGCAGTGAGAAGAGAATAACC
PsAOMT–L87A-forward	TCTCACTGCCGCTGCTGCTCTGCCT
PsAOMT–L87A-reverse	GCAGCGGCAGTGAGAAGAGAATAACC
AOMT-qPCR-forword	TAAGAAGGCTGGAGTGGAGCATAAG
AOMT-qPCR- reverse	GGCATAGTTTTCCTTGTCAGCATCC
Actin-forward (For *Paeonia*)	GCAGTGTTCCCCAGTATT
Actin-reverse (For *Paeonia*)	TCTTTTCCATGTCATCCC
Actin-forward (For transgenic lines)	GCCAACAGAGAGAAAATGACCC
Actin-reverse (For transgenic lines)	TCATGGATGGCTGGAAGAGGACTTC

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

	Paeonia suffruticosa cv. 'Gunpohden'	*P. tenuifolia*	*P. suffruticosa* cv. 'Fengdan' (flower disc)	*P. suffruticosa* cv. 'Hongling'	*P. suffruticosa* cv. 'Liuguangyi cai'	*P. hybrid* 'Hexie'	*P. lactiflora* cv. 'Dafugui'	*P. suffruticosa* cv. 'Chaoyi'	*P. suffruticosa* cv. 'Qinglongwo mochi	*P. lactiflora* cv. 'Zixiayingri'
Flower color	purple-red	vivid red	purple-red	purple-red	Purple-red	Purple-red	Purple-red	purple-red	purple-red	red-purple
RHSCC	77B	61A	—	61C	82C	72B	71D	71B	71A	61A
*L**	35.5	31.5	75.9	51.9	75.8	35.3	46.1	33.94	25.26	22.47
*a**	36.2	41.5	-7.4	40.7	-0.7	47.1	45.2	47.35	33.51	34.37
*b**	-16.0	9.4	5.6	-7.2	5.6	-17.0	-13.1	-10.60	-4.15	0.64
*C**	39.7	42.6	9.3	41.4	5.8	50.2	47.2	48.55	33.85	34.37
Hue	-23.9	-12.9	-10.2	-10.15	-15.05	-20.2	-16.5	-12.59	-6.86	1.07
Total anthocyanins (mg/g DW)	10.73	13.25	2.32	13.05	2.54	7.64	4．86	27.62	45.25	15.93
TF	32.70	0.72	—	42.28	33.1	38.23	23.37	24.18	41.71	23.55
CI	3.05	0.05	—	3.24	13.06	5.28	4.81	0.88	0.92	1.48
Methylation%	94	24	95	91	86	87	89	55	67	68
Amino acid at 87 position	Leucine	Arginine	Leucine	Leucine	Leucine	Leucine	Leucine	Leucine	Leucine	Leucine

The data of chroma parameters (RHSCC, *L**, *a**, *b**, *C** and hue), TA, TF and CI were cited from Li CH (2010, The flavonoid composition in tree peony petals and their effects on the coloration, PhD dissertation) and Jia N (2008, Studies on petal coloration mechanism and chemotaxonomy of herbaceous peony, Master dissertation).

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

Substrate (μM) and reaction duration (min)	Wavelength nm	Retention time min	*m/z* positive mode	*m/z* negative mode	Identification
Cy3G5G 25 μM-10 min	525	4.9	287.1(3.59),449.1(8.97), 611.1(100)		Cyanidin 3,5-di-*O*-glucoside
		6.2	301.0(3.39), 625.1(100)		Peonidin 3,5-di-*O*-glucoside
Cy3G 25 μM-10 min	525	6.4	287.0(69.94), 449.1(54.29)		Cyanidin 3-*O*-glucoside
		7.8	301.1(98.70), 463.1(100)		Peonidin 3-*O*-glucoside
Dp3G 25 μM-10 min	525	6.1	303.1(100), 465.1(75.44)		Delphinidin 3-*O*-glucoside
		7.6	317.0(98.05), 479.1(100)		Petunidin 3-*O*-glucoside
		8.9	331(74.91), 493.1(100)		Malvidin 3-*O*-glucoside
Qu3R 25 μM-10 min	350	9.8	303.0(100), 465.1(35.01), 611.1(28.62)		Quercetin 3-*O*-rutinoside
		11.9	317.1(100), 479.0(30.55), 625.1(22.67)		Isorhamnetin 3-rutinoside
Qu 25 μM-10 min	350	14.6	303.0(100)	300.9(100)	Quercetin
		18.1	149.0(100), 317.1(33.64)	311.0(100)	Isorhamnetin
Lu7G 25 μM-10 min	350	10.2	287.0(100), 449.1(49.36)		Luteolin 7-*O*-glucoside
		12.4	301.1(100), 463.1(46.08)		Chrysoderol 7-*O*-glucoside
Lu 25 μM-10 min	350	15.1	287.0(100)		Luteolin
		18.2	301.0(100)		Chrysoderol
Cy 50 μM-10 min	525	11.4	287.0(100)		Cyanidin
			301.2(31.10), 287.0(20.42), 276.0(72.62), 149.0(100)		Peonidin
Dp 50 μM-10 min	350	8.1	393.0(100),149.0(79.49), 132.0(75.05), 471.8(23.54)	468.9(100), 409.0(26.44),376.9(22.32),276.8(14.04)	Unknown
		11.4	132.0(100), 276.1(85.16), 395.0(76.54), 471.9(52.31)	392.8(84.76), 468.8(100)	Unknown
		13.8	132.0(100),149.0(81.39), 276.0(37.42), 309.7(89.57), 319.0(42.72)	297.0(100), 316.9(99.49)	Unknown

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

Substrates	PtAOMT-R87L				
	K_m μM	V_{max} $nM\ s^{-1}$	$K_{cat} \times 10^{-3}$ s^{-1}	K_{cat}/K_m $M^{-1}s^{-1}$	Specific Activity $pkat\ mg^{-1}$
Cyanidin 3,5-di-*O*-glucoside	8.51 (1.39)	14.94 (0.73)	106.71 (5.21)	13010 (1428)	1494 (73)
Cyanidin 3-*O*-glucoside	3.53 (0.31)	17.59 (0.30)	125.66 (2.16)	36058 (3045)	1759 (30)
Delphinidin 3-*O*-glucoside	27.27 (1.49)	19.06 (0.88)	0.14 (0.01)	4999 (82)	1906 (88)
Quercetin 3-*O*-rutinoside	8.09 (0.25)	9.31 (0.97)	0.07 (0.01)	8253 (923)	931 (97)
Luteolin 7-*O*-glucoside	48.88 (2.74)	1.26 (0.24)	0.01 (0.00)	189 (47)	126 (24)

Data are expressed as means (± SE) of triplicate assays.

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

Peak No.	Compound	Peak No.	Compound
a1	Pg 3,5-di-*O*-glucoside	a4	Cy 3-*O*-malonylglucoside
a2	Pg 3-*O*-glucoside	a5	Pg 3-*O*-malonylglucoside
a3	Pg 3-*O*-rutinoside	a6	Pn 3-*O*-malonylglucoside