

# Unraveling the Mechanism Underlying the Glycosylation and Methylation of Anthocyanins in Peach<sup>1[C][W]</sup>

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Modification of anthocyanin plays an important role in increasing its stability in plants. Here, six anthocyanins were identified in peach (*Prunus persica*), and their structural diversity is attributed to glycosylation and methylation. Interestingly, peach is quite similar to the wild species *Prunus ferganensis* but differs from both *Prunus davidiana* and *Prunus kansuensis* in terms of anthocyanin composition in flowers. This indicates that peach is probably domesticated from *P. ferganensis*. Subsequently, genes responsible for both methylation and glycosylation of anthocyanins were identified, and their spatiotemporal expression results in different patterns of anthocyanin accumulation in flowers, leaves, and fruits. Two tandem-duplicated genes encoding flavonoid 3-O-glycosyltransferase (F3GT) in peach, *PpUGT78A1* and *PpUGT78A2*, showed different activity toward anthocyanin, providing an example of divergent evolution of F3GT genes in plants. Two genes encoding anthocyanin O-methyltransferase (AOMT), *PpAOMT1* and *PpAOMT2*, are expressed in leaves and flowers, but only *PpAOMT2* is responsible for the O-methylation of anthocyanins at the 3' position in peach. In addition, our study reveals a novel branch of *UGT78* genes in plants that lack the highly conserved intron 2 of the *UGT* gene family, with a great variation of the amino acid residue at position 22 of the plant secondary product glycosyltransferase box. Our results not only provide insights into the mechanisms underlying anthocyanin glycosylation and methylation in peach but will also aid in future attempts to manipulate flavonoid biosynthesis in peach as well as in other plants.

Anthocyanins are important secondary metabolites and serve to protect plants against pathogenic attack and UV radiation and provide flowers and fruits with pigmentation to attract pollinators and seed dispersers (Winkel-Shirley, 2001). Anthocyanins are usually stored as glycosylated forms in vacuoles. The basic structure of anthocyanins is composed of an anthocyanidin aglycone and one or more sugar moieties linked to hydroxyl groups 3, 5, 7, 3', and 5', with the 3 position on the C-ring being dominant. The most common sugar moieties are Glc, Gal, Xyl, Ara, and Fru. With different types and/or numbers of sugar moieties attached to various positions, the structural diversity of anthocyanins increases significantly. Besides glycosylation, modifications such as methylation and acylation also contribute

to the structural diversity of anthocyanins. To date, there have been more than 550 anthocyanins identified in nature (Kong et al., 2003).

The formation of glycosides is catalyzed by UDP-Glc:flavonoid glycosyltransferase (UGT). Both monoglycosides and diglycosides are common in plants. For example, 3RT, 3GGT, and F3GGT1, responsible for further glycosylation of anthocyanidin 3-O-glycosides, have been reported in petunia (*Petunia hybrida*; Kroon et al., 1994), Japanese morning glory (*Ipomoea nil*; Morita et al., 2005), and kiwifruit (*Actinidia chinensis*; Montefiori et al., 2011), respectively. Glycosylation plays at least two roles in the accumulation of anthocyanins. One is to stabilize anthocyanins by attaching sugar moieties to the unstable anthocyanidin aglycones. Deficiency in UF3GT activity results in a significant reduction of anthocyanin accumulation in maize (*Zea mays*; Fedoroff et al., 1984) and Arabidopsis (*Arabidopsis thaliana*; Tohge et al., 2005), as anthocyanidins are highly unstable and easily susceptible to degradation. Another is to serve as a signal for transport of the anthocyanins to vacuoles (Ono et al., 2006). Anthocyanins are synthesized on the cytoplasmic face of the endoplasmic reticulum by metabolons, which are likely to function as a multienzyme complex (Winkel, 2004). Once synthesized, anthocyanins need to be transported to vacuoles by transporters such as multidrug and toxin extrusion transporters (Zhao et al., 2011). Vacuole

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localization is crucial for anthocyanins to function as pigments to attract pollinators and seed dispersers or as antioxidants to prevent photooxidation of the photosynthetic apparatus by lowering light intensity and screening out UV irradiation (Marrs et al., 1995). In short, glycosylation is essential for the accumulation of anthocyanins in plants.

UFGT belongs to family 1 glycosyltransferases, which are characterized by the presence of a conserved C-terminal domain termed the plant secondary product glycosyltransferase (PSPG) box (Yonekura-Sakakibara and Hanada, 2011). The PSPG motif is involved in binding to the UDP moiety of the sugar donor and thus plays an important role in anthocyanin biosynthesis (Caputi et al., 2012). To date, genes encoding UFGTs have been identified in a range of plant species, and they can be divided into two groups based on their specificity for sugar acceptors. The first group catalyze the glycosylation of anthocyanidins, including anthocyanidin 3-*O*-glycosyltransferase (F3GT; Fedoroff et al., 1984; Ford et al., 1998; Kubo et al., 2004; Tohge et al., 2005) and anthocyanidin 5,3-*O*-glucosyltransferase (Ogata et al., 2005). The second group are responsible for the further glycosylation of anthocyanins, including anthocyanidin 3-*O*-glucoside 6''-*O*-rhamnosyltransferase (F3GGT; Kroon et al., 1994), anthocyanidin 3-*O*-glucoside 2''-*O*-glucosyltransferase (F3GGT; Morita et al., 2005), anthocyanidin 3-*O*-glucoside 2''-*O*-glucuronosyltransferase (Sawada et al., 2005), and anthocyanidin 3-*O*-glucoside 2''-*O*-xylosyltransferase (F3GGT; Montefiori et al., 2011; Yonekura-Sakakibara et al., 2012). Among these UFGT genes, F3GTs can also catalyze the glycosylation of flavonols in some plant species, such as *Arabidopsis* (Tohge et al., 2005), *petunia* (Yamazaki et al., 2002), and grape (*Vitis vinifera*; Ford et al., 1998).

Methylation is known to affect both the stability and water solubility of anthocyanins; thus, it plays an important role in the accumulation of anthocyanins in plants (Sarni et al., 1995). Anthocyanin methylation is catalyzed by anthocyanin *O*-methyltransferase (AOMT), and AOMTs are responsible for the *O*-methylation of anthocyanins at either the 3' or 5' position (Wiering and deVlaming 1977). To date, two AOMT genes have been cloned and characterized in grapevine (Hugueney et al., 2009; Lückner et al., 2010; Fournier-Level et al., 2011).

Anthocyanin accumulation has a great impact on fruit quality, and many genes involved in the regulation of anthocyanin biosynthesis have been identified in fruit species such as grape (Kobayashi et al., 2004), apple (*Malus domestica*; Takos et al., 2006; Espley et al., 2007; Li et al., 2012), and pear (*Pyrus communis*; Feng et al., 2010). However, few studies have been reported on characterization of the UFGT and AOMT gene families in fruit trees. Functional characterization of UFGT and AOMT genes has so far only been reported in grape (Ford et al., 1998) and kiwifruit (Montefiori et al., 2011). Many questions related to the mechanism underlying the glycosylation and methylation of anthocyanins and the evolution of the UFGT and AOMT gene families in plants still remain unanswered.

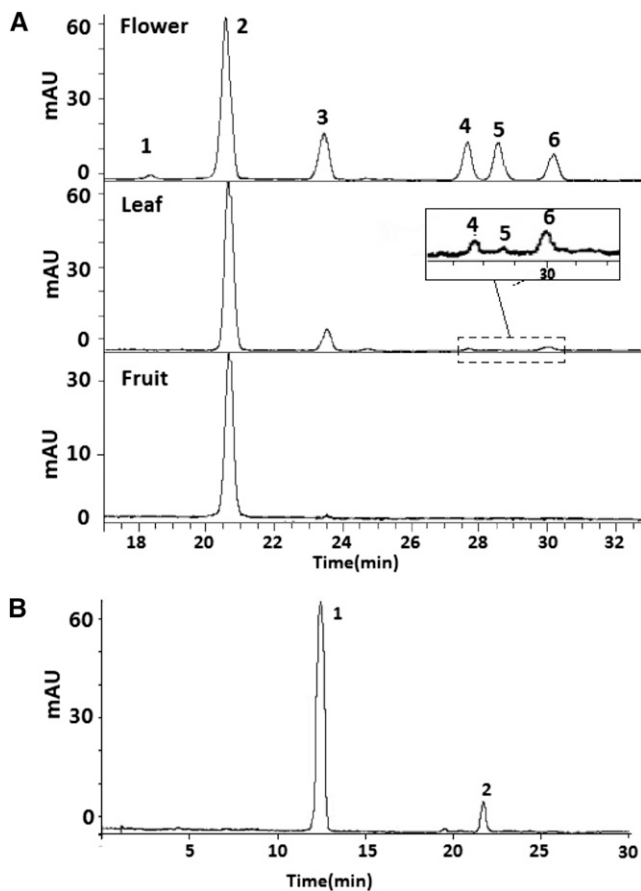
Peach (*Prunus persica*), a member of the Rosaceae family, is the third most important of deciduous fruit trees worldwide. Peach is a diploid with a small genome size of approximately 230 Mb (Arús et al., 2012). To date, there are few reports on genes involved in the regulation of anthocyanin accumulation in peach. Recently, we sequenced peach transcriptomes using the Illumina RNA sequencing (RNA-Seq) strategy, and the results provide an opportunity to investigate genes involved in anthocyanin accumulation in peach. Here, we report on the mechanisms underlying the methylation and glycosylation of anthocyanins in peach. Our study is not only helpful for understanding the molecular mechanism of anthocyanin accumulation in fruit trees but also useful for future research to manipulate flavonoid biosynthesis in peach as well as in other plants.

## RESULTS

### The Anthocyanin Component in Red-Colored Tissues of Peach and Its Wild Relatives

Peach cultivars Mantianhong (MT), Hongyetao (HY), and Wuhong9 (WH9) produce red-colored flowers, leaves, and fruits, respectively, and these red tissues were thus selected and subjected to an analysis of anthocyanin composition. As a result, a total of six peaks were identified in these three tissues (Fig. 1A). The flowers of cv MT contained all six peaks. The leaves of cv HY consisted of two major peaks, 2 and 3, and three traceable peaks, 4 to 6. The fruits of cv WH9 contained a major peak, 2, and a traceable peak, 3. Peaks 1 and 2 had the same mass spectrometry (MS) and tandem mass spectrometry (MS/MS) spectra, and peak 2 had the same retention time with the standard of cyanidin 3-glucoside (Cy-3-glu). Thus, peaks 1 and 2 were identified to be cyanidin 3-galactoside (Cy-3-gal) and Cy-3-glu, respectively (Table I). Based on the MS and MS/MS spectra, peaks 3 to 6 were deemed cyanidin 3-rutinoside (Cy-3-rut), peonidin 3-glucoside (Pn-3-glu), cyanidin 3-rhamnoside (Cy-3-rha), and peonidin 3-rutinoside (Pn-3-rut), respectively. Among these anthocyanins, Cy-3-rha was detected for the first time in Rosaceae, while Cy-3-gal, Pn-3-glu, and Pn-3-rut were identified for the first time in peach. To validate the anthocyanins identified in this study, anthocyanins in flowers of cv MT were further hydrolyzed to identify anthocyanidin composition using HPLC-electrospray ionization (ESI)-MS. Two peaks showing the same retention times with the standards of cyanidin and peonidin were detected (Fig. 1B). This is consistent with the results of HPLC-MS/MS analysis as mentioned above.

To determine if there are other anthocyanins in peach, the identification of anthocyanin composition in flowers was further performed for six more cultivars and three wild species. As a result, only the six anthocyanins mentioned above were detected (Table II). Overall, all the cultivars showed a similarity in anthocyanin composition. The major component of anthocyanins in flowers of all the tested cultivars was Cy-3-glu, with an average content of



**Figure 1.** HPLC scans of anthocyanin pigments in red-colored flower, leaf, and flesh from peach cv MT, cv HY, and cv WH9, respectively (A) and the component of anthocyanidins derived from red flower of cv MT (B). Individual anthocyanins corresponding to peaks 1 to 6 are listed in Table I. mAU, Milliabsorbance units.

52.17%. The remaining components, including Cy-3-gal, Cy-3-rut, Pn-3-glu, Cy-3-rha, and Pn-3-rut, showed 1.5%, 12.43%, 12.97%, 10.93%, and 10.42% of average contents, respectively. Interestingly, Cy-3-glu also represents the major anthocyanin in flowers of *Prunus ferganensis* and accounts for approximately 61% of the total anthocyanins. In addition, four and two anthocyanins were detected in flowers of *Prunus davidiana* and *Prunus kansuensis*, respectively. The most abundant anthocyanin in flowers of *P. davidiana* is Pn-3-glu (40.92%), followed by Pn-3-rut (35.19%), Cy-3-glu (15.92%), and Cy-3-rut (7.96%). Two anthocyanins, Cy-3-rut and Cy-3-glu, account for 56.14% and 43.86% of the total anthocyanins in flowers of *P. kansuensis*, respectively. In summary, *P. davidiana* and *P. kansuensis* accumulate mainly two peonidin-based and two cyanidin-based anthocyanins in flowers, respectively, whereas the common major anthocyanin Cy-3-glu is synthesized in flowers of peach and *P. ferganensis*.

In summary, there are six anthocyanins in peach, and their structural diversity is attributed to glycosylation and/or methylation.

## Identification of Genes Encoding UDP-Glycosyltransferase and O-Methyltransferase in the Peach Transcriptome

A total of 21.5 Gb of Illumina RNA-Seq reads, representing approximately 96 times the peach genome, were generated from different tissues, including red and green leaves, white- and red-fleshed fruits, and flowers (Wang et al., 2013). The RNA-Seq reads were assembled, and the consensus sequences were screened for the presence of the PSPG box, a characteristic of family 1 glycosyltransferases (UGTs). As a result, 79 genes encoding UGT were identified in the peach transcriptome (Supplemental Table S1). These peach UGT genes were named following the previously reported nomenclature system (Mackenzie et al., 1997), and they can be phylogenetically divided into eight orthologous groups according to the definition as described by Yonekura-Sakakibara and Hanada (2011). Of the eight orthologous groups, OG8 and OG23 consist of *F3GT* and *F3GGT* genes and are involved in the glycosylation of anthocyanins and anthocyanidins, respectively (Supplemental Fig. S1). Therefore, three *F3GT* genes, designated *PpUGT78A1*, *PpUGT78A2*, and *PpUGT78B*, and five *F3GGT* genes, designated *PpUGT79A*, *PpUGT79B*, *PpUGT94A2*, *PpUGT94A3*, and *PpUGT94A5*, were selected and subjected to expression profiling analysis.

Similarly, 22 genes encoding O-methyltransferase (OMT) were identified in the peach transcriptome (Fig. 2). Phylogenetic analysis indicated that two OMT genes, designated *PpAOMT1* and *PpAOMT2*, are closely related to *VvAOMT*, which is responsible for the methylation of anthocyanins in grapevine (Huguency et al., 2009). In addition, an OMT gene (*ppa010674m*) also shows a close relationship with *VvAOMT*. However, RNA-Seq and reverse transcription-PCR analysis demonstrated that *ppa010674m* was highly expressed in fruits that accumulate only nonmethylated anthocyanins but was not detectable in flowers (Supplemental Fig. S2), and it was not included in further analysis.

## Identification of Gene Families Encoding F3G, F3GGT, and AOMT in the Peach Genome

First, we screened the genome of peach cv Lovell (Verde et al., 2013), and two more UGT78 homologs designated *PpUGT78A3* and *PpUGT78A4* were identified. Of

**Table I.** Identification of individual anthocyanins in peach

Peaks indicated with letters a and b are identified in Rosaceae and peach species, to our knowledge, for the first time, respectively. *m/z*, Mass-to-charge ratio.

Peak	Retention Time	MS	MS/MS	Anthocyanin
	<i>min</i>	<i>m/z</i>		
1 b	18.41	449	449/287	Cy-3-gal
2	20.58	449	449/287	Cy-3-glu
3	23.44	595	595/449/287	Cy-3-rut
4 b	27.66	463	463/301	Pn-3-glu
5 a	28.56	433	433/287	Cy-3-rha
6 b	30.16	609	609/463/301	Pn-3-rut

**Table II.** Comparison of anthocyanin components in flowers between peach and its wild relatives

The relative content of each anthocyanin is calculated based on the value of the peach area. The major components are highlighted in boldface. ND, Not detectable.

Species	Accession	Percentage					
		Cy-3-gal	Cy-3-glu	Cy-3-rut	Pn-3-glu	Cy-3-rha	Pn-3-rut
Peach	Juhuatao	ND	<b>50.00</b>	14.00	17.00	8.00	11.00
	Fenhuashouxintao	ND	<b>48.54</b>	9.22	19.42	6.31	16.50
	Hongcuizhi	2.13	<b>53.19</b>	9.57	13.30	13.30	8.51
	Honghuashouxintao	0.51	<b>51.28</b>	15.90	11.28	8.21	12.82
	MT	1.56	<b>52.08</b>	15.10	11.46	11.46	8.33
	Hongbaihuatao	1.51	<b>50.25</b>	10.05	13.57	13.07	11.56
	HY	1.80	<b>59.88</b>	13.17	4.79	16.17	4.19
<i>P. ferganensis</i>	Xinjiangtao	ND	<b>60.98</b>	18.29	8.54	5.49	6.71
<i>P. davidiana</i>	Shantao	ND	15.92	7.96	<b>40.92</b>	ND	<b>35.19</b>
<i>P. kansuensis</i>	Gansutao	ND	<b>43.86</b>	<b>56.14</b>	ND	ND	ND

the five *PpUGT78* genes, four (*PpUGT78A1–PpUGT78A4*) are clustered in a 56-kb region on chromosome 1, and one *PpUGT78B* is located on chromosome 2 (Fig. 3A). All the *PpUGT78* genes except *PpUGT78A3* have intact coding frames. *PpUGT78A3* is a pseudogene due to a frameshift mutation induced by a 5-bp insertion in the coding region. The five *PpUGT78* genes share 56% to 86% identities among their DNA coding sequences (CDSs; Fig. 3B). Of the four *PpUGT78A* genes, *PpUGT78A1*, *PpUGT78A2*, and *PpUGT78A3* share 79% to 86% CDS identity, with approximately 66% CDS identity to *PpUGT78A4*. *PpUGT78B* shows the lowest CDS identity (56%–60%) with the four *PpUGT78A* genes. These results suggest that the *PpUGT78A* cluster is likely derived from two rounds of tandem gene duplication, while the ancestor of the cluster and *PpUGT78B* are probably derived following a genome duplication event during the speciation process of peach (Fig. 3C).

All four genes, *PpUGT78A1*, *PpUGT78A2*, *PpUGT78A4*, and *PpUGT78B*, contain a conserved 44-amino acid residue motif known as the PSPG box (Fig. 3D). The *PpUGT78A1* and *PpUGT78A2* proteins are similar, with equal length and 80% identity in amino acid sequences. The *PpUGT78A1* and *PpUGT78A2* proteins both show higher identities in amino acid sequences with *PpUGT78A4* (53%) than with *PpUGT78B* (47%). The *PpUGT78B* and *PpUGT78A4* proteins show a high degree of divergence and share the lowest identity (43%) in amino acid sequences.

Second, five copies of *PpUGT94A* genes are clustered in a 79-kb region on chromosome 2 (Fig. 3A). All five *PpUGT94A* genes show an average of 86% CDS identity, and *PpUGT94A1* and *PpUGT94A2* share the highest degree of CDS identity (97%).

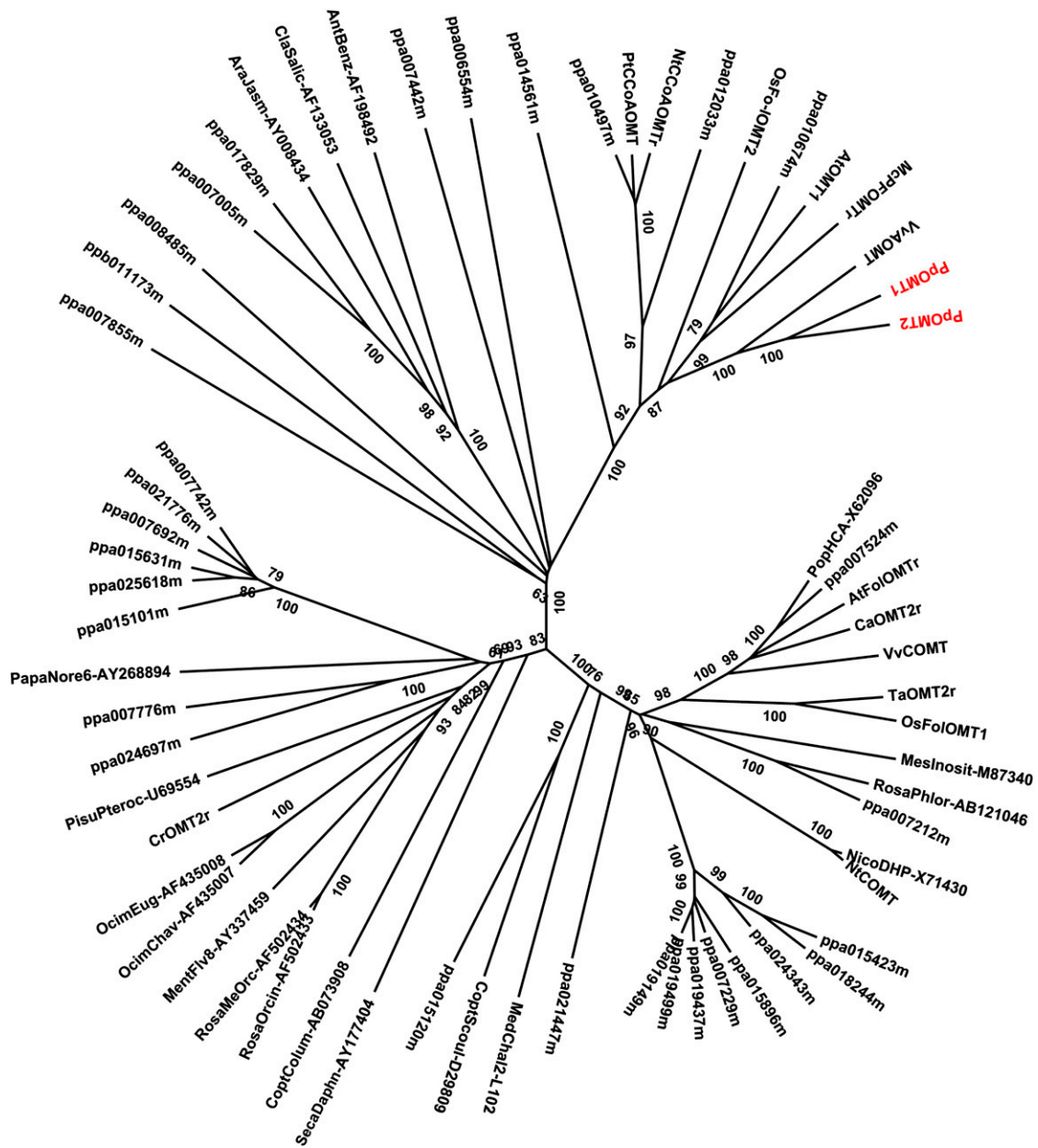
Finally, both *UGT79* and *AOMT* gene families contain two copies in the peach genome. *PpUGT79A* and *PpUGT79B* are both located on chromosome 4 and share 58% CDS identity. *PpAOMT1* and *PpAOMT2* are located on chromosomes 1 and 2, respectively, and share 79% CDS identity.

### Expression Profiling of *F3GT*, *F3GGT*, and *AOMT* Genes in Peach

Gene expression levels were initially estimated using fragments per kilo bases per million reads values (Supplemental Fig. S2). Overall, *F3GGT* homologous genes showed very low levels of expression in all tested tissues, including red- and white-fleshed fruits, red flowers, and green and red leaves. In contrast, three *F3GT* homologous genes showed high levels of expression in red tissues. Two *AOMT* homologous genes were highly expressed in flowers at early stages of development but not expressed in fruit tissues. These results were subsequently validated using real-time PCR analysis (Fig. 4).

*PpUGT78A1* was expressed in fruits, leaves, and flowers. The expression levels of *PpUGT78A1* increased during flower development and reached a maximum at full-bloom stage. Interestingly, *PpUGT78A1* was expressed in both red and green leaves, and its level of expression in green leaves was higher than in red leaves. However, the transcripts of *PpUGT78A1* were not detectable in red-fleshed fruits. *PpUGT78A2* was predominantly expressed in flowers, and its level of expression in red flowers was significantly higher than in pale pink flowers. *PpUGT78A2* transcripts were weakly accumulated in young red leaves but undetectable in green leaves and fruits. In contrast, *PpUGT78B* was mainly expressed in leaves and fruits, and its expression level in flowers was extremely low. *PpUGT78B* transcripts were significantly higher in red tissues than in nonred tissues. For example, the expression level of *PpUGT78B* in red-fleshed fruits of cv WH9 was 18.5-fold higher than that in white-fleshed fruits of cv Baifeng (BF). *PpUGT78B* transcripts were expressed at a 25.5-fold higher level in red leaves of cv HY than in green leaves of *P. davidiana*. In addition, *PpUGT78A4* was not expressed in all analyzed tissues. In short, *PpUGT78A1* was expressed in both green and red tissues, whereas *PpUGT78A2* and *PpUGT78B* were predominantly expressed in red tissues.

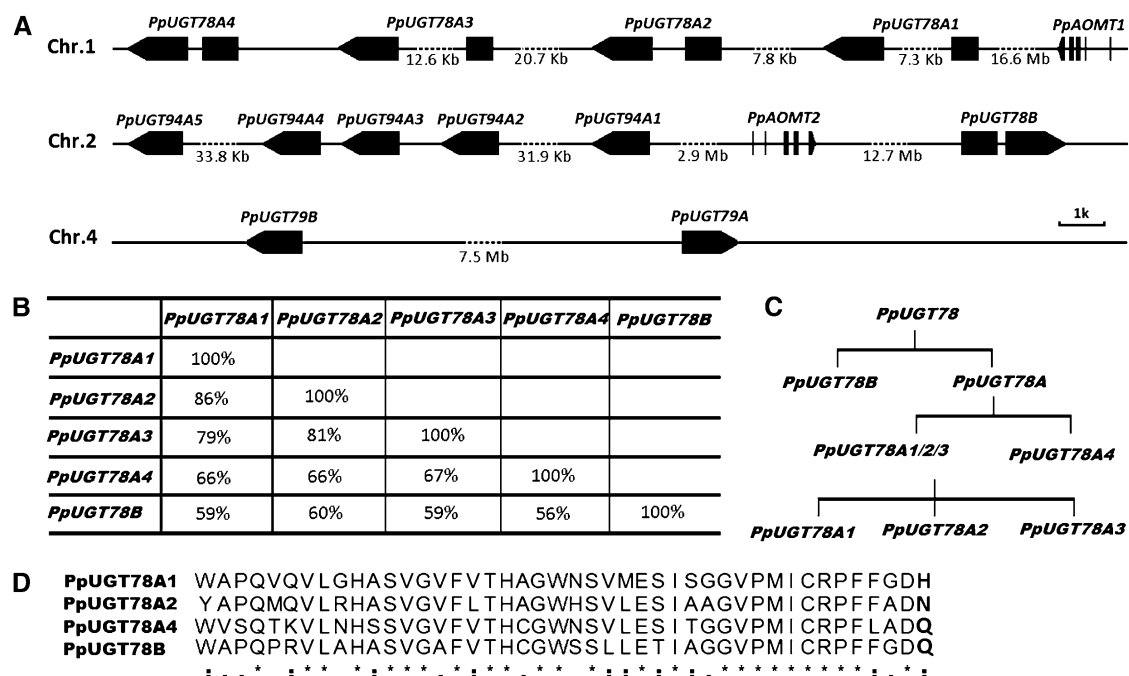
*PpUGT79A* was exclusively expressed in flowers of *P. davidiana*. *PpUGT79B* transcripts were expressed in



**Figure 2.** A phylogenetic tree of *PpOMT* genes in plants. Genes involved in anthocyanin methylation in peach are highlighted in red. The GenBank accession numbers are as follows: grapevine anthocyanin OMT (VvAOMT, FJ460168), grapevine caffeic acid OMT (VvCOMT, AF239740), tobacco caffeic acid OMT (NtCOMT, AF484252), *Triticum aestivum* OMT (TaOMT2r, DQ223971), *Catharanthus roseus* flavonoid OMT (CrOMT2r, AY127568), *Chrysosplenium americanum* OMT (CaOMT2r, U16793), Arabidopsis flavonol OMT (AtFolOMT, U70424), rice flavonol OMT (OsFolOMT1, DQ288259), grapevine caffeoyl-CoA-OMT (VvCCoAOMT, Z54233), tobacco caffeoyl-CoA-OMT (NtCCoAOMT, U38612), *Populus trichocarpa* caffeoyl-CoA-OMT (PpCCoAOMT, AJ224896), rice flavonol OMT (OsFo-IOMT2, XM\_483167), *Mesembryanthemum crystallinum* OMT (McPFOMT, AY145521), and Arabidopsis OMT (AtOMT1, AY087244). [See online article for color version of this figure.]

leaves and flowers, with high expression in nonred tissues, but were not detectable in fruits. For the *PpUGT94A* gene family, all three members were expressed in red-fleshed fruits, although both Cy-3-rut and Pn-3-rut were almost undetectable in red-fleshed fruits. Moreover, these *PpUGT94A* genes were highly

expressed in flowers at full-bloom stage, but their expression levels were extremely low or undetectable in flower buds at pink and bloom stages. These results strongly suggest that the *PpUGT94A* gene family is unlikely to be related to the glycosylation of anthocyanins in peach.



**Figure 3.** Genes involved in glycosylation and methylation in peach. A, Structural features and chromosomal positions of *PpUGT78*, *PpUGT79*, *PpUGT94*, and *PpAOMT* genes. Black boxes represent exons. B, DNA sequence identity for the coding regions of the *PpUGT78* genes. C, Duplication pattern of *PpUGT78* genes in the peach genome. D, Alignment of the conserved PSPG motif sequences of the *PpUGT78* genes. The amino acid residues at position 44 responsible for the sugar donor recognition are highlighted in boldface. Asterisks indicate that the residues in that column are identical; colons and periods indicate that conserved and semiconserved substitutions have been observed, respectively.

The two *PpAOMT* genes were highly expressed in flowers but weakly or not expressed in leaves and fruits. The expression levels of *PpAOMT1* in flowers had a maximum at pink stage and then decreased during flower development. The expression level of *PpAOMT2* in flowers increased during flower development and reached a peak at bloom stage, then decreased at full-bloom stage. These results suggest that methylation of anthocyanidin would occur during flower development but prior to the full-bloom stage.

#### Functional Analysis of *PpAOMT* and *PpUGT78* Genes

Enzyme activity in vitro was initially tested to determine the functionality of the *PpAOMT* genes. The recombinant proteins of both *PpAOMT1* and *PpAOMT2* were strongly induced in *Escherichia coli*, with an expected molecular size of approximately 29 kD (Fig. 5A). The recombinant protein was purified on an Ni<sup>2+</sup>-charged resin that binds the protein’s N-terminal His tag, and the activity was then assayed using Cy-3-glu as a substrate. No product was detected for the reaction of *PpAOMT1* with Cy-3-glu (Fig. 5B). In contrast, the product of the reaction of *PpAOMT2* with Cy-3-glu showed the same MS and MS/MS spectral properties as Pn-3-glu (Fig. 5B). This result shows that, in vitro, *PpAOMT2* works well on Cy-3-glu, leading to the generation of the methylated anthocyanin Pn-3-glu.

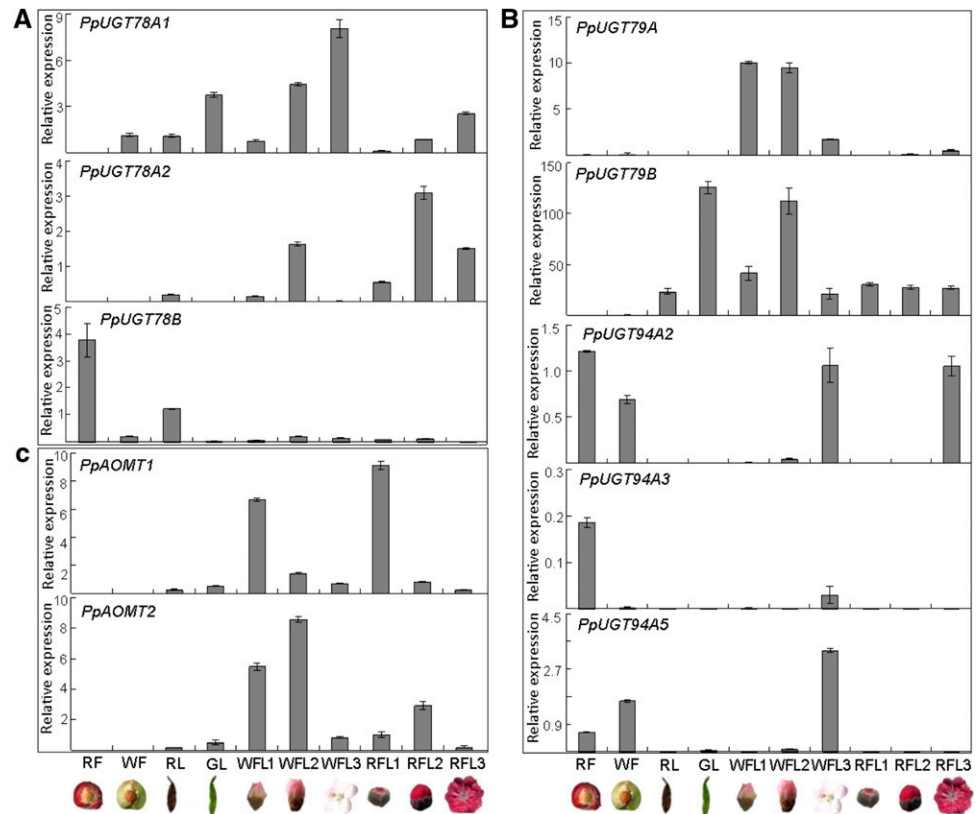
The functionality of *PpAOMT2* was further validated by transient expression in tobacco (*Nicotiana tabacum*). Two other discrete anthocyanins were detected in tobacco leaves coexpressing *NtAN2* and *PpAOMT2* genes (Fig. 5C). HPLC-MS/MS analysis indicated that two discrete anthocyanins were malvidin 3-glucoside and malvidin 3-rutinoside. This demonstrates that *PpAOMT2* is functional in vivo.

The functionality of *PpUGT78* genes was also tested in vitro using cyanidin as acceptor and UDP-Glc or UDP-Gal as sugar donor (Fig. 6). *PpUGT78A1* showed a very low activity toward cyanidin with either UDP-Glc or UDP-Gal as sugar donor. In contrast, both *PpUGT78A2* and *PpUGT78B* had activity toward cyanidin, with a higher level of glucosyl transfer activity than galactosyl transfer activity.

#### Phylogenetic Relationship between the Peach *UGT78* Genes and Their Homologs in Other Plants

The *UGT78* homologs were identified in sequenced genomes of plants, including monocots such as rice (*Oryza sativa*), maize, millet (*Pennisetum americanum*), and sorghum (*Sorghum bicolor*), and eudicots such as grapevine, poplar (*Populus spp.*), castor bean (*Ricinus communis*), cassava (*Manihot esculenta*), *Medicago truncatula*, soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), apple, strawberry (*Fragaria spp.*), cucumber

**Figure 4.** Expression levels of *UGT78A* and *UGT78B* in different tissues of peach. The tissue samples are listed as follows: RF, fruits of red-fleshed cv WH9 at ripening stage; WF, fruits of white-fleshed cv BF at ripening stage; RL, young red leaves of cv HY; GL, young green leaves of cv BF; WFL1, flower buds of cv Shantao at pink stage; WFL2, flower buds of cv Shantao at balloon stage; WFL3, flowers of cv Shantao at full-bloom stage (open flowers); RFL1, flower buds of cv MT at pink stage; RFL2, flower buds of cv MT at balloon stage; RFL3, flowers of cv MT at full-bloom stage. [See online article for color version of this figure.]



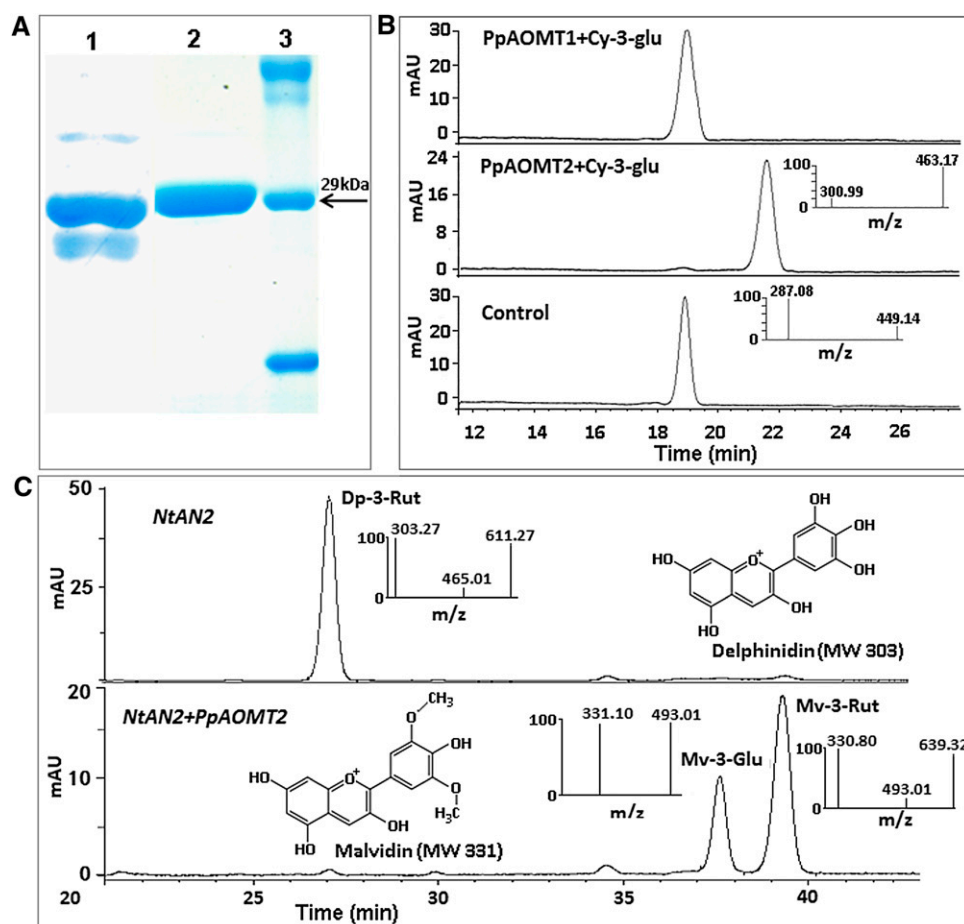
(*Cucumis sativus*), orange (*Citrus* spp.), papaya (*Carica papaya*), *Eucalyptus globulus*, *Arabidopsis*, *Thellungiella halophila*, *Capsella rubella*, *Brassica rapa*, tomato (*Solanum lycopersicum*), and potato (*Solanum tuberosum*). Phylogenetic analysis indicates that *UGT78* genes in plants are grouped into monocot and eudicot clades (Fig. 7). All *UGT78* genes identified in eudicots can be divided into two groups, denoted *UGT78I* and *UGT78II*, and the *UGT78I* group is composed of two subgroups, designated *UGT78Ia* and *UGT78Ib*. The three peach genes *PpUGT78A1*, *PpUGT78A2*, and *PpUGT78A3* belong to the *UGT78Ia* subgroup, while the peach gene *PpUGT78B* belongs to the *UGT78Ib* group. The *UGT78Ia* subgroup consists of characterized *UGT78* genes showing activity toward flavonols, including *VvF3GT1a-1* and *VvF3GT1a-2* in grapevine (Ono et al., 2010), *MtF3GT1a-1* in *M. truncatula* (Modolo et al., 2007), and *RhF3GT1a-3* in rose (*Rosa* spp.; Fukuchi-Mizutani et al., 2011). However, *PtF3GT1a-2*, which does not accept any flavonoids as a substrate, is also grouped into the *UGT78Ia* subgroup (Veljanovski and Constabel, 2013). The *UGT78Ib* group contains characterized *UGT78* genes showing activity toward anthocyanins and flavonols, including *VvF3GT1b* in grapevine (Ford et al., 1998), *AtF3GT1b-4* in *Arabidopsis* (Kubo et al., 2007), *RhF3T1b* in rose (Fukuchi-Mizutani et al., 2011), and *FaF3GT1b* in strawberry (Griesser et al., 2008), but two *Arabidopsis* genes, *AtF3GT1b-1* and *AtF3GT1b-3*, within the same subgroup accept only flavonols (Jones et al., 2003; Yonekura-Sakakibara et al., 2008).

Moreover, all the *UGT78II* genes except *GmF3GT2-2* lack introns, while both *UGT78Ia* and *UGT78Ib* genes have one intron. The *GmF3GT2-2* gene contains a single intron. The *UGT78I* genes are present in all analyzed eudicot plants, while the *UGT78II* genes are identified only in five eudicot plant species: poplar, castor bean, cassava, common bean, and soybean (Table III). To investigate whether specific amino acid residues within the PSPG motif could serve as sequence logos to differentiate one *UGT78* gene group from another in eudicots, logos for the conserved 44-amino acid residue motif of each *UGT78* gene group were analyzed using the online WebLogo program (<http://weblogo.berkeley.edu>). Interestingly, the amino acid residue at position 22 is very highly conserved in both *UGT78Ia* and *UGT78Ib* subgroups, whereas it is subjected to variation to Ala (A), Ser (S), or Cys (C) in the *UGT78II* gene group (Fig. 8). Thus, it seems that the residue Trp (W) at position 22 is a sequence logo for the *UGT78I* gene group.

## DISCUSSION

### The Complexity of Glycosylation and Methylation Leads to the Diversification of Anthocyanins in Peach

F3GTs catalyze the glycosylation of anthocyanidins. In this study, three F3GT genes, *PpUGT78A1*, *PpUGT78A2*, and *PpUGT78B*, are identified in the peach transcriptome. Of these genes, *PpUGT78B* is expressed in red-fleshed fruits, where only one anthocyanin Cy-3-glu



**Figure 5.** Functional analysis of the *PpAOMT* genes. A, Expression of *PpAOMT1* and *PpAOMT2* in *E. coli*. Lane 1, purified recombinant *PpAOMT1* enzyme; lane 2, purified recombinant *PpAOMT2* enzyme; lane 3, molecular mass marker proteins. B, HPLC-ESI-MS/MS analysis of the products catalyzed by the *PpAOMT* enzymes. C, Transient expression of *PpAOMT2* in *N. benthamiana*. mAU, Milliabsorbance units. [See online article for color version of this figure.]

accumulates predominantly. *PpUGT78B* contains the residue Gln (Q) at position 44 of the PSPG box, suggesting that it has glucosyl transfer activity (Kubo et al., 2004). This deduced functionality also has been validated in vitro. Thus, there is no doubt that *PpUGT78B* is involved in the synthesis of Cy-3-glu. *PpUGT78A1* is closely related to the rose *RhF3GT1a-1*, which can use both flavonols and anthocyanins as substrates, but with extremely low activity toward anthocyanins (Fukuchi-Mizutani et al., 2011). We also found that *PpUGT78A1* has very low activity toward cyanidin. Moreover, *PpUGT78A1* contains the residue His (H) at position 44 of the PSPG box, which is required for recognition of the sugar donor UDP-Gal (Kubo et al., 2004). Therefore, the low activity of *PpUGT78A1* for anthocyanidins is likely responsible for the traceable amount of Cy-3-gal in peach flowers.

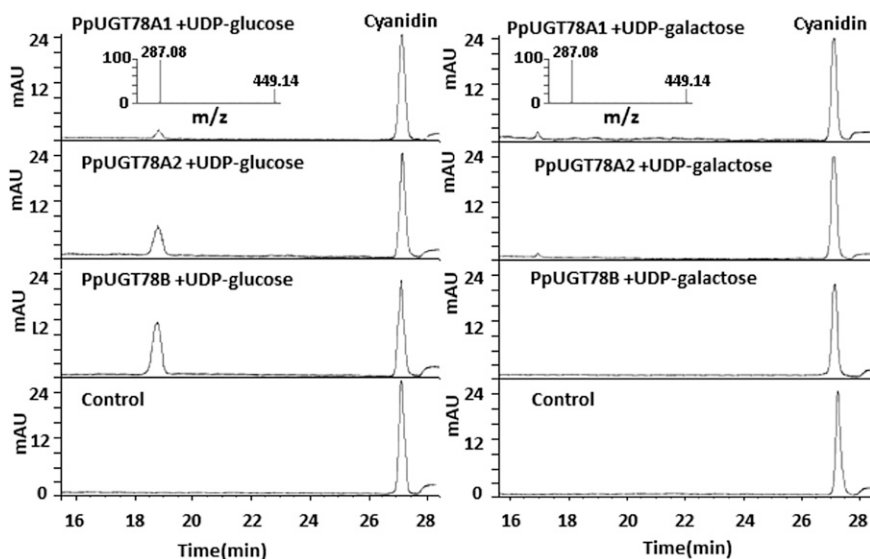
In flowers, both *PpUGT78A1* and *PpUGT78A2* are expressed, but the transcripts of *PpUGT78B* are almost undetectable. As mentioned above, *PpUGT78A1* has very low activity toward cyanidin, while *PpUGT78A2* has high activity toward cyanidin with UDP-Glc as sugar. Therefore, *PpUGT78A2* is responsible for the accumulation of Cy-3-glu in flowers. However, more studies are

still needed to address the mechanism of functional divergence between the two clustered *UGT78* genes *PpUGT78A1* and *PpUGT78A2*. In addition, *PpUGT78A2* contains the residue Asn (N) at position 44 of the PSPG box, whose activity toward the recognition of the sugar donor has not been reported. It is unclear if *PpUGT78A2* also can recognize the UDP-Rha donor, leading to the accumulation of Cy-3-rha in flowers of peach.

*PpUGT79A* and *PpUGT79B* are identified in the peach transcriptome, and they are homologs of the kiwifruit and petunia *F3GGTs* responsible for the glycosylation of anthocyanidin 3-*O*-glycosides (Morita et al., 2005; Montefiori et al., 2011). *PpUGT79B* is not expressed in fruits, but its transcripts are abundant in leaves and flowers. Both flowers and leaves accumulate Cy-3-rut and Pn-3-rut, but they are almost undetectable in fruits. Therefore, there is no doubt that *PpUGT79B* is involved in the accumulation of Cy-3-rut and Pn-3-rut in peach. Moreover, transcripts of *PpUGT79A* are also found in the flowers of *P. davidana*, but its expression is not in accordance with the accumulation of Cy-3-rut and Pn-3-rut in red-colored tissues (Supplemental Fig. S3A). In contrast, the expression of *PpUGT79B* is well in accordance with the accumulation of Cy-3-rut and Pn-3-rut.



**Figure 6.** Functional assay of the PpUGT78 enzyme activity toward cyanidin with UDP-Glc or UDP-Gal as sugar donor. mAU, Milli-absorbance units.



Taken together, these results suggest that *PpUGT79B* is involved in the biosynthesis of Cy-3-rut and Pn-3-rut in peach. It is worth noting that functional redundancy is often observed for secondary metabolite genes in plants (Hanada et al., 2011). We cannot exclude the possibility that *PpUGT79A* also can catalyze the glycosylation of anthocyanins in *P. davidana*.

Two genes in the peach genome, *PpAOMT1* and *PpAOMT2*, are closely related to *VvAOMT*, which has been shown to participate in the methylation of anthocyanins in grapevine (Hugueney et al., 2009). A significant correlation is observed between the expression level of *PpAOMT2* and the accumulation of peonidin-based anthocyanins in red-colored tissues, but not for *PpAOMT1* (Supplemental Fig. S3B). Moreover, *PpAOMT2* has been shown to be functional in vitro and in vivo. These results strongly suggest that *PpAOMT2* is responsible for anthocyanin methylation in peach. Besides *PpAOMT2*, *PpAOMT1* also is expressed in flowers and leaves. However, the functionality of *PpAOMT1* remains unclear, as it shows no activity toward cyanidin in vitro.

Taking all the results above, we propose a model underlying the structural diversification of anthocyanins in peach (Fig. 9).

#### Differential Expression Pattern of Genes Involved in Anthocyanin Modification in Plants

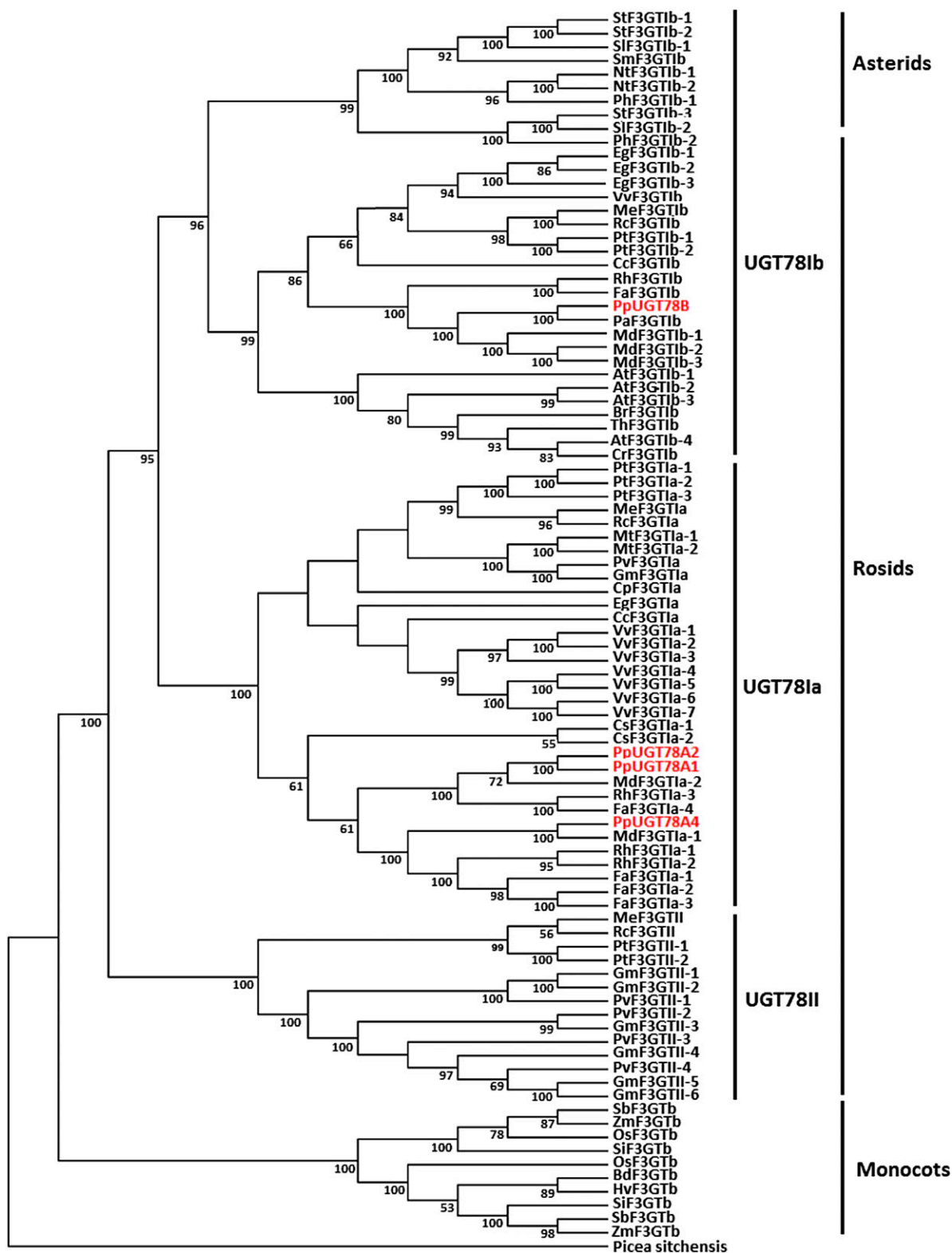
Tissue-specific expression profiles of regulatory genes involved in anthocyanin biosynthesis have been reported in previous studies. For example, four R2R3 MYB transcription factors (TFs), *Anthocyanin2* (*AN2*), *AN4*, *PURPLE HAZE* (*PHZ*), and *DEEP PURPLE* (*DPL*), have been isolated in petunia. *AN2* and *AN4* are responsible for the coloration of floral tissues, including petal limb, flower tube, and anther (Quattrocchio et al., 1999),

while *PHZ* and *DPL* mainly control anthocyanin accumulation in vegetative tissues such as leaves and stems (Albert et al., 2011). Similarly, two basic helix-loop-helix genes, *B-I* and *B-Peru*, confer anthocyanin pigmentation on vegetative tissues and seeds in maize, respectively (Selinger and Chandler, 1999). However, there are few reports of tissue-specific expression profiles of structural genes involved in anthocyanin modification.

Here, we characterize the expression profiles of gene families encoding F3GT, F3GGT, and AOMT in peach. Like the R2R3 MYB TFs in petunia and maize, two F3GT genes, *PpUGT78A2* and *PpUGT78B*, also show tissue-specific expression. *PpUGT78A2* is predominantly expressed in flowers, while *PpUGT78B* is mainly expressed in leaves and fruits. Similarly, two F3GGT genes, *PpUGT79A* and *PpUGT79B*, are not expressed in fruits but are expressed in leaves and/or flowers. Two AOMT genes, *PpAOMT1* and *PpAOMT2*, are mainly expressed in flowers, but with no or low expression in fruits and leaves. Spatiotemporal control of the expression of genes involved in the methylation and glycosylation of anthocyanins results in the differences of anthocyanin composition in flower, leaf, and fruit tissues of peach. Moreover, *UFGT* is a key gene in anthocyanin biosynthesis, and its expression is induced by MYB TFs (Kobayashi et al., 2002). To date, seven R2R3 MYB TFs have been identified in the peach genome (Rahim et al., 2014; Uematsu et al., 2014). It is worth studying whether the expression of *PpUGT78* genes is induced by specific MYB TFs, leading to the spatial and temporal patterns of anthocyanin synthesis in peach.

#### The *UGT78II* Genes Represent a Novel Branch of the *UGT78* Gene Family in Plants

In this study, we have comprehensively investigated the evolutionary development of *UGT78* genes in 20



**Figure 7.** Phylogenetic tree derived from amino acid sequences of UGT78 genes in both monocots and eudicots. The GenBank accession numbers of previously reported *F3GT* genes are as follows: petunia PhF3GT1b-2 (Q9SBQ8) and PhF3GT1b-1 (BAA89008); potato StF3GT1b-1 (AAX63403) and StF3GT1b-2 (AEN83502); tobacco NtF3GT1b-1 (BAM37965) and NtF3GT1b-2 (BAM37964); *Solanum melongena* SmF3GT1b (Q43641); *Rosa × hybrida* RhF3GT1b (BAF80946), RhF3GT1a-1 (BAF80947), RhF3GT1a-2 (BAK09602), and RhF3GT1a-3 (BAE72453); and *Hordeum vulgare* HvF3GTa (P14726). The genome sequences of

**Table III.** Copy numbers of intact *UGT78* homologs identified in the sequenced genomes of eudicots

Eudicots		Species	<i>UGT78la</i>	<i>UGT78lb</i>	<i>UGT78II</i>		
Rosids	Vitales	Vitaceae	Grapevine	7	1	0	
		Eurosids I	Salicaceae	Poplar	3	2	2
	Euphorbiaceae		Castor bean	1	1	1	
			Cassava	1	1	1	
			Fabaceae	Soybean	1	0	3
	Common bean			1	0	4	
	<i>M. truncatula</i>			2	0	0	
	Cucurbitaceae		Cucumber	2	0	0	
	Rosaceae		Apple	2	3	0	
			Peach	3	1	0	
			Strawberry	4	1	0	
	Eurosids II		Rutaceae	Orange	1	1	0
			Myrtaceae	<i>E. globulus</i>	1	3	0
			Caricaceae	Papaya	1	0	0
		Brassicaceae	Arabidopsis	0	4	0	
			<i>C. rubella</i>	0	1	0	
			<i>B. rapa</i>	0	1	0	
	Asterids	Solanaceae	<i>T. halophila</i>	0	1	0	
			Tomato	0	2	0	
Potato			0	3	0		

sequenced plant genomes. In eudicots, *UGT78* genes can be divided into two groups, *UGT78I* and *UGT78II*. It is well known that the Arabidopsis *UGT* intron 2 is highly conserved among the *UGT78* gene family (Li et al., 2001; Paquette et al., 2003). Interestingly, a single intron equivalent to the Arabidopsis *UGT* intron 2 has been detected in the *UGT78I* group but is nearly absent in the *UGT78II* group. Only one gene, *GmF3GT2-2*, in the *UGT78II* group contains intron 2. Moreover, the PSPG motif of the *UGT78I* group contains a highly conserved amino acid residue, Trp (W), at position 22, while residue 22 has great variation in the *UGT78II* group. In monocots, the *UGT78* genes, like the eudicot *UGT78I* genes, also contain intron 2 and the conserved amino acid residue Trp at position 22. This suggests that the *UGT78I* genes in eudicots and the *UGT78* genes in monocots are derived from a common ancestor. However, the *UGT78II* genes can be well distinguished from other *UGT78* genes in plants based on the combination of intron 2 and the amino acid residue at position 22. Thus, the *UGT78II* genes represent a novel branch of the *UGT78* gene family in plants.

As mentioned above, four of the five characterized *UGT78Ia* genes show activity toward flavonols (Modolo et al., 2007; Ono et al., 2010; Fukuchi-Mizutani et al., 2011), while four of the six characterized *UGT78Ib* genes accept both anthocyanins and flavonols (Ford et al., 1998; Kubo et al., 2007; Griesser et al., 2008; Fukuchi-Mizutani et al., 2011). To date, two *UGT78II* genes, *GmF3GT2-6* in soybean and *PtF3GTII-2* in poplar, have

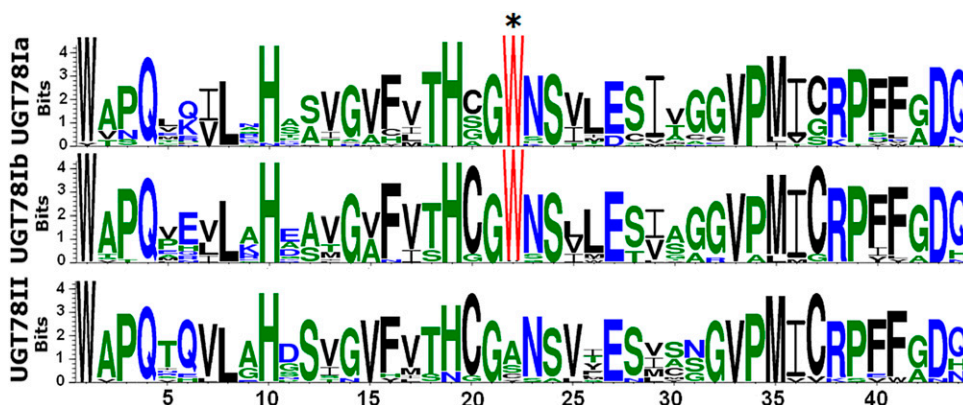
been characterized to function as a 3-*O*-glycosyltransferase in anthocyanin and flavonol biosynthesis (Kovinich et al., 2010; Veljanovski and Constabel, 2013). This indicates that the *UGT78II* genes are more closely related to the *UGT78Ib* genes than to the *UGT78Ia* genes. Thus, it is reasonable to speculate that the common ancestor of *UGT78I* and *UGT78II* groups is likely to have activity toward anthocyanins and flavonols. The *UGT78II* group retains the ancestral function, while the *UGT78I* group may have undergone functional divergence, dividing genes into the two subgroups *UGT78Ia* and *UGT78Ib*. In addition, the *UGT78II* genes are only found in five of 10 plant species in eurosids I and are absent in the genomes of 10 plant species in eurosids II, vitales, and asterids. It is unclear if the *UGT78II* genes tend to be lost in eudicots.

#### The Difference of Anthocyanin Composition between Peach and Its Wild Relatives and Its Implications for Inferring Peach Domestication History

In this study, six anthocyanins are identified in flowers of peach and its wild relatives. Interestingly, peach and *P. ferganensis* show a similar anthocyanin composition in flowers. However, peach is different from both *P. davidiana* and *P. kansuensis* in terms of anthocyanin composition in flowers. These results clearly demonstrate that peach is closely related to *P. ferganensis*, native to Xinjiang Province of northwestern China and the Fergana and Zeravshan valleys in central Asia (Scorza and Okie,

#### Figure 7. (Continued.)

grapevine, poplar, castor bean, cassava, *M. truncatula*, soybean, common bean, cucumber, papaya, *E. globulus*, Arabidopsis, *T. halophila*, *C. rubella*, *B. rapa*, tomato, potato, rice, maize, sorghum, citrus, apple, and strawberry were searched for *UGT78* homologs through the Phytozome version 5.0 database (<http://www.phytozome.net>). The peach *UGT78* genes are highlighted in light gray. [See online article for color version of this figure.]



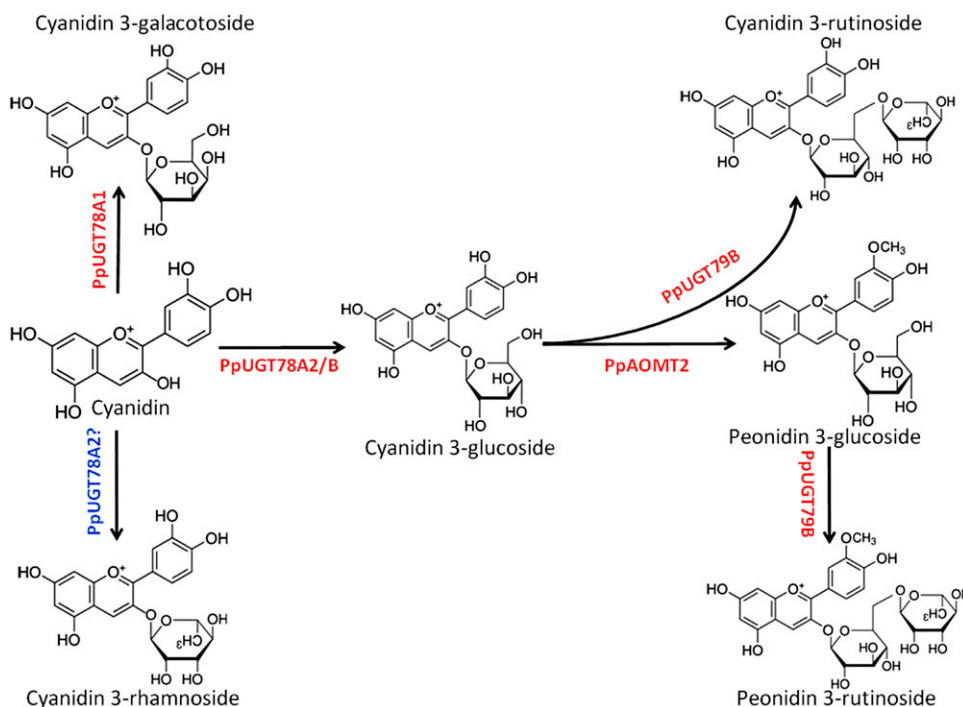
**Figure 8.** WebLogo of the plant-specific PSPG motif constructed from the *UGT78* genes encoding UDP-Glc:flavonoid 3-O-glycosyltransferase in eudicots. Letter size is proportional to the degree of amino acid conservation. The star indicates a sequence logo that differentiates the *UGT78II* genes from both *UGT78Ia* and *UGT78Ib* genes. [See online article for color version of this figure.]

1990). Peach was first domesticated in the region of northwestern China between the Tarim basin and the north slopes of the Kunlun Shan Mountains (Faust and Timon, 1995). Thus, it seems likely that peach is domesticated from *P. ferganensis*. This finding is supported by the latest study of peach genome sequencing, which indicates that *P. ferganensis* represents an intermediate genome in peach domestication (Verde et al., 2013). In addition, all seven of the tested peach cultivars share a similar pattern of anthocyanin composition in flowers, which is consistent with the fact that peach cultivars have a very narrow genetic base (Scorza et al., 1985).

The peach blossom has always been a sacred symbol in Chinese culture. Peach and *P. ferganensis* produce red or purple flowers, while both *P. davidiana* and *P. kansuensis* have pink or pale pink flowers. In other words, the flowers of peach and *P. ferganensis* are more attractive than those of *P. davidiana* and *P. kansuensis*. If

peach is indeed domesticated from *P. ferganensis*, then the question arises of the purpose of the original domestication of peach. Both *P. davidiana* and *P. kansuensis* bear edible fruits, and *P. kansuensis* is also native to Xinjiang Province of northwestern China. The original cultivation of peach might not have been very important as a source of fruits but could be important as a source of valuable ornamental plants due to the beauty of flowers. Moreover, our study shows that the flowers of peach and *P. ferganensis* are richer in anthocyanin components than those of *P. davidiana* and *P. kansuensis*. Thus, the complexity of anthocyanin composition is likely related to the diversity of flower color in peach and its wild relatives.

Peach flowers are a common ingredient used in traditional Chinese medicine. Here, we show that peach flowers accumulate a variety of anthocyanins, whereas the fruits and leaves predominantly accumulate a single



**Figure 9.** A proposed model of the glycosylation and methylation of anthocyanins in peach. [See online article for color version of this figure.]

anthocyanin, Cy-3-glu. Several studies have demonstrated that both peonidin-based and cyanidin-based anthocyanins can inhibit cancer cell growth (Chen et al., 2005; Ho et al., 2010). Therefore, peach flowers are a more suitable ingredient to use in medicine than leaves and fruits in terms of the anthocyanin benefits.

## CONCLUSION

Methylation and glycosylation contribute to the diversification of anthocyanins in peach. Peach and *P. ferganensis* share a similar anthocyanin composition in flowers, suggesting that they are closely related to each other. Four *UGT* genes, *PpUGT78A1*, *PpUGT78A2*, *PpUGT78B*, and *PpUGT79B*, are likely responsible for the glycosylation of anthocyanins, while one gene, *PpAOMT2*, is involved in the methylation of anthocyanins in peach. The spatiotemporal control of expression of these anthocyanin modification genes is responsible for the different patterns of anthocyanin accumulation in flower, leaf, and fruit tissues. In addition, our study reveals the divergent evolution of both *UGT78* and *AOMT* genes in peach.

## MATERIALS AND METHODS

### Plant Materials

Peach (*Prunus persica*) accessions used in this study consisted of seven ornamental cultivars, Juhuaotao, Fenuashouxintao, Hongcuizhi, Honghuashouxintao, MT, Hongbaihuatao, and HY; two other cultivars, red-fleshed WH9 and white-fleshed BF; and three wild relatives, *Prunus davidiana*, *Prunus ferganensis*, and *Prunus kansuensis*. All the peach accessions are maintained at the Wuhan Botanical Garden of the Chinese Academy of Sciences in Hubei Province. Leaves were collected during juvenile stage in spring. Fruits were harvested at mature stage. Flowers were collected at pink, balloon, and blossom stages. All samples were immediately frozen in liquid nitrogen and then stored at  $-75^{\circ}\text{C}$  until use.

### Extraction and HPLC-ESI-MS/MS Analysis of Anthocyanins

The anthocyanins were extracted according to a previously reported method (Huang et al., 2009). Briefly, an approximately 0.5-g sample was ground in liquid nitrogen and then added to 25 mL of extraction solution (80:20 [v/v] methanol:water mixture containing 1.18 mM HCl). The mixture was incubated on a steam bath in the dark at  $30^{\circ}\text{C}$  with gentle shaking for 16 h, sonicated for 15 min, and centrifuged at  $10,000g$  for 10 min. An aliquot of 10 mL of supernatant was collected and evaporated under vacuum at  $30^{\circ}\text{C}$  using a rotary evaporator. The residual was resuspended in acidified water (1.18 mM HCl) and added to a CNWBOND LC-C18 SPE column that was preconditioned with 5 mL of methanol followed by 5 mL of acidified water. The SPE column was then washed three times with acidified water.

Anthocyanins were eluted with 1 mL of methanol, filtered through 0.22- $\mu\text{m}$  Millipore membranes, and analyzed using an HPLC-ESI-MS/MS system (ThermoFisher Scientific). The analytical column was Zorbax Extend C18,  $4.6 \times 250$  mm, with a particle size of 5  $\mu\text{m}$  (Agilent Technologies). The analytical column was sequentially eluted using mobile phase A (formic acid:water, 5:95, v/v) and mobile phase B (methanol) with a flow rate of  $0.8 \text{ mL min}^{-1}$ . The linear gradient of phase B was as follows: 0 to 2 min, 5%; 2 to 7 min, 5% to 15%; 7 to 20 min, 15% to 20%; 20 to 25 min, 20% to 27%; 25 to 32 min, 27%; 32 to 41 min, 27% to 35%; and 41.01 to 43 min, 5%. A UV-visible light detector wavelength was set at 520 nm. Mass spectra were acquired in positive ion mode. Ion was scanned from 200 to 1,000 mass-to-charge ratio at a speed of  $1,000$  atomic mass units  $\text{s}^{-1}$ . The flow rate of nebulizing gas was  $1.5 \text{ L min}^{-1}$ , and the drying gas ( $\text{N}_2$ ) pressure was 0.1 MPa. The components of anthocyanins were determined according to the

retention time of standards and  $M_r$  and subsequently quantified according to cyanidin equivalents.

## Hydrolyzation of Anthocyanins

Anthocyanins were hydrolyzed into anthocyanidins under acidic and high-temperature conditions according to a previously reported procedure (Pinho et al., 2011). Approximately 1 g of ground sample was mixed with 10 mL of 6 M HCl and 40 mL of methanol. The mixture was flushed with nitrogen and incubated in a water bath at  $100^{\circ}\text{C}$  for 2 h. After acid hydrolysis, the mixture was cooled, mixed with 50 mL of methanol, sonicated for 15 min, and centrifuged at  $10,000g$  for 10 min. The supernatant was purified using the SPE column as mentioned above. The components of anthocyanidins were analyzed using the HPLC-ESI-MS system. The mobile phase was composed of 3:57:40 (v/v/v) formic acid:water:methanol solution. The column flow rate was  $0.8 \text{ mL min}^{-1}$ . The sample running time was 35 min. Anthocyanidins were recalled according to MS data, retention time, and  $M_r$  of internal standards.

## Expression of the Peach *AOMT* and *UGT78* Genes in *Escherichia coli*

The full complementary DNA (cDNA) sequences of *PpAOMT1* and *PpAOMT2* and were amplified using two pairs of primers (the XbaI and XhoI sites at the 5' end of the forward and reverse primers, respectively, are indicated in bold), 5'-GGGAATCCATATGATGGCAGAGAAGCAACAACA-3' / 5'-AGCCGCTCGAGCCACATTATTTGAAGACAACAGC-3' and 5'-GGG-AATCCATATGTTGGCTCTCTGTTCAACATGG-3' / 5'-AGCCGCTCGAGC-TAGTATCCTAATAGAGACGCCTGC-3', respectively, and cloned individually into pET-28b(+) vector (Novagen). Clone authenticity was confirmed by sequencing. The constructed vector was then transferred into *E. coli* BL21 (DE3) cells (Transgen). The transformed cells were cultured at  $37^{\circ}\text{C}$  in Luria-Bertani medium containing  $25 \mu\text{g mL}^{-1}$  kanamycin to an optical density at 600 nm of 0.4 to 0.6. The culture was adjusted to  $18^{\circ}\text{C}$ , and recombinant *AOMT* expression was induced by the addition of isopropylthio- $\beta$ -galactoside to a final concentration of 5 mM. The culture was incubated for 20 h at  $18^{\circ}\text{C}$  (250 rpm) prior to harvesting the cells. Then, recombinant *AOMT* was purified using His $\bullet$ Bind Resin (Novagen). The activity of purified recombinant *AOMT* (5  $\mu\text{g}$ ) was measured in a final volume of 200  $\mu\text{L}$  containing 200  $\mu\text{M}$  S-adenosyl-L-methionine, 200  $\mu\text{M}$  anthocyanin substrate, 0.1 M HEPES, pH 7.5, 10 mM  $\text{MgCl}_2$ , and 14 mM 2-mercaptoethanol. The reaction was incubated for 30 min at  $35^{\circ}\text{C}$  and stopped with 400  $\mu\text{L}$  of methanol containing 200 mM HCl. All reactions were filtered through 0.22- $\mu\text{m}$  Millipore membranes and analyzed by HPLC-ESI-MS/MS. Assays were performed in triplicate, and experiments were repeated three times. Likewise, the full coding sequences of three genes, *PpUGT78A1*, *PpUGT78A2*, and *PpUGT78B*, were cloned into pET-28b(+) vector using three pairs of primers, 5'-GGGGATCCATGGTAGGAAACCCAAACCC-3' / 5'-GCCTCGAGCGTTTTGGACCTAGATCTAAGAAGC-3', 5'-GGGAATTCATGG-CAGGAAACCCCAAACA-3' / 5'-AGCTCGAGCCCTTTTCACTGCCATTCA-3', and 5'-CCGAATTCATGGCACCACAACCGATT-3' / 5'-GCCTCGAGGCCG-GATAAATAGTAGAAGCAAAGC-3', respectively. The activity of recombinant *UGT78* was measured in a final volume of 200  $\mu\text{L}$  containing 5 mM UDP-Glc or UDP-Gal, 250 mM substrate, 0.1 M HEPES, pH 7, 14 mM 2-mercaptoethanol, and raw protein extract with approximately 1  $\mu\text{g}$  of recombinant protein. Enzyme assays were incubated for 30 min at  $30^{\circ}\text{C}$  and stopped by the addition of 250  $\mu\text{L}$  of 0.6 M HCl.

## Transient Expression in Tobacco

For *Agrobacterium tumefaciens*-mediated transient expression, *PpAOMT2* from peach and *NtAN2* from tobacco (*Nicotiana tabacum*) were amplified using two pairs of primers, 5'-GGGAATCCATATGATGGCAGAGAAGCAACAACA-3' / 5'-AGCCGCTCGAGCCACATTATTTGAAGACAACAGC-3' and 5'-CCGCTCGAGATGAATATTTGTACTAATAAAG-3' / 5'-GCTCTAGATC-AACTGAGAAGTGGCATTTC-3', respectively, and inserted individually into the modified binary vector pSAK277. All constructs were introduced into *A. tumefaciens* strain GV3101 by electroporation. *Nicotiana benthamiana* leaves were infiltrated with *A. tumefaciens* cultures (optical density at 600 nm of 0.1–0.3). Discs were punched from *N. benthamiana* leaves 1 week after *A. tumefaciens* infiltration and analyzed for anthocyanin content.

## Gene Expression Analysis Using Real-Time PCR

Total RNA was extracted using the ZP401 kit (Beijing Zoman Biotechnology) according to the manufacturer's instructions and was treated with DNase I (Takara) to remove any contamination of genomic DNAs. Approximately 3  $\mu\text{g}$  of total RNA per sample was then used for cDNA synthesis. A SYBR Green-based real-time PCR assay was carried out in a total volume of 20  $\mu\text{L}$  of reaction mixture containing 10  $\mu\text{L}$  of 2 $\times$  SYBR Green I Master Mix (Takara), 0.2  $\mu\text{M}$  of each primer, and 100 ng of template cDNA. An actin gene encoding glyceraldehyde-3-phosphate dehydrogenase, *PpGAPDH*, in peach was used as a constitutive control.

Amplifications were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). The amplification program consisted of one cycle of 95°C for 3 min followed by 40 cycles of 95°C for 30 s and 60°C for 34 s. The fluorescent product was detected at the second step of each cycle. Melting curve analysis was performed at the end of 40 cycles to ensure the proper amplification of target fragments. Fluorescence readings were consecutively collected during the melting process from 60°C to 90°C at the heating rate of 0.5°C s<sup>-1</sup>. All analyses were repeated three times using biological replicates. Primer sequences used for real-time PCR are listed in Supplemental Table S2.

## Sequence Alignment and Phylogenetic Analysis

The amino acid sequences of genes encoding UGT and AOMT from various plants were used for phylogenetic analysis. Sequences were aligned using ClustalX and adjusted manually as necessary. The resulting data were analyzed using equally weighted neighbor joining. Neighbor-joining trees were sought using the heuristic search strategies of MEGA version 5. Bootstrap values (Felsenstein, 1985) were calculated from 1,000 replicate analyses, and only those values compatible with the 50% majority-rule consensus tree were recorded. A *UGT* gene of *Picea sitchensis* (GenBank accession no. ABK24936) was used as an outgroup.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Phylogenetic tree derived from amino acid sequences of genes encoding UGT in the peach transcriptome.

**Supplemental Figure S2.** Estimation of gene expression levels of F3GT, F3GGT, and AOMT homologs using fragments per kilobases per million reads values in peach.

**Supplemental Figure S3.** Correlation between the expression of the anthocyanin modification genes and anthocyanin accumulation in different tissues of peach.

**Supplemental Table S1.** Genes encoding UDP identified in the peach transcriptome.

**Supplemental Table S2.** Primers used for real-time PCR analysis of genes encoding UGT78, UGT79, UGT94, and OMT in peach.

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