Plant Biotechnology 28, 239–244 (2011)
DOI: 10.5511/plantbiotechnology.10.1220a

Gene Note

Biochemical and molecular characterization of anthocyanidin/flavonol 3-glucosylation pathways in *Rosa×hybrida*

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Received November 12, 2010; accepted December 20, 2010 (Edited by M. Nishihara)

Abstract Rose petals contain 3-glucosylated anthocyanidins and flavonols. We isolated three flavonoid 3-glucosyltransferase (UF3GT) homolog genes (*RhUF3GT1*, *RhUF3GT2*, and *RhUF3GT3*) from rose. *RhUF3GT1* encoded an amino acid sequence that is almost identical to the reported rose partial *UF3GT* homologs and highly homologous to strawberry and apple UF3GTs. Recombinant RhUF3GT1 expressed in yeast catalyzes 3-glucosylation of anthocyanidins but not flavonols. *RhUF3GT1* was not expressed in the petals of many cultivars even when anthocyanin biosynthesis was active, while it was expressed in the mature petals of cultivars that synthesize cyanidin 3-glucoside in the mature petals. RhUF3GT2 and RhUF3GT3, sharing 79% identity, exhibit only 42% and 41% identities to RhUF3GT1, respectively, and are distantly related to strawberry and apple UF3GTs. They were expressed in coordination with the *flavonol synthase* gene in the petal. The recombinant RhUF3GT2 expressed in yeast catalyzed 3-glucosylation of flavonol much more efficiently than that of anthocyanidins. We suggest that RhUF3GT2 catalyzes flavonol 3-glucosylation in rose petals and that it also contributes to accumulation of anthocyanidin 3-glucoside in the petals.

Key words: Anthocyanidin, flavonoid, flavonol, glucosyltransferase, *Rosa×hybrida*.

Flavonoids and their class of colored compounds, anthocyanins, are major flower color constituents and are distributed in various organs in seed plants. The pathway leading to anthocyanidin 3-glucosides is well conserved in seed plants and all enzymatic genes involved in the pathway have been isolated and characterized (Tanaka et al. 2008). Anthocyanidins are the first colored compounds in the pathway and are 3-glucosylated by the action of UDP-glucose: flavonoid 3-glucosyltransferase (UF3GT). Anthocyanidin 3-glucosides are usually further modified by glycosylation, acylation, and methylation in a species specific manner (Tanaka et al. 2008) and then transported in vacuoles (Zhao and Dixon 2010). The genes encoding UF3GT have been isolated from many plant species including gentian (Tanaka et al. 1996), petunia (Yamazaki et al. 2002), and iris (Yoshihara et al. 2005). They form a cluster in family 1 glycosyltransferase (Fukuchi-Mizutani et al. 2003), which is one of 91 subfamilies of glycosyltransferases classified on the basis of sequence similarity, catalytic mechanisms, and presence of conserved sequence motifs (for review, see Yonekura-Sakakibara 2009). Recombinant petunia UF3GT was shown to catalyze glucosylation of anthocyanidins as efficiently as that of flavonols (Yamazaki et al. 2002), while recombinant gentian UF3GT catalyzes glucosylation of anthocyanidins more efficiently than that of flavonols (Tanaka et al. 1996).

Rosa×hybrida (cultivated rose) petals uniquely contain anthocyanidin 5,3-glucosyltransferase (A53GT) that catalyzes 5-glucosylation of anthocyanidins and then 3-glucosylation to accumulate anthocyanidin 3,5-diglucoside, the dominant anthocyanin in *Rosa×hybrida* petals (Ogata et al. 2005). On the other hand, many wild rose petals contain cyanidin or peonidin 3-glucoside in addition to 3,5-diglucoside. Some contain 3-sophoroside or 3-rutinoside and *Rosa rugosa* 'Salmon Pink' contains 3-glucoside as the dominant anthocyanin (Mikanagi et al. 2000). Genetic analysis of the biosynthesis of cyanidin 3-glucoside was carried out in *Rosa×hybrida* 'Frensham' and its progenies (Arisumi et al. 1977). *Rosa×hybrida* petals also contain small amount of cyanidin or pelargonidin 3-glucoside (Biolley and Jay 1993). Transgenic *Rosa×hybrida* petals contain

Abbreviations: A53GT, anthocyanidin 5,3-glucosyltransferase; DFR, dihydroflavonol 4-reductase; DIG, digoxigenin; F5GT, flavonoid 5-glucosyltransferase; FLS, flavonol synthase; GAPDH, glyceroaldehyde 3-phosphate dehydrogenase; GT, glucosyltransferase; UF3GT, UDP-glucose: flavonoid 3-glucosyltransferase.
This article can be found at http://www.jspcmb.jp/

delphinidin 3-glucoside as well as delphinidin 3, 5-diglucoside (Katsumoto et al. 2007). The petal color of *Rosa*×*hybrida* 'Charleston' changes from yellow to red due to accumulation of cyanidin 3-glucoside after the flower opens (Hennayake et al. 2006b). These results indicate that rose petals should have a pathway leading to anthocyanidin 3-glucoside in addition to the pathway catalyzed by A53GT.

Culture cells derived from leaves treated with UV-B radiation and media stress produced cyanidin 3-glucoside (Hennayake et al. 2006b). Mature petals of 'Charleston' and 'Ehigasa' accumulate cyanidin 3-glucoside and cyanidin 3,5-diglucoside (Hennayake et al. 2006a). The gene fragments homologous to UFGT were also isolated and their expression was shown to correlate with the accumulation of cyanidin 3-glucoside both in 'Charleston' petals (Hennayake et al. 2007) and culture cells (Hennayake et al. 2006b). However, their full-length sequences and enzymatic function have not been revealed, and temporal regulation of anthocyanin accumulation in 'Charleston' petals is different from that in typical cultivars where anthocyanin biosynthesis is most active and the biosynthetic genes are expressed most strongly at the flower opening stage (Tanaka et al. 1995). Rose flavonols are also 3-glucosylated (Mikanagi et al. 1995) and *Rosa*×*hybrida* 'Madam Violet' petals contain quercetin 3-glucoside and quercetin 3-rutinoside (unpublished results), but the gene or the enzyme involved in the glucosylation has not been studied.

These observations prompted us to clarify 3-glucosylation of anthocyanidins and flavonols in *Rosa*×*hybrida* petals. The clarification may bring us clues to engineer rose anthocyanin biosynthetic pathway and flower color in addition to delphinidin accumulation reported previously (Katsumoto et al. 2007). We isolated three *UF3GT* genes and heterologously expressed two of them. One of them only catalyzed anthocyanidin 3-glucosylation and the other favored flavonols.

The *Rosa*×*hybrida* cultivars used in this study were commercially available and grown in a glasshouse in our institute (Osaka, Japan). The flowers of 'Ehigasa' that accumulate anthocyanins in the petals after flower opening were classified into four stages (I–IV, Figure 1A), and the petals were collected from each stage for anthocyanin analysis and RNA preparation. The flowers of the other cultivars that accumulate anthocyanins at earlier stages than 'Ehigasa' were classified into five developmental stages as previously described [stage 1, closed buds, petals are not pigmented; stage 2, pigmented closed buds; stage 3, sepals are opening and petals are pink; stage 4, petals are opening; stage 5, opened flower (Tanaka et al. 1995)], and the petals of each stage were collected for RNA preparation. Petals of 'Black Baccara', 'Rote Rose', 'Lavande', 'Medeo', and 'Madame Violet' at stage 3, when anthocyanin biosynthesis is most active, were also collected. Total RNA was prepared using RNeasy kit (Qiagen Inc., Valencia, USA). Genomic DNA isolated from young leaves of 'Lavande' using Phytopure (GE Healthcare UK Ltd., Little Chalfont, UK) was used to construct the genomic DNA library with *Xho*I digested λBlueStar (Agilent Technologies, Santa Clara, USA) according to the manufacturer's protocol.

Flavonoid analysis and general molecular procedures have been described previously (Fukuchi-Mizutani et al. 2003; Katsumoto et al. 2007). DNA sequences corresponding to the amino terminal portions of perilla, gentian, snapdragon, and petunia UF3GT were amplified with the pairs of primers P3GTF, 5′-CACATTGGCGTTCTAGCATTC-3′ and P3GTR: 5′-GCCTTCGGGGGTCCCGTCCCA-3′, G3GTF: 5′-CATGTTGCAGTGCTTGCATTT-3′ and G3GTR: 5′-TCCCTCCGGCGAGCCATCCCA-3′, S3GTF: 5′-CACATTGGCGTGCTAGCGTTC-3′ and S3GTR: 5′-GCCCTCTGGGGTGCCATCCCA-3′, and Ph3GTF: 5′-CACATTGCACTTCTTGCTTTC-3′ and Ph3GTR: 5′-TGTTTCAGTGACACCATCCCA-3′, respectively, using the plasmids containing each cDNA as the template. PCR consisted of 25 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplified DNA fragments were digoxigenin (DIG)-labeled (Roche Diagnostics, Mannheim, Germany) using the same set of primers and were then used as molecular probes to isolate a rose UF3GT gene from the genomic library.

A directional cDNA library derived from young colored leaves of *Rosa*×*hybrida*. 'Samba' was constructed using Uni-ZAP XR vector (Agilent Technologies). The constructed library was screened with the DIG-labeled perilla and gentian UF3GT fragments described above. The resultant UF3GT sequence was amplified with RT-PCR using a pair of primers (RhF3GT-FW: 5′-ATGAGCCACAAACTTGCTAGT-3′, RhF3GT-RV: 5′-CTACCAGACTTTCAAGTCTTG-3′) and 'Lavande' petal cDNA as the template.

The UF3GT cDNAs were inserted into a yeast expression vector, pYE22m (Tanaka et al. 1996), where the inserted cDNA was regulated by a constitutive glyceroaldehyde 3-phosphate dehydrogenase (GAPDH) promoter. Crude extract of transformed yeast was prepared as previously described (Tanaka et al. 1996) and subjected to UF3GT assay. The reaction mixture (100 μl) consisted of the substrate solution (final concentration of 200 μM), 50 μl of crude extract, 5 μl of 10 μM UDP-glucose and 10 μl of 1 M potassium phosphate buffer, pH 7.5. The reaction mixture was incubated at 30°C for 15 min for anthocyanidins and 30 min for flavonols. The reaction was terminated by addition of 100 μl of 50% acetonitrile containing 0.1% TFA and 10 μl of TFA, and then subjected to flavonoid analysis.

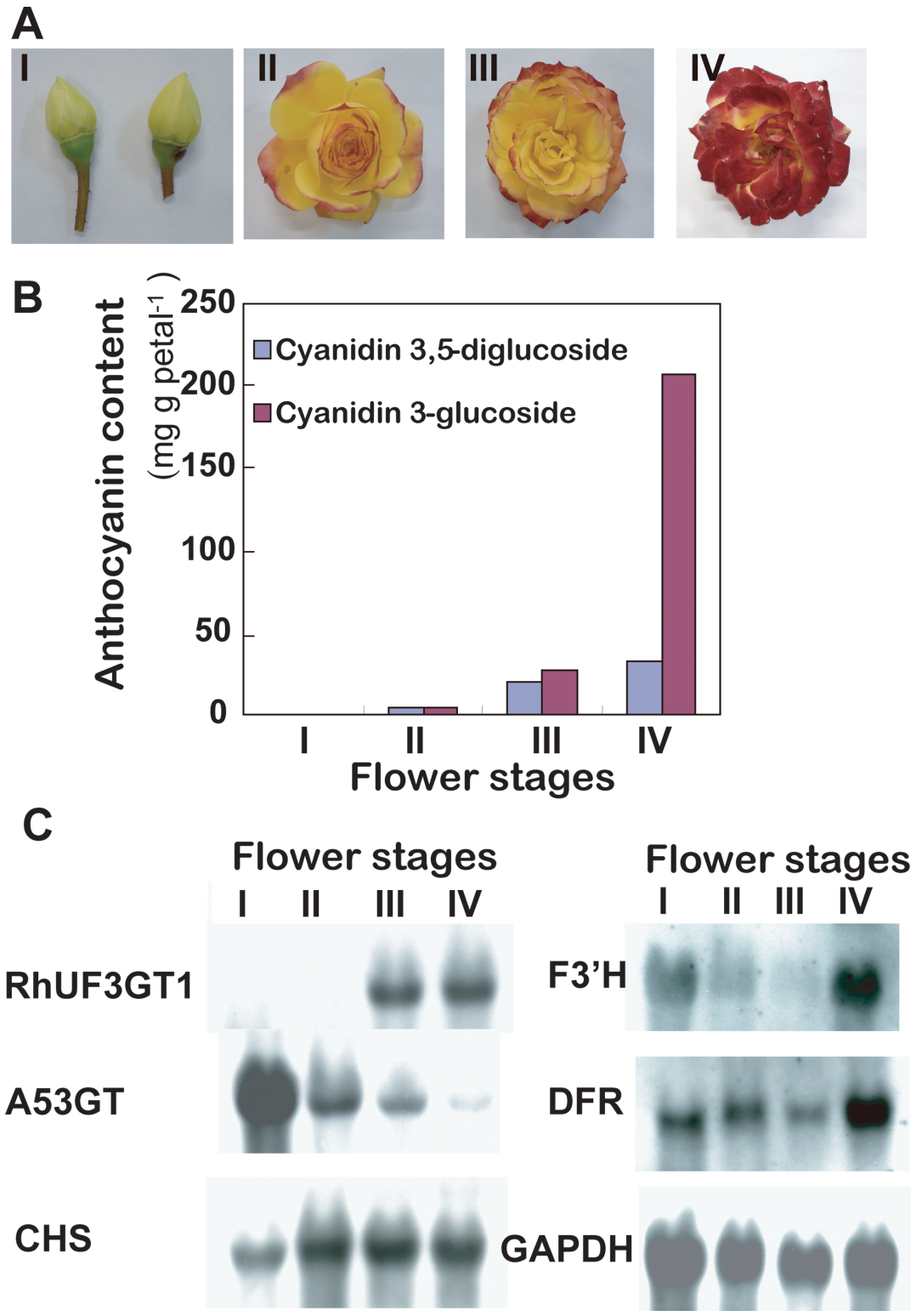

Figure 1. Analysis of anthocyanin synthesis in mature petals of *Rosa×hybrida* 'Ehigasa'. (A) Petal developmental stages (I–IV). (B) Accumulation of anthocyanins at the four petal developmental stages. Cyanidin 3-glucoside is the major anthocyanin. No anthocyanins were detected at stage I. (C) Expression profile of *RhUF3GT1*, *anthocyanidin 5,3-glucosyltrasferase (A53GT), chalcone synthase (CHS), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR),* and *glyceroaldehyde phosphate dehydrogenase (GAPDH)* genes in the petals at stage I to IV.

DIG-labeled *chalcone synthase* (AB038246), *flavonoid 3′-hydroxylase* (AR576387), *dihydroflavonol 4-reductase* (*DFR*, Tanaka et al. 1995), *A53GT* (Ogata et al. 2005), and *RhUF3GT1* (this study), *GAPDH* (AB370120) cDNAs were used as probes for Northern analysis of 'Ehigasa' petals using 10 μg of RNA

obtained from stages I–IV. Ten micrograms of RNA from various cultivars was subjected to Northern analysis to detect rose UF3GT genes.

RT-PCR analysis to detect *DFR*, *flavonol synthase* (*FLS*), *RhUF3GT1*, *A53GT*, rose flavonoid 5GT (*F5GT*) *homolog*, *RhUF3GT2* and *GAPDH* was carried out using RNAs as templates and pairs of primers (DFRcommonF2: 5′-GGCTACIIICTCCGAGCCACCG-TGCGA-3′ (I is inosine) and DFRcommonR2: 5′-CTCIA/GA/CICC/TGTATTTGAACTCGAACCC-3′, RFL-FW: 5′-ATGGGGGTAGAGAGAGTTCAAG-3′ and RFL-RV: 5′-TTACTGGGGGATCTTGTTGAGC-3′, A3GT16-NcoI: 5′-GACTCCATGGCACCAGCATCAA-ATC-3′ and A3GT16-KpnI: 5′-CGTTGAGGTACCTC-TTGAATTTGG-3′, RhA53GT-FW: 5′-ATGGGTGGTG-ATGCTATAGTTTTG-3′ and RhA53GT-RV: 5′- TCAT-TTTTGCTTCCACAGCTGAGC-3′,UF5GT-F1: 5′-TTC-CTACTGGCTTGACTTTTGCTC 3′ and UF5GT-R1: 5′-GAACCTCCACTTGACTACACCACG-3′, RhGAPDH-F: 5′-TGTCATCTCTGCCCCAAGTAAGG-3′ and RhGAPDH-R: 5′-CAACATCCTCATCGGTGTAACCC-3′, the pair of primers for RhUF3GT2 was as described above). PCR consisted of 30 cycles at 95°C for 30 s, at 55°C for 30 s, and at 72°C for 60 s.

Genomic library screening yielded one plasmid containing a DNA sequence encoding an UF3GT homolog. A subcloned plasmid containing an UF3GT-like open reading frame was sequenced (database accession number AB292796). The sequence contained a 496-bp first exon, 187-bp intron, and 908-bp second exon. The open reading frame (RhUF3GT1) showed 99.7% and 97.3% identity to the reported partial UF3GT amino acid sequence (BAF96591) from rose cell culture (Hennayake et al. 2006b) and BAF35998 from ‘Charleston’ petal (Hennayake et al. 2007), respectively. The sequence was also closely related to strawberry (Rosaceae) UF3GT (AAU12366, 87%) and apple (Rosaceae) flavonoid 3-galactosyltransferase (BAI44431, 56%). Their close relationship reflects their taxonomy. A phylogenetic tree consisting of UF3GT from various plants is shown in Figure 2.

The intron in the genomic sequence of *RhUF3GT1* was removed by PCR and the constructed cDNA was expressed in yeast. The recombinant RhUF3GT1 only catalyzed anthocyanidin glucosylation and flavonol glucosylation activity was not detected (Table 1).

Mature petals of the ‘Ehigasa’ mainly accumulated cyanidin 3-glucoside (Figure 1B), as in ‘Charleston’ (Hennayake et al. 2007), while cyanidin 3,5-diglucoside is the dominant anthocyanin in the petals of most rose cultivars. Northern analysis showed that *RhUF3GT1* expression correlated with anthocyanin accumulation (Figure 1C). Together with the specificity of RhUF3GT1 to anthocyanidins, RhUF3GT1 functions as anthocyanidin 3GT *in vivo*, at least in mature petals of

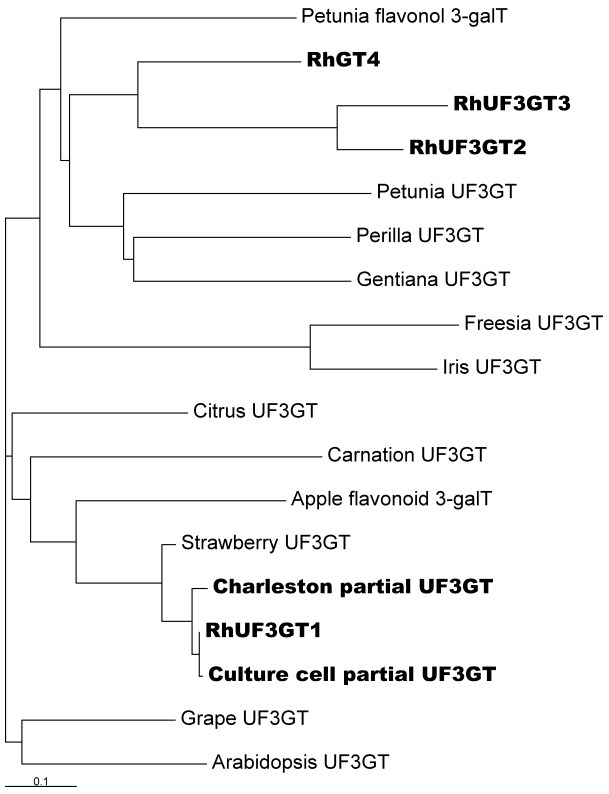

Figure 2. Non-rooted phylogenetic tree containing RhUF3GT1, RhUF3GT2, RhUF3GT3, and various reported UF3GT homolog amino acid sequences. Rose glucosyltransferases are shown in bold letters. The bar indicates 0.1 substitutions/site. The amino acid sequences of these glycosyltransferases are aligned using the CLUSTALW program (available at DDBJ http://clustalw.ddbj.nig.ac.jp/top-e.html) in and the tree is constructed using TREEVIEW (Page 1996). Petunia flavonol 3-galT, *Petunia×hybrida* UDP-galactose: flavonol 3-galactosyltransferase (AAD55985); RhGT4, *Rosa×hybrida* RhGT4 (BAE72453); petunia UF3GT, *Petunia×hybrida* UDP-glucose: flavonoid 3-glucosyltransferase (UF3GT, BAA89008); Perilla UF3GT, *Perilla frutescens* UF3GT (BAA19659); Gentian UF3GT, *Gentiana triflora* UF3GT (BAA12737); Freesia UF3GT, *Freesia hybrida* UF3GT (ADK75021); Iris UF3GT, *Iris hollandica* UF3GT (BAD83701); Citrus UF3GT, *Citrus paradise* UF3GT (ACS15351); Carnation UF3GT, *Dianthus caryophyllus* DicGT1 (BAD52003); Apple flavonoid 3-galT, *Malus×domestica* flavonoid 3-galactosyltransferase (BAI44431); Straweberry, *Fragaria ananassa* UF3GT (AAU12366); Charleston partial UF3GT, rose UF3GT partial sequence from *Rosa×hybrida* ‘Charleston’ (BAF35998, Hennayake et al. 2006a); Culture cell partial UF3GT, UF3GT partial sequence from *Rosa×hybrida* culture cells (BAF96591, Hennayake et al. 2006b); Grape UF3GT, *Vitis vinifera* FIUFGT2 (BAB41026); Arabidopsis UF3GT, *Arabidopsis* thaliana UF3GT (AAM91139).

‘Ehigasa’ and ‘Charleston’. On the other hand, the absence of *RhUF3GT1* mRNA at stage 3 in ‘Black Baccara’, ‘Rote Rose’, ‘Lavande’, ‘Medeo’, and ‘Madame Violet’ shown by Northern and RT-PCR analyses (data not shown) indicates that RhUF3GT1 is unlikely to contribute to cyanidin 3-glucosylation in these cultivars. Northern analysis of ‘Ehigasa’ (Figure 1C) also showed interesting results: Expression of *DFR* and *F3′H* genes decreased at stage III from those at stages I and II and increased at stage IV, in contrast to

Table 1. Relative activities (%) of RhUF3GT1 and RhUF3GT2 expressed in yeast for various flavonoids

	RhUF3GT1	RhUF3GT2
Pelargonidin	100*	0.91
Cyanidin	57	0.60
Delphinidin	89	0.34
Kaempferol	n. d.	42
Quercetin	n. d.	100**
Myricetin	n. d.	43
Cyanidin 3-glucoside	n. d.	n. d.
Dihydroquercetin	n. d.	n. d.
Apigenin	n. d.	n. d.

The activity was measured three times and their averages were shown. Amount of anthocyanidin or flavonol 3-glucosides were quantified. A reaction mixture contained 1.6 mg or 1.8 mg of protein derived from yeast expressing *RhUF3GT1* or *RhUF3GT2*. * 65 pmole min^{-1} mg $protein^{-1}$, ** 27 pmole min^{-1} mg $protein^{-1}$, n. d., not detected.

A53GT, whose expression peaked only at stage I. This is in contrast to the expression profile of the DFR gene in 'Kardinal'; the DFR transcripts are most abundant in stages 3 and 4 and decrease in the petals of open flowers (Tanaka et al. 1995). Regulation of anthocyanin biosynthetic gene in roses varies depending on cultivars. Such variation may originate from different ancestral wild rose species or from long and complex breeding activities.

Screening of the 'Samba' leaf cDNA library with perilla and gentian *UF3GT* genes yielded a UF3GT homolog (RhUF3GT2) (the sequence was deposited in a DNA database as AB292797). To determine whether the *RhUFGT2* gene is expressed in petals, RT-PCR using RhF3GT-FW and RhF3GT-RV as the pair of primers and 'Lavande' petal cDNA as the template was carried out, which resulted in two sequences. One matched completely with RhUF3GT2 and the other (RhUF3GT3) exhibited 79% amino acid sequence identity to RhUF3GT2 (the DNA sequence was deposited in database as accession number AB599928). Phylogenetic analysis shows that RhUF3GT2 (42% identity to RhUF3GT1), RhUF3GT3 (41% identity to RhUF3GT1), and RhGT4 (BAE72453) are distantly related to Rosaceae UF3GT, which indicates their earlier gene duplication from the *RhUF3GT1* gene before speciation of *Rosa* or rapid amino acid residue substitution in RhUF3GT2 and RhUF3GT3.

RhUF3GT2 and *RhUF3GT3* were expressed in yeast. Recombinant RhUF3GT2 showed higher activity for flavonols than for anthocyanidins (Table 1). We were unable detect UF3GT activity in the yeast expressing *RhUF3GT3*. *RhUF3GT2* and *RhUF3GT3* transcripts were detected in stage 3 petals of *Rosa*×*hybrida* cultivars (Figure 3A). RT-PCR analysis showed that *RhUF3GT2* and *RhUF3GT3* expression was well coordinated with the expression of the *FLS* gene in 'Lavande' (Figure 3B). The results indicate that

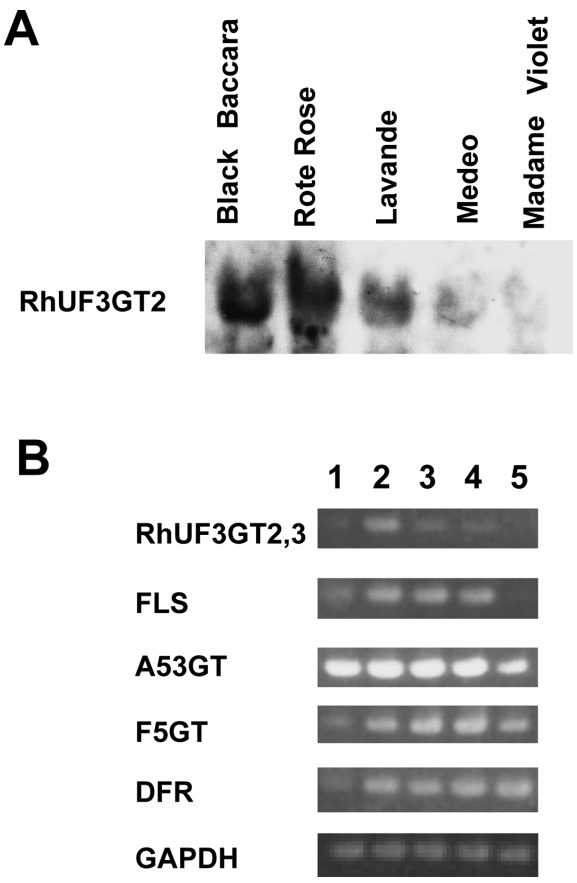

Figure 3. Expression profile of the flavonoid biosynthetic genes in *Rosa*×*hybrida*. (A) Northern analysis of *RhUF3GT2* in the stage 3 petals of some cultivars. Since *RhUF3GT2* and *RhUF3GT3* are homologous, the transcripts of *RhUF3GT3* were possibly detected in this analysis. (B) Developmental profile of *RhUF3GT2/RhUF3GT3*, *flavonol synthase* (*FLS*), *dihydroflavonols 4-reductase* (*DFR*), *anthocyanidin 5, 3-GT* (*A53GT*), *flavonoid 5GT* (*F5GT*) homolog and glyceroaldehyde 3-phosphate dehydrogenase (GAPDH) genes in 'Lavande' obtained by RT-PCR analysis. The primers used for RT-PCR of *RhUF3GT2* also amplified *RhUF3GT3*. *RhUF3GT1* transcripts were not detected (data not shown). Stage 1, closed buds, petals are not pigmented; stage 2, pigmented closed buds; stage 3, sepals are opening and petals are pink; stage 4, petals are opening; stage 5, opened flower.

RhUF3GT2 (and possibly RhUF3GT3 as well) catalyzes flavonol 3-glucosylation *in vivo*. Low activity of RhUF3GT2 for anthocyanidins may result in the small amount of cyanidin 3-glucoside found in the petals of *Rosa*×*hybrida* cultivars. Strong and consistent expression of A53GT confirmed its major role in anthocyanidin glucosylation in rose petals.

Rose F5GT homolog was shown to express in the late stage in 'Charleston' (Hennayake et al. 2007) and 'Lavande' petals (Figure 3B). In pear, cDNA encoding a 5GT-like sequence has been shown to encode flavonoid 7GT (Fischer et al. 2007). Assuming anthocyanidin 5-glucosylation is catalyzed by A53GT (Ogata et al.), the rose F5GT homolog may catalyze flavonoid 7-glucosylation in petals because rose petals accumulate 7-glucosylated flavonols (Mikanagi et al. 1995) and

transgenic 'Lavande' petals expressing torenia *flavone synthase* gene contain 7-glucosylated flavones (unpublished results). It is also possible that the rose F5GT homolog catalyzes 5-glucosylation of cyanidin 3-glucoside at later stages in 'Charleston' (Hennayake et al. 2007) and 'Ehigasa' (Figure 1B), because a significant amount of cyanidin 3,5-diglucoside accumulates in those petals and the expression of A53GT is low (Figure 1C).

It is intriguing to consider how *Rosa*×*hybrida* and wild roses temporarily and spatially regulate multiple UF3GT genes to glucosylate anthocyanidin. It would also be interesting to know when and how *Rosa*×*hybrida* acquired A53GT and multiple rose UF3GT genes. Rose fruits and leaves often contain anthocyanins and as per our knowledge their biosynthesis has not been studied. Further extensive analysis is necessary to obtain a complete picture of flavonoid glucosylation in *Rosa* species.

Acknowledgements

The authors are grateful to Bio-oriented Technology Research Advancement Institution for giving us an opportunity to write this paper.

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