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Original Paper

Flower color modification in *Rosa hybrida* by expressing the *S*-adenosylmethionine: anthocyanin 3′,5′-*O*-methyltransferase gene from *Torenia hybrida*

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Abstract We isolated a cDNA encoding *S*-adenosylmethionine: anthocyanin 3′,5′-*O*-methyltransferase (A3′5′OMT) from a cDNA library derived from *Torenia hybrida* petals that mainly accumulated malvidin type anthocyanins using the petunia A3′OMT cDNA as a probe. The torenia A3′5′OMT shared 52–72% amino acid sequence identity with previously reported AOMTs and belongs to the Group A1 methyltransferase family that also include caffeoyl CoA *O*-methyltransferase. The recombinant A3′5′OMT produced by *Escherichia coli* efficiently catalyzed methylation of the 3-glucoside and 3,5-diglucoside of delphinidin and cyanidin, but it did not catalyze the methylation of anthocyanidins, flavonols, or flavones. The torenia *A3′5′OMT* gene was expressed in *Nierembergia* sp., the petals of which naturally accumulate anthocyanins derived from delphinidin. The resultant transgenic petals produced methylated anthocyanins, based on malvidin and petunidin, in addition to delphinidin, which indicated that the torenia *A3′5′OMT* gene was functional in a heterologous plant. Rose petals rarely contain methylated anthocyanins. Transgenic rose petals expressing both a pansy *flavonoid 3′,5′-hydroxylase* (*F3′5′H*) and the torenia *A3′5′OMT* genes accumulated methylated anthocyanins based upon malvidin, petunidin, and peonidin, which comprised up to 88% of the total anthocyanidins, and their magenta color was more brilliant than that of the petals that accumulated delphinidin type anthocyanins by expressing the *F3′5′H* gene alone. These results indicate that the torenia *A3′5′OMT* gene is a useful molecular tool for altering and diversifying flower color.

Key words: Anthocyanin, anthocyanin methyltransferase, flower color, rose, torenia.

Flower color is mainly attributable to flavonoids, carotenoids, and betalains (Tanaka et al. 2008). Anthocyanins, a colored class of flavonoids, confer a wide range of flower colors such as red, magenta, violet, and blue. Anthocyanin color variations are due to versatile anthocyanin structures, coexisting copigments, metal ions, and the pH in the vacuoles where anthocyanins are localized (Yoshida et al. 2009). The structural diversity of anthocyanins is related to the species-specific glycosylation, acylation, and methylation of anthocyanins. The glycosylation and acylation of anthocyanins are catalyzed by specific glycosyltransferases and acyltransferases, respectively, and the genes encoding these enzymes have been isolated from many plants (Tanaka et al. 2008). However, the methylation of anthocyanins has been less studied in terms of molecular biology.

The methylation of anthocyanins is catalyzed by *S*-adenosylmethionine (SAM): anthocyanin *O*-methyltransferase (AOMT). Peonidin type anthocyanins are biosynthesized from cyanidin-based anthocyanins, and petunidin- and malvidin-type anthocyanins are biosynthesized from delphinidin-based anthocyanins (Figure 1), where methylation results in a slight reddening of the anthocyanins (Harbourne 1958). Many horticultural plants, including petunia, torenia, cyclamen and grape, accumulate anthocyanins derived from malvidin in their flowers or fruit skins. In cyclamen, a mutation in the *AOMT* gene changes the flower color from purple to red–purple (Kondo et al. 2009). Therefore, it should be possible to increase the flower color varieties of horticultural crops by engineering anthocyanin methylation.

Genetic and biochemical analyses of *Petunia hybrida*, a model species used to study flower color and anthocyanin biosynthesis, have suggested that

Abbreviations: AOMT, *S*-adenosylmethionine: anthocyanin *O*-methyltransferase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; F3′5′H, flavonoid 3′,5′-hydroxylase.

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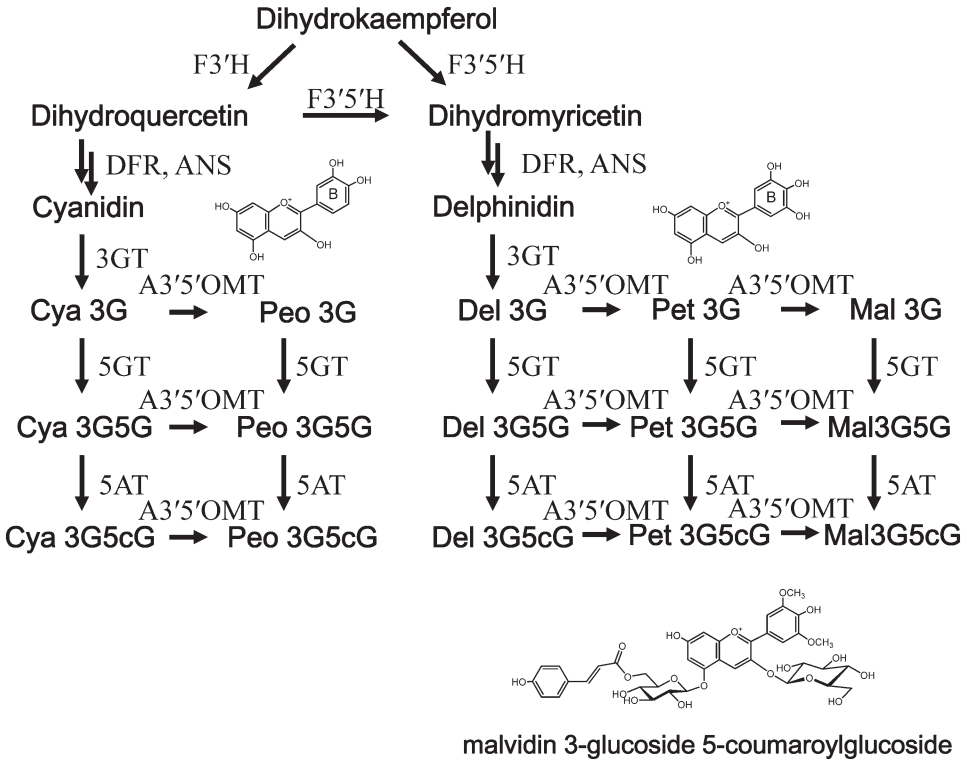

Figure 1. Part of the anthocyanin biosynthetic pathway of *Torenia hybrida*. The most abundant anthocyanin in the torenia petals is included. Cya, cyanidin; Peo, peonidin; Del, delphinidin; Pet, petunidin; Mal, malvidin; G, glucoside; cG, coumaroylglucoside; B, B-ring; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose: anthocyanidin 3-glucosyltransferase; 5GT, UDP-glucose: anthocyanin 5-glucosyltransferase; 5AT, aromatic acyl CoA: anthocyanin 5-acyltransferase; A3′5′OMT, *S*-adenosylmethionine: anthocyanin 3′,5′-*O*-methyltransferase.

petunia has two pairs of *A3′5′OMT* and *A3′OMT* loci: METHYLATION AT THREE (*MT1* and *MT2*) and METHYLATION AT FIVE (*MF1* and *MF2*) (Wiering 1974; Wiering and de Vlaming 1977). Recently, it was shown that the *A3′OMT* gene is encoded by *MT2*, the *A3′5′OMT* genes are encoded by *MF1* and *MF2*, and *MT1* is nonexistent (Provenzano et al. 2014). A grape *A3′5′OMT* gene was isolated and the recombinant grape *A3′5′OMT* in in vitro assays was shown to catalyze the 3′- or 3′,5′-methylation of cyanidin 3-glucoside, delphinidin 3-glucoside, quercetin 3-glucoside, cyanidin, quercetin, and myricetin; however, pelargonidin 3-glucoside, catechin, and epicatechin were not suitable substrates (Hugueney et al. 2009; Lucker et al. 2010). Tobacco leaves that transiently expressed the genes for grape *A3′5′OMT* and an *Arabidopsis* transcriptional factor that upregulates anthocyanin biosynthesis were found to accumulate malvidin 3-rutinoside (Hugueney et al. 2009). The cyclamen *A3′5′OMT* gene (*CkmOMT2*) has been isolated and the recombinant enzyme was shown to catalyze the methylation of delphinidin 3,5-diglucoside, cyanidin 3-glucoside, and quercetin 3-glucoside (Akita et al. 2011). The potato AOMT3 gene was shown to encode A3′5′OMT because *Nicotiana benthamiana* leaves that coexpressed the *AOMT3* gene and a potato transcriptional factor gene that regulates anthocyanin biosynthesis were found to accumulate petunidin 3-rutinoside and malvidin 3-rutinoside (Payyavula et al. 2013). Tomato A3′5′OMT catalyzes the methylation of quercetin, myricetin, quercetin 3-glucoside, quercetin 3-rutinoside, delphinidin 3-glucoside, and cyanidin 3-glucoside, and its strong preference for delphinidin 3-glucoside was demonstrated (Roldan et al. 2014). These AOMTs are closely related to the caffeoyl CoA OMT (CCoOMT) clade (Group A1) in the plant OMT family (Lam et al. 2007; Provenzano et al. 2014) in terms of their amino acid sequences and they are designated as CCoOMT-like proteins, together with *Arabidopsis* spermidine methyltransferase (Fellenberg et al. 2008) and an ice plant OMT, which has a broad substrate specificity (Ibdah et al. 2003).

It is rare for a single species to have a full range of flower colors, mainly because each species produces a limited range of anthocyanins due to its genetic constraints (Tanaka and Brugliera 2013; Tanaka and Brugliera 2014). Roses, carnations, and chrysanthemums account for half of the cut flower market, and they are economically important floricultural crops. Although there has been intensive hybridization breeding to increase flower color varieties, which are based on

anthocyanidins, roses (Mikanagi et al. 2000; Mikanagi et al. 1995) and carnations (Nakayama et al. 2000) can only produce those derived from pelargonidin and cyanidin, while chrysanthemums produce cyanidin based anthocyanins (Nakayama et al. 1997) but not delphinidin, while they rarely accumulate methylated anthocyanins. The expression of the flavonoid 3′,5′-hydroxylase (*F3′5′H*) gene (Figure 1) in these crops successfully generated delphinidin in the petals and novel flowers with blue hues were obtained, which is not possible by hybridization breeding (Tanaka and Brugliera 2013; Tanaka and Brugliera 2014). However, the production of methylated anthocyanins via genetic engineering has not been achieved in these crops. The expression of the *AOMT* gene in addition to the *F3′5′H* gene is expected to increase the varieties of anthocyanins, thereby diversifying the flower colors of these species.

Torenia hybrida is a popular garden plant. Its blue petals mainly contain malvidin 3-glucoside 5-coumaroylglucoside and its flavonoid biosynthetic pathway has been characterized in terms of molecular biology and biotechnology (Nakamura et al. 2010). In this study, we isolated torenia cDNA and characterized the recombinant torenia A3′5′OMT, which was expressed in *Escherichia coli*. Transgenic roses that expressed torenia *A3′5′OMT* and pansy *F3′5′H* genes accumulated high levels of malvidin type anthocyanins. Their flower colors were clearly different from those of the host or transgenic roses that only expressed the *F3′5′H* gene. These results indicate that the torenia *A3′5′OMT* gene is a useful molecular tool for modifying and diversifying flower color.

Materials and methods

Plant materials

Torenia hybrida cv. Summerwave Blue was obtained from Suntoryflowers Ltd. (Japan). *Nierembergia* spp. NB18, a Solanacae floricultural crop, has been described previously (Ueyama et al. 2006). *Rosa hybrida* cv. WKS124 was kindly provided by Keisei Rose Nurseries (Japan).

Molecular procedures and analysis

The torenia petal cDNA library and standard molecular procedures, including gene isolation and plasmid construction, have been described previously (Ueyama et al. 2002). The amino acid sequences of the AOMTs were aligned using CLUSTALW (available at DDBJ http://clustalw.ddbj.nig.ac.jp/top-e.html) and the phylogenetic tree was constructed with TREEVIEW (Page 1996).

Expression of TMT5 with a His-tag sequence in E. coli

The DNA sequence encoding the TMT5 coding region and restriction sites was amplified by PCR using the primers: 5′-GCCATATGAAA GAT AAG TTC TAT GGC A-3′ (the *Nde*I site is underlined) and 5′-ATCTCGAGTTT GAG ACG TTT GCA C-3′ (the *Xho*I site is underlined), using pTMT5 plasmid DNA as template. The amplified DNA was cloned into pET15b (Merck Millipore, Tokyo, Japan) using the *Nde*I and *Xho*I sites. The resultant plasmid encoded the recombinant torenia A3′5′OMT with a His-tag in its amino-terminus. The constructed plasmid was introduced into *E. coli* BL21 (DE3). The transformant was cultured in 2 ml of Lucia–Bertani broth containing 50 μg/ml ampicillin, which supplemented with 2% (v/v) solution 1 (Overnight Express Autoinduction Systems, Merck Millipore), 5% (v/v) solution 2, and 0.1% (v/v) solution 3, with shaking at 37°C for 4 h. The culture was transferred to 300 ml of the same medium and shaken at 27°C for 16 h.

Harvested cells suspended in methyltransferase (MT) sonication buffer (40 mM sodium phosphate, pH 7.5, 2 mM $MgCl_2$, 500 μM EDTA, 50 μM APMSF, 1 μM SAM, 0.1% (v/v) 2-mercaptoethanol) were disrupted by sonication. After centrifugation at 15,000 rpm at 4°C for 15 min, the supernatant (soluble fraction) was applied to a HisTrap HP column (1 ml, GE Healthcare LifeSciences, Hino, Japan), which had been equilibrated with MT sonication buffer containing 20 mM imidazole. The recombinant fusion protein was eluted with MT sonication buffer containing 200 mM imidazole. The eluted proteins were precipitated by adding ammonium sulfate (80% saturation) and the precipitant was dissolved in 500 μl of MT sonication buffer and desalted using a NAP5 column (GE Healthcare BioSciences), which had been equilibrated with the buffer. The purity of the eluted protein was determined by SDS-PAGE.

The enzyme assay mixture comprised 50 mM KPi, pH 7.5, 2–40 μM of a flavonoid, 1 mM SAM, and 2 μg of recombinant protein in a total volume of 100 μl. The reaction mixture was pre-incubated for 2 min at 37°C and the reaction was started by adding the enzyme solution. The mixture was incubated at 37°C for 5 min and terminated by adding 50% acetonitrile (v/v) containing 1% TFA, which was followed by centrifugation at 10,000 rpm for 10 min at 4°C. The HPLC analysis was performed as described previously (Nakamura et al. 2010) and decrease of the substrate was quantified. K_m values were determined with a substrate concentration ranging from 2 to 300 μM by using Lineweaver–Burk plot.

Binary vector construction and plant transformation

Binary vectors were constructed to overexpress the torenia *A3′5′OMT* gene (pSPB1530), a pansy *F3′5′H* gene (Katsumoto et al. 2007) (pSFL207), and both the pansy *F3′5′H* and the torenia *A3′5′OMT* genes (pSPB1532) using the enhanced cauliflower mosaic virus 35S promoter derived from plasmid pBE2113-GUS (Mitsuhara et al. 1996), as described previously (Katsumoto et al. 2007). The binary vector pBinPLUS (van Engelen et al. 1995) containing a neomycin phosphotransferase gene as a selection marker for plants was used as the backbone.

The transformations of *Nierembergia* (Ueyama et al. 2006)

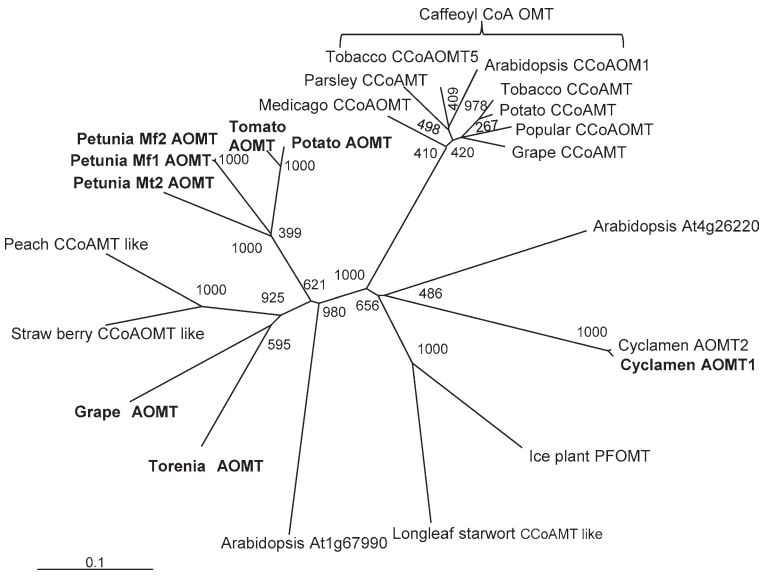

Figure 2. Phylogenetic tree of Group A1*O*-methyltransferase amino acid sequences (modified from Provenzano et al. 2014). Functionally identified AOMTs are shown in bold letters. The numbers on the branches indicate the bootstrap support values (1000 replicates). *Torenia* AOMT (this study), grape AOMT (ACO52469), strawberry CCoAOMT like (XP_00431007), peach CCoAOMT like (EMJ22806), petunia Mt2AOMT (AIE77045), potato AOMT (AIE77045), petunia Mf1 (AIE77044), Mf2 petunia M2 (AIE77046), potato AOMT (AGC31679), tomato (XP_004247599), cyclamen AOMT1 (AB618482), cyclamen AOMT2 (AB618483), ice plant PFMOT (AAN61072), *Arabidopsis* At1g67990 (Q9C9W4), longleaf starwood CCoAOMT like (AAB61680), parsley CCoAOMT (AAA3385), tobacco CCoAOMT5 (AAB80931), *Medicago* CCoAOMT (Q40313), *Arabidopsis* CCoAOMT (O49499), ice plant (Q40313), grape CCoAOMT(CAA90969), tobacco CCoAOMT (AAC49913), potato CCoAOMT (Q8H9B6), polular CCoAOMT (ACC63876), *Arabidopsis* At4g26220 (AAM64800).

and rose (Katsumoto et al. 2007) were performed as described previously using *Agrobacterium tumefaciens* strain AGL0 (Lazo et al. 1991), which harbored a binary vector.

Measurement of flower color and flavonoid analysis

The rose petal color (hue and reflectance (color lightness)) was measured using colorimetric values in a CIE L*a*b* system measured with a CM-2022 spectrophotometer (Minolta Co., Ltd.) as described previously (Katsumoto et al. 2007).

Anthocyanidins derived from the rose petals were prepared by acid hydrolysis in 6 N HCl at 100°C for 20 min (Katsumoto et al. 2007). Anthocyanins, anthocyanidins, and flavonoids were analyzed by HPLC as described previously (Nakamura et al. 2010). To confirm the identities of the anthocyanins in rose petals, the petal extracts were subjected to LC-FTICR-MS analysis (Iijima et al. 2008), as described previously (Nakamura et al. 2010). The metabolites of the transgenic and host plants were compared by determining the molecular formulae of the anthocyanins using Xcalibur (Thermo Electron, Rockford, IL, USA).

Results and discussion

Isolation of the torenia AOMT homolog

The torenia AOMT homolog (clone TMT5, the nucleotide sequence is registered as LC005089 in *DDBJ*/*ENA*/GenBank databases) was obtained from a torenia petal cDNA library by screening with DIG-labeled petunia AOMT *MT2*cDNA (Provenzano et al. 2014). The open reading frame of the AOMT homolog comprised 239 amino acid residues. The amino acid sequence shared 72% identity with the grape A3′5′OMT, as well as sharing similarity with petunia *Mf2*A3′5′OMT (68%), *Mf1*A3′5′OMT (67%), *Mt2*A3′OMT (64%), and cyclamen A3′5′OMT (AOMT2) (52%). Figure 2 shows the phylogenetic tree, which includes various group A1 OMT sequences (Lam et al. 2007). In the group A1 OMT family, CCoAOMTs formed a closely related group whereas AOMTs did not. The CCoA OMT-like group includes an *Arabidopsis* CCoAOMT-like sequence (At1g67990), which is involved in spermidine methylation in flowers and pollen (Fellenberg et al. 2008); an iceplant (*Mesembryanthemum crystallinum*) OMT (PFOMT) that catalyzes the methylation of caffeoyl CoA, flavonols, and caffeoyl glucose (Ibdah et al. 2003); and functionally unidentified peach, strawberry, and logleaf starwood CCoAOMT-like proteins (Figure 2). This suggests that the CCoAOMT-like group contains enzymes with various activities.

AOMTs are not always closely related, as shown in Figure 2, and a limited number of species produce methylated anthocyanins, thus the genes that encode AOMTs might have evolved independently several times during plant evolution, possibly via the duplication of CCoAOMT genes and their neofunctionalization. The isolation of *AOMT* genes from more plant species will help to clarify the molecular evolution of *AOMT*

genes. Gene duplication and the neofunctionalization of flavonoid biosynthetic pathway genes is not uncommon and have been reported previously (Rausher 2006; Seitz et al. 2006).

Biochemical characterization of recombinant torenia AOMT

The recombinant TMT5 protein was produced in *E. coli* and purified by Ni column chromatography. Figure 3A shows that the TMT5 protein was expressed successfully in *E. coli* and purified almost to homogeneity.

The reaction between the purified protein and delphinidin 3-glucoside produced new peaks with retention times and spectra that matched those of petunidin 3-glucoside and malvidin 3-glucoside (Figure 3B). The reaction with delphinidin 3,5-diglucoside yielded petunidin 3,5-diglucoside and malvidin 3,5-diglucoside (Figure 3C). These results demonstrate that *TMT5* encodes A3′5′OMT. The protein also catalyzed the synthesis of peonidin 3-glucoside and peonidin 3,5-diglucoside from cyanidin 3-glucoside and cyanidin 3,5-diglucoside, respectively (data not shown).

The K_m values for delphinidin 3-glucoside, delphinidin 3,5-diglucoside, cyanidin 3-glucoside, and cyanidin 3,5-diglucoside were determined as 103±6.83 μM, 89±9.44 μM, 65.9±5.08 μM, and 123±17.8 μM. These K_m values are in a similar range to the grape A3′5′OMT (i.e., the K_m values for cyanidin 3-glucoside and delphinidin 3-glucoside are 43 and 44 μM, respectively (Hugueney et al. 2009)), but higher than the K_m value for delphinidin 3-glucoside with tomato A3′5′OMT, i.e., 12 μM (Roldan et al. 2014).

The similar K_m values for the recombinant torenia A3′5′OMT with the 3-glucosides and 3,5-diglucosides of cyanidin and delphinidin support the assumption that the anthocyanin biosynthesis pathway forms metabolic grids in torenia (Nakamura et al. 2010), as found in the perilla (Gong et al. 1997) and gentian (Fukuchi-Mizutani

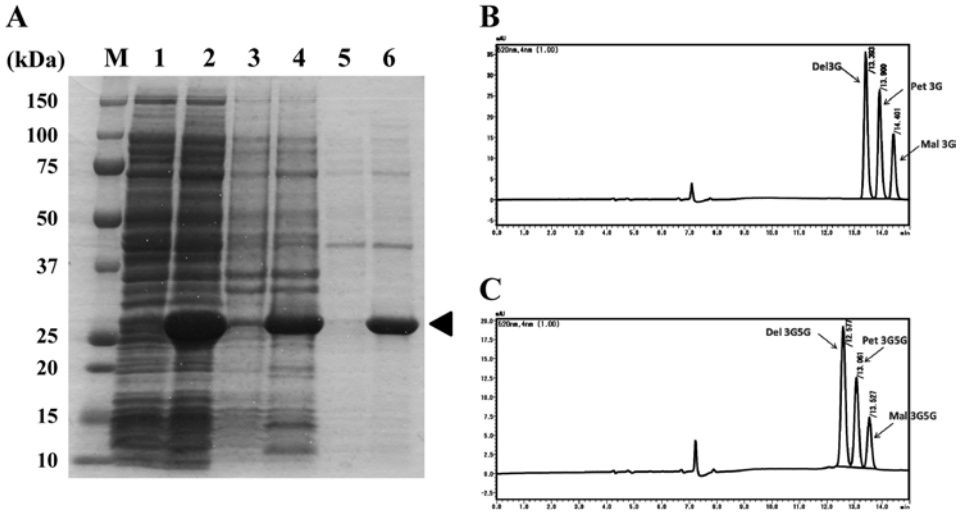

Figure 3. Enzymatic characterization of the recombinant torenia A3′5′OMT. (A) Expression of the torenia A3′5′OMT in *E. coli* and its purification; M, Precision Plus Protein Standards (Bio Rad); 1, a soluble fraction of *E. coli* harboring pET15b; 2, a soluble fraction of *E. coli* expressing torenia A3′5′OMT; 3, an insoluble fraction of *E. coli* harboring pET15b; 4, an insoluble fraction of *E. coli* expressing torenia A3′5′OMT; 5, a fraction eluted from the HisTrap HP column with 200 mM imidazole; 6, a fraction eluted from the column with 200 mM imidazole. HPLC analysis of the reaction between recombinant torenia A3′5′OMT and delphinidin 3-glucoside (B), delphinidin 3,5-diglucoside (C). Petunidin and malvidin glucosides were detected in both reactions.

Table 1. Anthocyanidin analysis in *Nierembergia* petals that expressed the torenia *A3′5′OMT* gene.

Line	Del (mg/g petal)	Pet (mg/g petal)	Mal (mg/g petal)	Total (mg/g petal)	Methyl (%)
Host	1.13	n.d.	n.d.	1.13	n.d.
NB18/1530-1	0.49	0.05	0.19	0.72	32
NB18/1530-2	0.54	0.03	0.12	0.69	22
NB18/1530-9	0.41	0.02	0.12	0.55	27
NB18/1530-12	0.61	0.03	0.18	0.82	26
NB18/1530-13	0.56	0.04	0.18	0.78	28
NB18/1530-17	0.47	0.03	0.14	0.64	27
NB18/1530-19	0.74	0.04	0.30	1.08	32
NB18/1530-20	0.37	0.02	0.07	0.46	19
NB18/1530-22	0.60	0.04	0.23	0.87	31

Del, delphinidin; Pet, petunidin; Mal, malvidin. n.d., not detected. Methyl (%)=percentage of peonidin, petunidin, and malvidin/total anthocyanidins.

Figure 4. Flowers of *Nierembergia* spp. NB18 and its transgenic plants expressing the torenia *A3′5′OMT* gene. The transgenic plants exhibited a slightly paler color. Malvidin was detected in the transgenic petals.

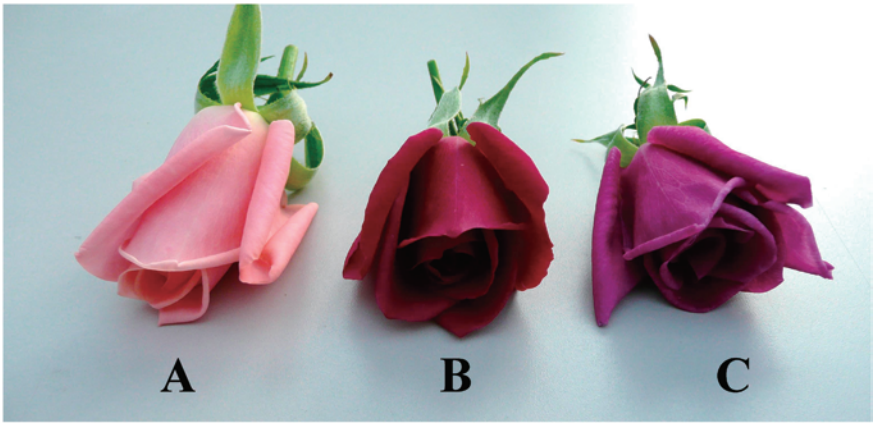

Figure 5. Flowers of WKS124, WKS124/207-2-2, and WKS124/1532-12-1. WKS124/207-2-2 expressing the pansy *F3′5′H* gene (B) and WKS124/1532-12-1 expressing the pansy *F3′5′H* and the torenia *A3′5′OMT* genes (C) are darker than WKS124 (A) due to their higher levels of anthocyanins while WKS124/1532-12-1 had a more vivid color than WKS124/207-2-2 due to the accumulation of malvidin-type anthocyanins.

et al. 2003) anthocyanin biosynthetic pathways. Although delphinidin 3-glucoside 5-coumaroylglucoside can be a precursor of malvidin 3-glucoside 5-coumaroylglucoside (Figure 1), the major anthocyanin in the torenia petals (Nakamura et al. 2010), the activity of the torenia A3′5′OMT with delphinidin 3-glucoside 5-coumaroylglucoside could not be determined because of its unavailability. Petunia and tomato

Table 2. Anthocyanidin analysis of rose petals from the host rose (WKS124), those expressing pansy *F3′5′H* gene (207-lines), and those expressing both the pansy *F3′5′H* and torenia *A3′5′OMT* genes (1532-lines).

Line	Hue (°)	Ref	Pel (mg/g petal)	Cya (mg/g petal)	Peo (mg/g petal)	Del (mg/g petal)	Pet (mg/g petal)	Mal (mg/g petal)	DPM (%)	Methyl (%)
WKS124	31.1	30.8	0.07	0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
207-01-02	353	2.38	0.00	0.07	n.d.	1.23	n.d.	n.d.	95	n.d.
207-05-03	373	0.55	0.00	0.09	n.d.	1.59	n.d.	n.d.	94	n.d.
207-02-02	360	2.91	0.00	0.09	n.d.	1.17	n.d.	n.d.	93	n.d.
207-06-02	351	3.45	0.00	0.10	n.d.	1.29	n.d.	n.d.	93	n.d.
207-19-4	350	3.74	0.00	0.13	n.d.	1.36	n.d.	n.d.	91	n.d.
Average	352	6.87	0.01	0.08	n.d.	0.75	n.d.	n.d.	86	n.d.
1532-5-2	341	2.32	0.01	0.03	0.05	0.20	0.18	0.80	89	82
1532-7-4	345	1.45	0.00	0.02	0.11	0.23	0.19	1.02	91	84
1532-11-2	348	1.35	0.00	0.04	0.09	0.49	0.37	0.94	93	72
1532-12-1	347	0.84	0.00	0.01	0.07	0.13	0.11	0.86	93	88
1532-25-1	345	0.79	0.00	0.01	0.09	0.21	0.19	0.83	92	83
Average	347	3.34	0.02	0.06	0.07	0.43	0.23	0.38	87	57

Ref, reflectance; Pel, pelargonidin; Cya, cyanidin; Peo, peonidin; Del, delphinidin; Pet, petunidin; Mal, malvidin. n.d., not detected. DPM (%)=percentage of delphinidin, petunidin, and malvidin/total anthocyanidins. Methyl (%)=percentage of peonidin, petunidin, and malvidin/total anthocyanidins.

A3′5′OMT catalyze the methylation of delphinidin 3-coumaroylrutinoside 5-glucoside (Provenzano et al. 2014; Roldan et al. 2014).

The activity of the torenia A3′5′OMT was not detected with delphinidin, cyanidin, pelargonidin, pelargonidin 3-glucoside, kaempferol, quercetin, myricetin, kaempferol 3-glucoside, quercetin 3-glucoside, apigenin, and luteolin. These results suggest that torenia A3′5′OMT has a stricter substrate specificity than the grape A3′5′OMT, which utilizes cyanidin, quercetin 3-glucoside, and myricetin as substrates (Hugueney et al. 2009; Lucker et al. 2010); the cyclamen A3′5′OMT, which utilizes quercetin 3-glucoside (Akita et al. 2011); and the tomato A3′5′OMT, which utilizes quercetin, myricetin, and quercetin 3-glucoside (Roldan et al. 2014).

Expression of TMT5 cDNA in Nierembergia

Nierembergia spp. NB18 accumulates anthocyanins derived from delphinidin but not petunidin or malvidin (Table 1). Ten transgenic *Nierembergia* plants that harbored T-DNA derived from pSPB1530 were obtained. Nine of them accumulated petunidin and malvidin (up to 30% of the total anthocyanidins), which were not present in the host. The results indicate that the expression of a torenia *A3′5′OMT* gene modified the anthocyanin biosynthetic pathway in the petals of a heterologous plant. The petal color of the plants that expressed the torenia *A3′5′OMT* gene was slightly paler and their total anthocyanin contents were lower than that of their host (Table 1), but the reason for this decrease was not clear.

Expression of TMT5 cDNA in rose

A rose cultivar that mainly accumulated pelargonidin-based anthocyanins (WKS124) was transformed with *Agrobacterium* containing pSFL207 or pSPB1532, thereby obtaining 43 and 76 transgenic rose lines, respectively. The petals of the transgenic lines exhibited clear color changes compared with their hosts (Figure 5) and the results of the anthocyanidin analyses are shown in Table 2. There were color changes to darker levels (lower reflectance values, Table 2) and increases in the anthocyanin amount due to the dominant accumulation of delphinidin (up to 94.5%, Table 2) in the transgenic roses that expressed the pansy *F3′5′H* gene, which indicated that the overexpression of the *F3′5′H* gene alone successfully converted the biosynthetic pathway to utilize delphinidin. This was presumably due to WKS124 lacking a F3′H activity to compete with the introduced F3′5′H.

The color of the petals of transgenic roses derived from pSPB1532, which expressed both pansy *F3′5′H* and torenia *A3′5′OMT* genes, were a more vivid magenta color than those that expressed the pansy *F3′5′H* gene alone, although there were not substantial differences in their hue and reflectance values. The petals contained malvidin, petunidin, and peonidin, which together comprised >80% of the total anthocyanidins. These results indicate that the torenia *A3′5′OMT* gene also functioned efficiently in the transgenic rose and that the color difference between roses containing the T-DNAs from pSFL207 and pSPB1532 were due to methylated anthocyanins (mainly malvidin type anthocyanins).

The anthocyanins of one line each (207-19-4 and 1532-12-1) that exhibited a stable flower color was subjected to FT-ICR-MS analysis to confirm the identities of the anthocyanins. The accurate mass and MS/MS spectra of the compounds in the petals of 1532–12-1 agreed with those of malvidin 3-glucoside (measured accurate mass=493.133834, MS/MS=331.15 (100)/332.09 (14.6) for malvidin, where the numbers in parentheses are the relative intensities), petunidin 3-glucoside

(479.11818, 317.12 (100), 332.09 (13.7)), malvidin 3,5-diglucoside (655.185441, 331.13 (70.6)/332.17 (13.2) for malvidin, 492.99 (100)/494.10 (20.3) for malvidin hexoside), petunidin 3,5-diglucoside (641.1709900, 317.12(80.3)/318.09(12.4) for petunidin, 479.03(100)/480.01(18.8) for petunidin hexoside), or peonidin 3,5-diglucoside (625.176239, 301.12 (67.7)/302.16 (10.4) for peonidin, 463.03 (100)/464.06 (17.6) for peonidin hexoside). These compounds were not detected in the petals of 207-19-4. These results confirm that the transgenic rose petals contained 3′- or 3′,5′-methylated anthocyanins, which were not produced by the host.

Thus, expression of the torenia *A3′5′OMT* gene in a rose successfully conferred the biosynthetic pathway for methylated anthocyanins, and flower color modifications were obtained. The torenia *A3′5′OMT* gene is a useful molecular tool for modifying and increasing the color variations in ornamental flowers.

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