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Original Paper

Generation of pink flower varieties from blue *Torenia hybrida* by redirecting the flavonoid biosynthetic pathway from delphinidin to pelargonidin

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Abstract Transgenic torenia plants with various pink petal tones were obtained from blue or violet cultivars by combining downregulation of endogenous *flavonoid 3′-hydroxylase* (*F3′H*) and *flavonoid 3′, 5′-hydroxylase* (*F3′5′H*) genes with expression of a heterologous gene. Knockdown of the *F3′H* and *F3′5′H* genes in a blue torenia cultivar resulted in pale pink lines by decreasing the cyanidin and delphinidin levels and increasing the pelargonidin-based anthocyanin levels. Additional expression of rose *dihydroflavonol 4-reductase* (*DFR*) gene elevated the level of pelargonidin and yielded darker pink petals. Expression of pelargonidin *DFR* gene instead of the rose *DFR* gene increased the level of pelargonidin and darkened the petal color. Introducing the two genetic constructs containing a *DFR* gene into a violet torenia cultivar, which had more anthocyanins and a darker color than the blue one, further increased the level of pelargonidin and pink color intensity. These results reveal that selection of a suitable gene source and host greatly affects the phenotypes of the resultant transgenic plants.

Key words: Anthocyanin, flower color, genetic engineering, torenia, pelargonidin.

Flower colors such as brick red, red, magenta, violet, and blue originate from anthocyanins, a colored class of flavonoids, which are usually localized in the epidermal cell vacuoles of petals (Tanaka et al. 2008). Despite the wide range of structures of anthocyanins, most of them are biosynthesized from three kinds of anthocyanidins: pelargonidin, cyanidin, and delphinidin (Figure 1). The hydroxylation pattern of the B-ring greatly affects the color of the anthocyanins and thus the petal color. Orange/brick red petals tend to contain anthocyanins derived from pelargonidin (pelargonidin-based anthocyanins), red/magenta petals contain anthocyanins derived from cyanidin or peonidin (cyanidin-based anthocyanins), and blue/violet petals contain anthocyanins derived from delphinidin, petunidin, or malvidin (delphinidin-based anthocyanins) (Figure 1). Modification of anthocyanins with aromatic acyl groups, presence of copigments (flavones and flavonols) or metal ions, and higher vacuolar pH also contribute to blue coloration (Yoshida et al. 2009).

The hydroxylation pattern of the B-ring is determined by two cytochrome P450 enzymes, flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′, 5′-hydroxylase (F3′5′H) (Tanaka 2006). Overexpression of exogenous genes and/or suppression of an endogenous gene in transgenic plants can change the pathway and thus change petal color, as reviewed recently (Tanaka and Ohmiya 2008; Tanaka et al. 2009). Expression of the *F3′5′H* gene in roses and carnations, which lack blue/violet varieties because of the lack of delphinidin, results in novel blue/violet varieties (Katsumoto et al. 2007; Tanaka et al. 2009).

Dihydroflavonol 4-reductase (DFR) is a key enzyme in anthocyanin biosynthesis and flower color. Petunia does not accumulate pelargonidin and therefore does not have orange color varieties because its DFR does not utilize dihydrokaempferol (DHK) (Forkman and Ruhnau 1987). Expression of genes for DFR, which catalyzes DHK to leucopelargonidin in petunia mutants that are deficient in F3′H and F3′5′H, results in pelargonidin production and orange color (Elomaa et al. 1995; Meyer et al. 1987; Tanaka et al. 1995). The gerbera *DFR* gene yields more

Abbreviations: ANS, anthocyanidin synthase; DFR, dihydroflavonol 4-reductase; DHK, dihydrokaempferol; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′, 5′-hydroxylase; FLS, flavonol synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LC-FTICR-MS, liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry.
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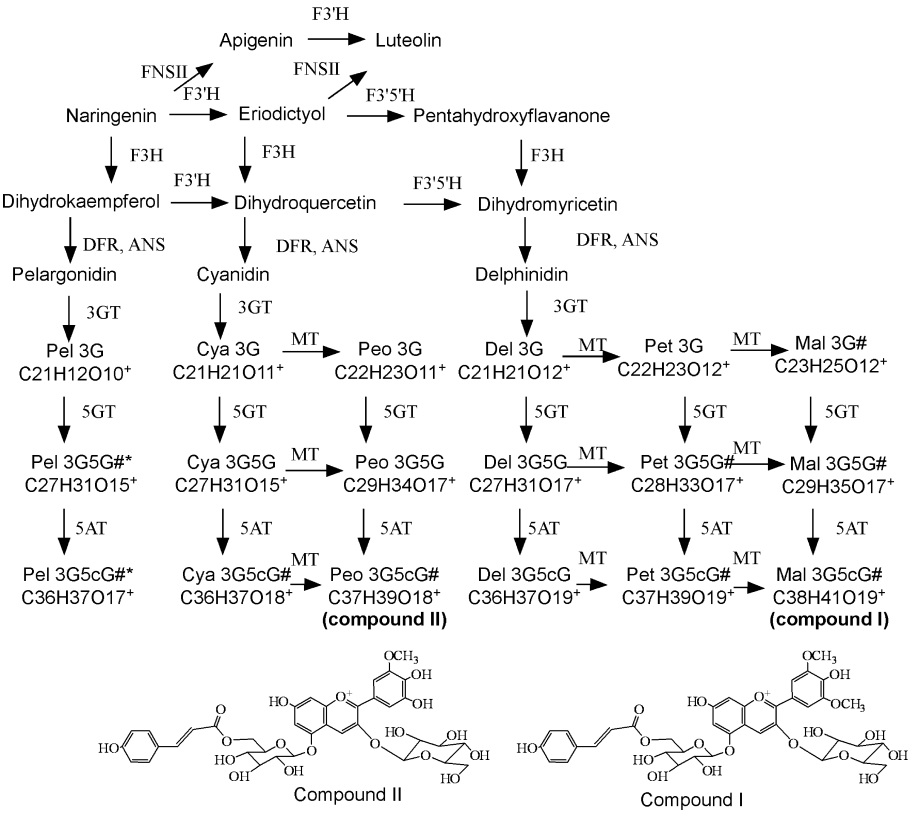

Figure 1. A proposed anthocyanin and flavone biosynthetic pathway for *Torenia hybrida*. Compound I and II are major anthocyanins in *Torenia hybrida* cv. Summerwave Blue and Summerwave Violet petals. # Anthocyanins whose molecular formula and MS/MS spectra agreed with compounds detected by liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry. * Anthocyanins detected only in transgenic plants. Pel, pelargonidin; Cya, cyanidin; Peo, peonidin; Del, delphinidin; Pet, petunidin; Mal, malvidin; G, glucoside; cG, coumaroyl glucoside; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; FNSII, flavone synthase II; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, anthocyanidin 3-glucosyltransferase; 5GT, anthocyanin 5-glucosyltransferase; 5AT, anthocyanin 5-acyltransferase; MT, anthocyanin methyltransferase.

intense flower color than the maize gene in petunia (Elomaa et al. 1995). Downregulation of the *F3′H* gene and expression of the rose *DFR* gene in red petunia accumulating cyanidin results in an orange petunia producing pelargonidin (Tsuda et al. 2004).

Downregulation of the *F3′H* and *flavonol synthase* (*FLS*) genes and expression of the gerbera *DFR* gene in tobacco results in pelargonidin production (Nakatsuka et al. 2007). Downregulation of the *F3′5′H* gene and overexpression of the gerbera *DFR* gene in *Osteospermum hybrida* successfully redirects anthocyanin synthesis from delphinidin to pelargonidin (Seitz et al. 2007). Pelargonium petals of all examined cultivars contain pelargonidin in addition to cyanidin and delphinidin (Kobayashi et al. 1998). In other words, pelargonium petals accumulate pelargonidin even in the presence of F3′H and F3′5′H, which leads us to hypothesize that the pelargonium *DFR* gene may be a good molecular tool to redirect the flavonoid pathway to pelargonidin.

Torenia, a popular bedding plant, is a good model for the study of biological phenomena including flavonoid biosynthesis and fertilization and can be used to alter flower color through genetic engineering (Aida 2009). White or partially white torenia plants have been obtained by downregulating the *chalcone synthase* gene, *DFR* gene (Aida et al. 2000; Suzuki et al. 2000), or *anthocyanidin synthase* (*ANS*) gene (Nakamura et al. 2006). A yellow torenia accumulating aurones can be generated by expressing the snapdragon *tetrahydroxychalcone 4′-glucosyltransferase* and *aureusidin synthase* genes and downregulating anthocyanin biosynthesis (Ono et al. 2006).

The blue petals of torenia contain mainly malvidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-*p*-coumaroyl-*β*-D-glucoside) (compound I, Figure 1), and a small level of peonidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-*p*-coumaroyl-*β*-D-glucoside) (compound II, Figure 1) as anthocyanins. A postulated biosynthetic pathway leading to these compounds is shown in Figure 1. Most of the biosynthetic genes shown in Figure 1 have been isolated. The delphinidin pathway, leading to compound I in blue torenia cultivar, has been redirected to the cyanidin pathway to yield pale pink flowers by downregulating the *F3′5′H* gene (Suzuki et

al. 2000) and magenta flowers have been produced by additional overexpression of the torenia *F3′H* gene (Ueyama et al. 2002). These results suggest that accumulation of pelargonidin-based anthocyanins is necessary to produce a more deep red or intense pink petal color.

In this study, we downregulated the *F3′5′H* and *F3′H* genes and/or overexpressed the rose or pelargonium *DFR* gene in a blue or violet torenia cultivar that mainly accumulate delphinidin-based anthocyanins. The resulting torenia exhibited pink flowers with various intensities as a result of the accumulation of various levels of pelargonidin-based anthocyanins.

Materials and methods

Plant materials and transformation

Torenia hybrida cv. Summerwave Blue (SWB) and Summerwave Violet (SWV) (Suntory Flowers Ltd., Tokyo, Japan), which are interspecies varieties *T. fournieri* and *T. concolor*, were used as hosts for genetic modification. Torenia transformation was performed as described previously (Aida and Shibata 1995), using *Agrobacterium tumefaciens* AGL0 (Lazo et al. 1991) harboring a binary vector. The transgenic plants were grown in a contained glasshouse for genetically modified plants to assess petal color changes and color stability. The temperature of the glasshouse was set at 25°C but it fluctuated depending on the outside temperature, and the day length was not controlled.

Molecular cloning and binary vector construction for flower color modification

The molecular biological procedures were described in previous studies (Fukuchi-Mizutani et al. 2003). Pelargonium *DFR* cDNA was obtained by screening a cDNA library derived from *Pelargonium zonale* (L.) petals with rose *DFR* cDNA (Tanaka et al. 1995). Torenia *F3′H* (Ueyama et al. 2002) and *F3′5′H* (Suzuki et al. 2000) cDNAs were used to downregulate their expression. The *F3′H* double stranded RNA, *F3′5′H* double stranded RNA, and the *DFR* genes were transcribed by constitutive promoters; the El235S promoter, which is an enhanced cauliflower mosaic virus 35S promoter from pBE2113-GUS (Mitsuhara et al. 1996) or the Mac1 promoter (Comai et al. 1990). Expression cassettes were inserted into multiple cloning sites of the pBinPLUS binary vector (van Engelen et al. 1995).

Quantitative RT-PCR analysis was performed using the TaqMan^{R} Gene Expression Master Mix (Applied Biosystems Inc., Foster City, CA, USA) and the ABI Prism ABI7000 Sequence Detection System (Applied Biosystems). The following specific primer sets were used to quantify transcripts: For *F3′H*, forward 5′-GGCGTTGGCTTGGGAATAC-3′ corresponding to bases 1351–1369 counting from the initiation codon, reverse 5′-GCAAGGTCCAGATCGAATGC-3′ (1424–1405), and Taq Man MGB probe 5′-TGGTCCAACTG-TTGACG-3′ (1373–1389); for *F3′5′H*, forward 5′-GGAGGAGGCCTTTGGGATT-3′ (1446–1464), reverse 5′-GGCAGGCGAGGAGTAACGA-3′ (1514–1496), and Taq Man MGB probe 5′-TCTGCAGAAGGCCACAC-3′ (1467–1483); rose *DFR*, forward 5′-CCGTGTCAAAGTCACAG-GCTAA-3′ (1029–1050), reverse 5′-TAGGCACACCTCTAA-AATCAAACAGT-3′ (1137–1112), and Taq Man MGB probe 5′-AGTATTGGTAACATTTGTATCTC-3′ (1063–1085); for pelargonium *DFR*, forward 5′-CATGTCAATGGAAAGGCTT-AAGTAATT-3′ (1003–1029), reverse 5′-GTTGATCCAACT-AACTTCCAAAACC-3′ (1141–1117), and Taq Man MGB probe 5′-TGATCATGTCTTCGTTATAAGT-3′ (1057–1078). The primers for endogenous *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) were described previously (Nakamura et al. 2006). The levels of *F3′5′H*, *F3′H*, and *DFR* transcripts were standardized by the ratio of the level of *GAPDH* transcript for each plant.

Analysis of flavonoid aglycones

Flavonoid analysis procedures were described previously (Fukui et al. 2003). Three petals from each plant were collected and lyophilized. Flavonoids were extracted from the petals with 50% acetonitrile (v/v) containing 0.1% trifluoric acid (TFA) and then dried. Flavonoids were dissolved in 6 N HCl (0.2 ml) and maintained at 100°C for 20 min to yield anthocyanidins. Anthocyanidins were extracted with 0.2 ml 1-pentanol and subjected to high performance liquid chromatography (HPLC) on an ODS-A312 column (15 cm×6 mm; YMC Co., Ltd., Kyoto, Japan) at a flow rate of 1 ml min^{-1} using an isocratic solvent (AcOH : MeOH : H_2O=15 : 20 : 65, v/v/v).

Petal extract (200 μl) was obtained as described above for flavone and flavonol aglycone analysis. It was then dried and subjected to enzymatic hydrolysis in 0.2 ml of 0.1 M potassium phosphate buffer (pH 4.5) with 6 units of β-glucosidase (Sigma, St. Louis, MO, USA) and 1 unit of naringinase (Sigma) for 16 h at 30°C. The reaction was terminated by adding 200 μl of 90% (v/v) acetonitrile in water containing 0.1% TFA, and then, was subjected to HPLC analysis. HPLC was performed using a Shim-pack FC-ODS column (15 cm×4.6 mm; Shimadzu Co., Ltd., Kyoto, Japan) with a linear gradient using solvent A (H_2O-TFA, 99.9 : 0.1, v/v)) and B (H_2O-acetonitrile-TFA, 9.9 : 90 : 0.1, v/v); from 18% solvent B to 63% solvent B in solvent A for 18 min, followed by isocratic elution using 63% solvent B in solvent A for 5 min at a flow rate of 0.6 ml min^{-1}. Flavonoids were detected at an absorbance ranging from 250–600 nm using a photodiode array detector (SPD-M20A, Shimadzu).

Flavonoid analysis by LC-FTICR-MS analysis

Flavonoids were subjected to liquid chromatography/Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR-MS) analysis to elucidate their structures by accurately determining *m/z* and MS/MS data as described previously (Iijima et al. 2008a; Iijima et al. 2008b). Metabolites of the transgenic and host plants were compared by searching the molecular formulas of the anthocyanins shown in Figure 1 using Xcalibur software (Thermo Electron, Rockford, IL, USA).

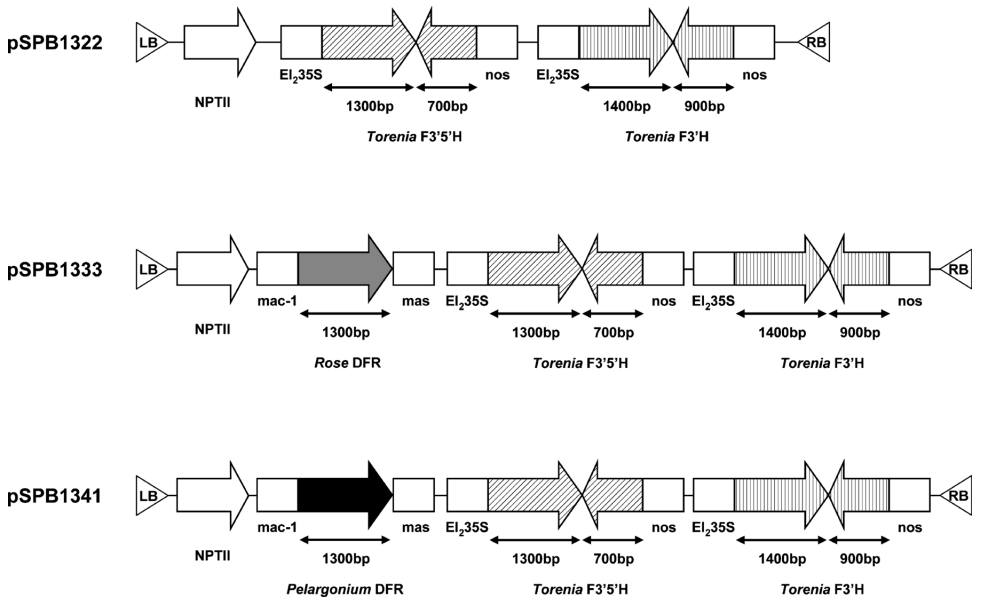

Figure 2. T-DNA region structures of the binary vectors constructed for color modification in this study. Arrows indicate transcriptional direction of cDNAs or a gene. Double-stranded RNA derived from *F3′H* and *F3′5′H* cDNA was expected to be transcribed in transgenic plants. Approximate DNA length of the cDNAs is shown. LB, left border; RB, right border; mac-1, mac-1 promoter; $El_2$35S, an enhanced cauliflower mosaic virus 35S promoter; nos, nopaline synthase.

Results

Cloning pelargonium DFR cDNA and binary vector construction

Screening of a quarter million plaques of the pelargonium petal cDNA library, using rose *DFR* cDNA as the molecular probe, yielded three positive clones. Sequence analysis revealed that they encoded amino acid sequences that were homologous to the DFR of other plant species. One of the clones (pSPB1407) was sequenced completely and used for further study (its accession number in DDBJ is AB534774). Its open reading frame contained 341 amino acid residues. The amino acid sequence exhibited 73% and 63% amino acid identity with rose and petunia DFRs, respectively.

The binary vectors constructed in this study are shown in Figure 2. The plasmid pSPB1322 was designed to downregulate the endogenous *F3′H* and *F3′5′H* genes. We constructed pSP1333 and pSPB1341 to additionally express rose and pelargonium *DFR* cDNAs, respectively.

Torenia transformation and color change

SWB was transformed with *A. tumefaciens* harboring binary vectors (pSP1322, pSP1333, and pSPB1341) and 67, 54, and 28 independent transgenic lines were obtained, respectively, from each transformation experiment. The numbers of transgenic plants with altered flower color were 27 (40%), 27 (50%), and 10 (36%), respectively.

The transgenic plants exhibiting a clear color change to pink were selected and subjected to flavonoid analysis. The selected plants were maintained in a glasshouse for 2 years. Blue color occasionally reappeared on the petals in most of the selected lines (data not shown), probably as a result of the release of suppression of the *F3′5′H* gene in the transgenic plants. Three lines showing altered petal color most consistently (SWB/1322-17, 45, and 90; SWB/1333-17, 34, and 55; SWB/1341-8, 13, and 15; SWV/1333-3, 6, and 8; and SWV/1333-9, 17, and 28) were selected from the transgenic plants derived from each construct and the petals of each plant were subjected to flavonoid and RT-PCR analyses.

Petals of SWB/1322 plants with pink petals mainly contained pelargonidin (Table 1). This result indicates that *F3′H* and *F3′5′H* gene expression was suppressed successfully, as shown in Figure 4 A and B, and that the torenia DFR can utilize DHK as a substrate. Additional expression of a heterologous *DFR* gene increased the level of pelargonidin in SWB/1333 and SWB/1341 plants. Transcripts of the *F3′H* and *F3′5′H* genes were also downregulated in these plants (Figure 4 A, B). Interestingly, the SWB/1341 plants expressing the pelargonium *DFR* gene produced more pelargonidin and darker petals than the SWB/1333 plants, indicating that the pelargonidin *DFR* gene produces pelargonidin more efficiently than the rose *DFR* gene. Their expression was confirmed as shown in Figure 4 C and D. The color-modified transgenic plants contained much lower levels of anthocyanidin (Table 1). The reason for this decrease is unclear. However, metabolic flux toward pelargonoidin based anthocyanins may not be efficient enough. The level and composition of flavones was not different in

Table 1. Level of anthocyanidins, and aglycones of flavones and a flavonol

Plant	Anthocyanidin			Flavone		Flavonol
	Del type*	Cya type**	Pelargonidin	Luteolin	Apigenin	Kaempferol
SWB	568 ± 160	86.3 ± 20.2	2.1 ± 1.6	350.7 ± 93.1	4580 ± 954	n. d.
SWB/1322	25.8 ± 4.4	15.1 ± 4.6	28.5 ± 3.5	24.5 ± 5.7	5300 ± 566	66.9 ± 4.3
SWB/1333	18.8 ± 1.7	16.6 ± 2.3	63.3 ± 9.0	24.1 ± 3.6	6030 ± 343	57.8 ± 6.1
SWB/1341	14.3 ± 3.7	16.7 ± 2.8	126 ± 11.3	26.9 ± 2.7	5440 ± 410	46.5 ± 3.3
SWV	849 ± 14.6	687 ± 134	n. d.	2120 ± 47.2	1660 ± 294	n. d.
SWV/1333	29.7 ± 10.5	87.1 ± 25.4	199 ± 59.6	44.6 ± 5.1	5110 ± 768	72.2 ± 6.0
SWV/1341	29.5 ± 13.0	120 ± 63.6	468 ± 267	57.7 ± 2.1	6450 ± 174	62.2 ± 4.7

Data are expressed as the mean±SD (μg^{-1} fresh weight) of the three selected lines described in the text. The level of each flavonoid in transgenic plants, except apigenin, differed significantly from that of *Torenia hybrida* cv. Summerwave Blue (SWB) and Summerwave Violet (SWV) at the 1% level.

* The total level of delphinidin, petunidin, and malvidin,

** The total level of cyanidin and peonidin, n. d. not detected.

Figure 3. Pictures of whole plants, flowers of hosts (*Torenia hybrida* cv. Summerwave Blue [SWB], Summerwave Violet [SWV]), and the selected transgenic plants (SWB/1322-45, SWB/1333-17, SWB/1341-15, SWV/1333-3, and SWV/1341-17). No significant morphological changes were observed.

these plants (Table 1).

Transformation of SWV by *A. tumefaciens* harboring pSPB1333 and 1341 generated 83 and 81 transgenic plants, respectively, and eight (9.6%) and 17 (21%) of them produced phenotypic changes, respectively. As expected, the transgenic plants had darker-colored flowers than plants derived from SWB. The transgenic plants derived from SWV also had a decreased level of anthocyanidins than SWV (Table 1). Expression of the pelargonium *DFR* gene (pSPB1333) also produced more pelargonidin than expression of rose *DFR* (pSPB1341), as in the case of SWB. Flavone composition also changed; the level of luteolin decreased and that of apigenin increased, indicating that the *F3′H* gene is suppressed in SWV transgenic plants and that F3′H determines the hydroxylation pattern of the flavone B-ring in SWV. This result is consistent with our previous study that the torenia F3′H can catalyze hydroxylation of apigenin to luteolin *in vitro* (Ueyama et al. 2002). Suppression of the *F3′H* and *F3′5′H* transcripts and expression of rose or pelargonium *DFR* gene in SWV transgenic plants were confirmed by RT-PCR analysis (Figure 4, E-H). The increased level of flavones in the transgenic plants may have been caused by a decrease in the level of anthocyanins.

Structural elucidation of anthocyanins and flavonoids by LC-FTICR-MS

Metabolites whose *m/z* values and MS/MS spectra matched those of anthocyanins in the torenia biosynthetic pathway are shown in Table 2. The transgenic plants contained compounds whose molecular formulas and MS/MS spectra matched those of pelargonidin 3-*O*-glucoside-5-*O*-coumaroyl glucoside and pelargonidin 3, 5-*O*-diglucoside. Detecting some anthocyanins in the analysis supports the pathway shown in Figure 1.

Discussion

By modulating gene expression involved in flavonoid biosynthesis in torenia, the pathway leading to delphinidin was successfully converted to that leading to

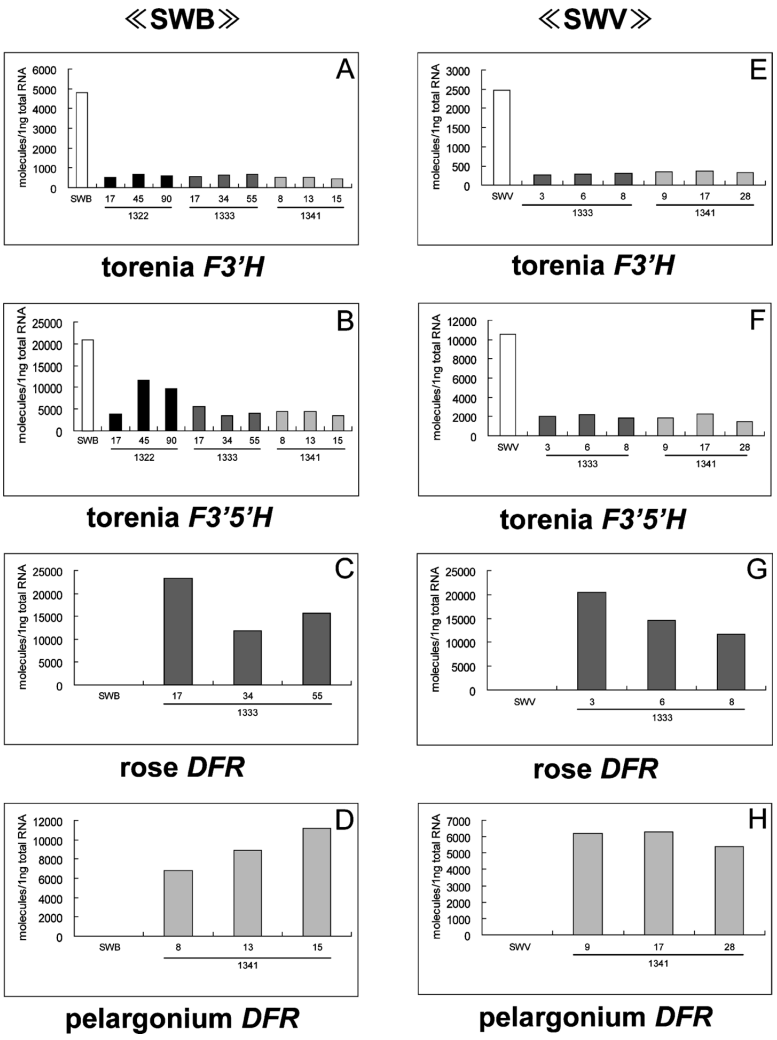

Figure 4. Results of quantitative RT-PCR analysis of *F3′H* (A, E), *F3′5′H* (B, F), and rose (C, G) or pelargonium (D, H) *DFR* transcripts in the torenia petals. Results of *Torenia hybrida* cv. Summerwave Blue (SWB) and its transgenic plants are shown in the left panel and those of *Torenia hybrida* cv. Summerwave Violet (SWV) and its transgenic plants are shown in the right panel. Each value on the vertical axis indicates relative levels of *GAPDH*. Data represent the average of three independent PCR reactions, and bars indicate standard deviations.

pelargonidin, and an accompanying color change from blue/violet to pink was achieved. These results reconfirm that pelargonidin production is an effective way to change flower color toward orange or bright red, as shown previously in petunia (Elomaa et al. 1995; Meyer et al. 1987; Tanaka et al. 1995; Tsuda et al. 2004), tobacco (Nakatsuka et al. 2007), and *Osteospermum* (Seitz et al. 2007). Our results confirm that downregulation of the *F3′5′H* and *F3′H* genes is insufficient and that addition of a suitable the proper *DFR* gene is necessary to generate a metabolic flux toward pelargonidin.

DFRs have different substrate specificities depending on plant species. The DFRs of petunia (Forkman and Ruhnau 1987) and *Cymbidium* (Johnson et al. 1999) do not catalyze DHK. Choosing a DFR with appropriate substrate specificity is crucial to successfully modifying flower color through the biosynthetic pathway engineering of flavonoids. For example, expression of petunia or pansy *F3′5′H* and the petunia *DFR* gene yields violet carnations accumulating delphinidin exclusively (Tanaka et al. 2009) and that of pansy F3′5′H and iris DFR produces similar roses (Katsumoto et al. 2007). It has been proposed that a portion of the DFR amino acid sequence (from 129–160 in petunia DFR) may be important for determining substrate specificity, and the petunia DFR has an Asp at 136 rather than an Asn as identified in other DFRs (Beld et al. 1989). Construction of chimeric DFRs from gerbera and petunia DFRs and a mutagenesis study of the gerberaDFR suggested that Asn 134 of the gerbera DFR (corresponding to Asn 136 of the pelargonium DFR) is critical for substrate specificity, i.e., the mutagenized gerbera DFR with Leu at that position preferably utilizes DHK over dihydroquercetin and cannot efficiently reduce dihydromyricetin (Johnson et al. 2001). Measuring

Table 2. Major anthocyanins detected by LC-FRICR-MS/MS analysis

Retention time (min)	λmax (nm)	[M]+	Δ p.p.m.	Molecular formula	MS/MS (relative intensity)/ assignment	Probable compound
14.65	520 322 276	641.171112	−0.178	C28H33O17	479.08(90.9)/Pet+hex 317.12(100)/Pet	Pet3G5G^{V}
15.33	516 522	595.165219	−0.886	C27H31O15	433.08(100)/Pel+hex 271.08(84.7)/Pel	Pel3G5G*^{T}
15.67	274 524	655.186695	−0.276	C29H35O17	493.11(93.5)/Mal+hex 331.15(100)/Mal	Mal 3G5G^{T}
17.49	360 272	493.134055	0.005	C23H25O12	331.15(100)/Mal	Mal3G^{V}
19.83	520 300 274	757.196883	−0.737	C36H37O18	595.10(100)/Cya+hex+c 449.05(21.4)/Cya+hex 287.11(84.8)/Cya	Cya3G5cG^{V}
19.86	526 322 274	787.207791	−0.272	C37H39O19	625.1(100)/Pet+hex+c 479.1(16.7)/Pet+hex 317.1(73)/Pet	Pet3G5cG^{V}
20.44	528 378 326 272	741.203037	−0.051	C36H37O17	579.07(100)/Pel+hex+c 433.22(23.7)/Pel+hex 271.05 (85.6)/Pel	Pel3G5cG*^{T}
20.49	544 398 330 274	801.2236	−0.07	C38H41O19	639.12(100)/Mal+hex+c 493.15(15.9)/Mal+hex 331.15(80.8)/Mal	Mal 3G5cG^{T} (compound I)
20.56	544 398 330 274	771.212751	−0.314	C37H39O18	609.09(100)/Peo+hex+c 463.09(20.3)/Peo+hex 301.10(48.5)/Peo	Peo3G5cG^{T} (compound II)

* Found only in transgenic plants; h, hexose. The other abbreviations are shown in the Figure 1 legend. The data shown in the table is derived from analysis of *Torenia hybrida* cv. Summerwave Violet (SWV) (^{V}) or SWV 1341 (^{T}). When both plants had the same compounds, the data showing the least Δ p.p.m. were adopted.

recombinant enzymes of three *Lotus japonicus* (DFR2 [Asn type], DFR3 [Asn type], and DFR5 [Asp type]) revealed that DFR2 and DFR3 preferably reduce DHK, and that DFR5 does so less efficiently (Shimada et al. 2005). Most DFRs from angiosperms have Asn in this position, and an Asp substitution occurs before divergence of the Gentianales and Solanales (Des Marais and Rausher 2008).

The crystal structure of the grape DFR supports the importance of the residue for substrate binding. However, substituting Asp at position 133 for Asn in the grape DFR does not change the substrate specificity (Petit et al. 2007). The pelargonium DFR isolated in this study has Asp in this position and catalyzes DHK. The DFR of *Verbena hybrida*, which accumulates pelargonidin, also contains Asp at this position (Togami et al. 2006). The DFRs (AB201759, AF029685, AY575027, AY695812, and AY695813) of *Fragaria x ananassa*, which accumulate pelargonidin, contain alanine in this position. These results agree with the view that residue 133 alone does not determine substrate specificity, as reported previously (Petit et al. 2007).

It is interesting that expression of plargonium *DFR* cDNA yielded more pelargonidin than that of rose *DFR* cDNA. The pelargonium DFR may have kinetic properties suitable for DHK catalysis such as a higher affinity for DHK. Alternatively, the gene and enzyme are more efficiently transcribed and translated, or the enzyme is more stable than the rose DFR, at least in torenia. The pelargonium *DFR* gene is a useful molecular tool to engineer pelargonidin-accumulating plants. The gerbera *DFR* gene is more efficient than the maize *DFR* for pelargonidin accumulation in petunia (Elomaa et al. 1995). Selection of a suitable plant as a gene source is also important for successful redirection of a pathway. Some floricultural species, such as the gentian and iris, lack an intense red cultivar because they lack pelargonidin. Intense red cultivars may be generated by downregulating the *F3′H* and *F3′5′H* genes and expressing the proper *DFR* gene.

It is interesting that only the transgenic torenia petals accumulate kaempferol, a flavonol. Flavonols are biosynthesized from corresponding dihydroflavonols by the action of FLS, a member of the 2-oxoglutarate dependent dioxygenase family, which ANS also belongs to. FLS and ANS are closely related in the family and ANS encodes FLS activity *in vitro* (Turnbull et al. 2004) and in *Arabidopsis* (Stracke et al. 2009). ANS may catalyze flavonol synthesis in the transgenic torenia in which dihydrokaempferol becomes available as a result

of F3′H and F3′5′H suppression.

The transgenic plants derived from SWV had darker flower color than those from SWB, which probably reflects the darker petal color derived from larger levels of anthocyanins in SWV than SWB. These results indicate that selection of a suitable host is an important consideration for producing transgenic plants with a desirable phenotype. A further increase in pelargonidin will lead to a stronger pink or red color, which could be achieved by switching biosynthesis from flavones to anthocyanins. Other tactics to yield red flowers can be to accumulate apigeninidin or 6-hydroxy pelargonidin, which have shorter absorption maxima than pelargonidin.

The petal color of host plants (blue or violet) often reappeared in the petals of transgenic plants, and only a fraction of the transgenic plants exhibited a stable altered color. This was probably the result of inconsistent downregulation of the endogenous *F3′5′H* gene. Although transcription of double-stranded RNA causes effective downregulation of a target gene (Nakamura et al. 2006; Waterhouse et al. 1998), a method producing more consistent downregulation is needed. Knockout of a target gene by homologous recombination (Terada et al. 2007) or the use of a zinc-finger nuclease (Shukla et al. 2009; Townsend et al. 2009) has been developed for some plant species. Until such technology is transferred to a wide variety of plant species, transcription of double-stranded RNA remains the best way to downregulate a target gene in plants. To obtain a transgenic plant in which an intended gene is consistently suppressed, it is practical to select a line with a stable phenotype from a large number of transgenic plants. In other words, an efficient plant transformation system needs to be developed for a given target species.

This study is another successful example of color modification by genetic engineering. The transgenic plants have morphology, flowering characters, and vigor similar to the hosts and may have commercial value. A field trial is currently being conducted in Australia to assess the performance of some of the transgenic plants obtained by us.

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