Journal of Plant Biology, December 2007, 50(6) : 626-631

Co-expression of *flavonoid 3', 5'-hydroxylase* and *flavonoid 3'-hydroxylase* Accelerates Decolorization in Transgenic Chrysanthemum Petals

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The *flavonoid 3',5'-hydroxylase (F3',5'H)* gene, derived from petunia, was introduced into chrysanthemum tissues by *Agrobacterium*-mediated genetic transformation. Cotyledon explants were co-cultured with *A. tumefaciens* LBA 4404 harboring the vector pMBP that carries *F3',5'H* under the control of the CaMV 35S promoter and *nptII* as a selectable marker gene. After 72 h of co-cultivation, the explants were placed on an MS medium supplemented with 4 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 400 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin. After 4 weeks, kanamycin-resistant adventitious shoots had developed at a frequency of 6.3%. These shoots were then rooted and acclimatized in potting soil. Integration of *F3',5'H* into the plant genome was confirmed by Southern blot analysis. Flower buds that had red petals did not differ between the transgenic and the wild-type plants. However, petal color did change from red to bright orange to yellow when the buds developed into fully opened flowers on the transgenics. Spectrometric analysis revealed that the content of flavonoid compounds was more rapidly reduced in the transgenic petals as floral development proceeded. RT-PCR analysis showed that *F3',5'H* and *flavonoid 3'-hydroxylase* (*F3'H*) were expressed simultaneously in the transgenic plants. Therefore, we suggest that this more rapid change in petal color results from 1) competition between levels of transgenic *F3',5'H* and endogenous *F3'H*, each of which uses the same substrate in the flavonoid biosynthetic pathway and 2) the intrinsic substrate specificity of chrysanthemum *DFR* (*dihydroflavonol 4-reductase*).

Key words: adventitious shoots; *Chrysanthemum*, flavonoid 3',5'-hydroxylase, flavonoid 3'-hydroxylase, genetic transformation

Chrysanthemum plants are one of the most commercially important ornamental crops worldwide. However, despite their efforts, conventional breeders have been unable to produce an abundance of new varieties because of heterogeneity and the lack of diverse floral features (Mol et al., 1989). These limitations have been partially overcome through a genetic transformation system that uses *Agrobacterium tumefaciens* (Chung and Park, 2005), and efforts with genetic engineering have been successful in modifying flower color (Ledger et al., 1991; Jong et al., 1994; Fukai et al., 1995; Sherman et al., 1998). For example, the introduction of a sense or antisense chalcone synthase gene into chrysanthemum has led to the production of fully white or very pale pink flowers at a low frequency (Courtney-Gutterson et al., 1994).

Flower color is determined by the ratio of anthocyanidins, subsequent modification of the anthocyanin structure, and other factors, such as vacuole pH and co-pigmentations.

Anthocyanin biosynthetic pathways are fairly well-known (Holton and Cornish, 1995; Shirley et al., 1995; Shirley, 1996; Dixon and Steele, 1999; Lee et al., 2005). In nature, chrysanthemums, roses, and carnations do not produce purple or blue colored flowers because those species lack a gene for the flavonoid 3', 5'-hydroxylase (F3',5'H), which is responsible for those hues (van der Meer et al., 1993; Holton et al., 1993). This enzyme is a member of the cytochrome P450 family. *Flavonol 3'-hydroxylase* (*F3'H*) catalyzes the hydroxylation of naringenin to dihydrokaemperol (DHK), which is then converted to dihydroquercetin (DHQ) by *F3'H*, while DHK and DHQ are hydroxylized to dihydromyricetin (DHM) by *F3',5'H* (Brugliera et al., 1999; Holton et al., 1993). *Dihydroflavonol 4-reductase* (*DFR*) reduces three components of dihydroflavonols to leucoanthocyanidins, the precursor of anthocyanidins. Those anthocyanidins -- pelargonidin, cyanidin, and delphinidin – are responsible for orange, red, and blue flower colors, respectively (Tanaka et al., 1998).

Here, we generated plants of transgenic chrysanthemums that were transformed with petunia *F3',5'H*. Our objective was to determine how tissues could be decolorized during flower development, and to investigate the relationship with *F3'H*.

MATERIALS AND METHODS

Plant Materials and Culturing Conditions

Seeds of chrysanthemum (*Dendranthema grandiflora* (Ramat.) Tzvelev) cv. Fashion Scarlet Shadow were aseptically germinated on an MS (Murashige and Skoog, 1962) basal medium. After 7 d, the cotyledons were excised from the seedlings and pre-cultured for 3 to 5 d on an MS medium supplemented with 4 mg L^{-1} 6-benzyladenine (BA) and 0.1 mg L^{-1} α-naphthalaneacetic acid (NAA) prior to co-cultivation with *Agrobacterium*.

The culture medium used for all experiments consisted of MS inorganic salts, 100 mg L^{-1} myo-inositol, 0.4 mg L^{-1} thiamine·HCl, 3% (w/v) sucrose, and 0.4% (w/v) Gelrite. Unless

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otherwise mentioned, all cultures were incubated at 25°C under a 16-h photoperiod provided from cool-white fluorescent lamps (approx. 30 μmol m^{-2} s^{-1}).

Chrysanthemum Transformation

Petunia *F3',5'H* cDNA (Florigene, Ltd.) was introduced into the plant expression vector pCAM (Invitrogen, USA), which carries *nptII* as a selectable marker and is under the control of the CaMV 35S promoter. This pMBP construct (Fig. 1) was then introduced into *Agrobacterium tumefaciens* LBA4404.

Cultured *A. tumefaciens* LBA4404 carrying pMBP was collected by centrifugation at 3,000 Xg for 20 min at room temperature and was re-suspended to a final OD_{600} of 0.3. Pre-cultured cotyledon explants were immersed into this bacterial suspension. After 30 min, they were blotted on filter paper and placed on a solid MS basal medium. Following 72 h of co-culturing in the dark, the explants were transferred to an MS medium supplemented with 4 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 400 mg L^{-11} carbenicillin, and 100 mg L^{-1} kanamycin.

Kanamycin-resistant adventitious shoots were excised from the explants and re-selected by transferring them to an MS medium containing 4 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 200 mg L^{-1} carbenicillin, and 150 mg L^{-1} kanamycin. They were then rooted on a half-strength MS medium with 0.1 mg L^{-1} NAA. Regenerated plantlets were acclimated, transplanted to potting soil, and maintained in a growth chamber (25°C, 16-h photoperiod from cool-white fluorescent lamps at approx. 100 μmol m^{-2} s^{-1}). To accelerate their flowering, transgenic plants were exposed to short-day conditions (30°C day/25°C night and 9-h photoperiod).

Southern Blot Analysis

For Southern blot analysis, genomic DNAs from both wild-type and putative transgenic chrysanthemum plants were purified with the DNeasy plant Mini Kit (Qiagen, Germany) according to the manufacturer's guidelines. As our probe, the 0.88-kb *F3',5'H* gene fragment was labeled with [α-^{32}P] dCTP. Purified genomic DNA was digested with *Bam*HI, separated on a 1% agarose gel, and transferred to a Zeta-Probe GT Blotting membrane (Bio-Red). The blots were probed overnight at 58°C in 0.25 M sodium phosphate (pH 7.2) buffer and 7% (w/v) SDS, then washed twice at 58°C for 10 min with 20 mM sodium phosphate (pH 7.2) buffer containing 5% SDS.

RT-PCR

To determine the expression of *F3'H* and *F3',5'H* in transgenic chrysanthemum plants, total RNA was purified with an RNeasy plant Mini Kit, then reverse-transcribed via the ThermoScript RT-PCR System (Invitrogen, USA). RT-PCR analysis was performed as follows: 94°C for 5 min; followed by 26, 28, or 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The primer pairs for petunia *F3',5'H* were 5'-GTT GCT TCT ACC CCT GAT GC-3' and 5'-TGG CCC CTC TCG ACT CAT ATC-3'; for chrysanthemum *F3'H*, 5'-CGC AAT GTC CGA ACT CAT TC-3' and 5'-CGA GGA GTC TGG ATC CCT TA-3'.

UV-Spectrometric Analysis of Anthocyanins

To quantitatively analyze anthocyanin contents, we divided the steps of petal development into four stages: flower bud formation, early flowering, mid-flowering, and fully opened flowers. Anthocyanidin pigments were extracted by incubating approximately 10 mg (fresh weight) of petals in 150 mL of an extraction solution (1% HCl and 70% methanol) at 25°C for 3 h, then centrifuging them at 12,000 rpm for 10 min. The supernatant was evaluated in a UV-Vis spectrometer.

RESULTS

Genetic Transformation of Chrysanthemum

Chrysanthemum normally lacks the activity of *F3',5'H* that is responsible for the conversion of both DHK and DHQ to DHM, and plants do not produce delphinidin-type anthocyanin. Therefore, to generate transgenic explants that expressed F3',5'H, we co-cultivated excised cotyledons with *A. tumefaciens* LBA4404 carrying pMBP (Fig. 1). Kanamycin-resistant adventitious shoots were obtained at a transformation frequency of 6.3% after 4 weeks of culture (Fig. 2A, B). To avoid wild-type escapes, these adventitious shoots were transferred to MS media containing 4 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 200 mg L^{-1} carbenicillin, and 150 mg L^{-1} kanamycin, and were sub-cultured at 3-week intervals (Fig. 2C). After 4 to 6 rounds, the surviving shoots were transferred to a half-strength MS basal medium, where they proliferated (Fig. 2D). These were then excised and rooted on a half-strength MS basal medium supplemented with 0.1 mg L^{-1} NAA (Fig. 1E). After 4 weeks, putative transgenic plants (approx. 5 cm tall) were successfully transplanted to potting soil and grown to maturity in a greenhouse (Fig. 2F, G).

Expression of Endogenous F3'H and Transgenic F3',5'H in Transgenic Plants

To verify integration of *F3',5'H*, genomic DNAs purified

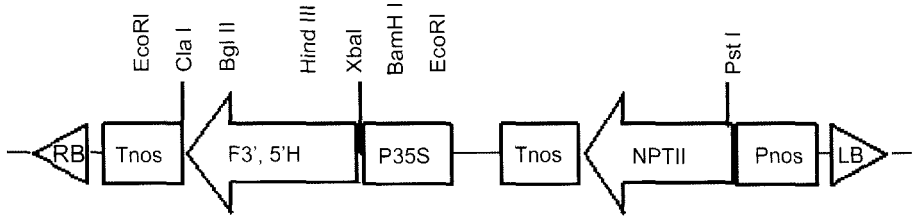

Figure 1. Schematic representation of binary plasmid pMBP. Petunia *F3', 5'H* gene was introduced into plant expression vector pCAM, which carries *nptII* as selectable marker and is under control of CaMV 35S promoter.

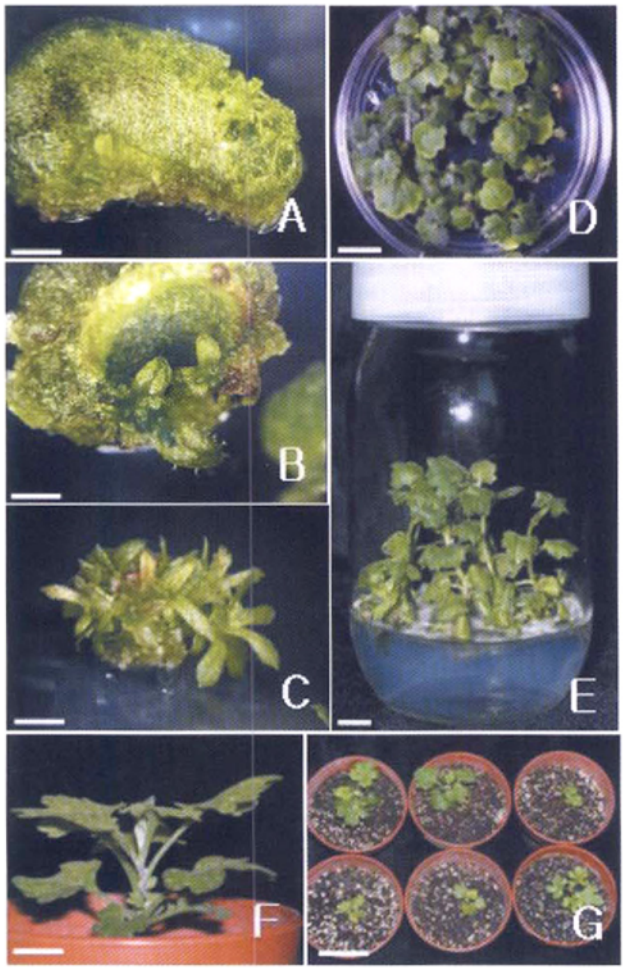

Figure 2. Generation of transgenic chrysanthemum transformed with petunia F3′,5′H. **A, B,** Kanamycin-resistant adventitious shoots formed on cotyledon explants cultivated on selection medium supplemented with 4 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 400 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin (scale bar = 2 mm); **C**, Production of kanamycin-resistant adventitious shoots (scale bar = 1 cm); **D**, Elongation of kanamycin-resistant adventitious shoots (scale bar = 1 cm); **E**, Rooting of transgenic adventitious shoots (scale bar = 1 cm); **F, G,** Transgenic plants after transfer to soil in glasshouse (scale bar = 1 cm).

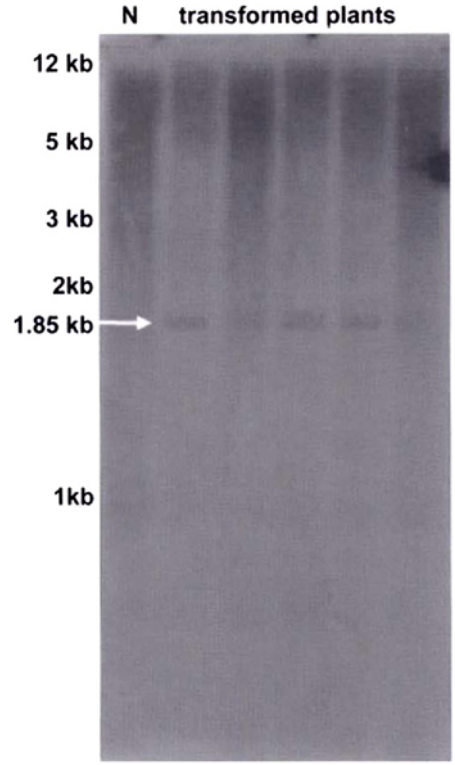

Figure 3. Southern analysis of genomic DNA extracted from 5 transgenic chrysanthemum lines. Purified genomic DNAs of wild-type and transgenic plants were digested with *Bam*HI, separated on agarose gels, and transferred to Zeta-Probe GT Blotting membranes. The 0.88-kb *F3′,5′H* gene fragment was labeled with [α-^{32}P] dCTP as probe. Lane N: wild-type plant; Lanes 2-6: transgenic plants. Each regenerated transgenic plant was derived from single transgenic cell line.

from the wild type (WT) and putative transgenics were digested with *Bam*HI and subsequently hybridized with the labeled *F3′,5′H* gene fragment. A single hit was detected in all tested transgenic lines in our Southern blot analysis (Fig. 3). This indicated that a single copy of that gene had been successfully incorporated into the chrysanthemum genome.

We performed RT-PCR to measure the expression of endogenous *F3′H* and the transgenic *F3′,5′H* gene in WT and transgenic plants. Expression of the former was dramatically decreased in the petals of both genotypes during flower development (Fig. 4). Moreover, *F3′,5′H* was expressed at a similar level in the transgenic plants, but not in the WT. These data demonstrated that both genes were expressed simultaneously in the transgenics, thereby suggesting that the biological properties of transgenic *F3′,5′H* and endogenous *F3′H* can affect the anthocyanin biosynthesis pathway as well as flower color in transformed chrysanthemums.

Analysis of Anthocyanin in Transgenic Plants

Flowers on plants that resulted from transformation with petunia *F3′,5′H* showed variations in coloring during their development. At floral bud-forming Stage 1, both WT and transgenic plants produced red-colored buds (Fig. 5). Petals from the WT then maintained that color throughout the remaining stages. In contrast, from Stage 2 through Stage 4, petal color on the transgenics dramatically changed from red to bright orange to, finally, yellow. This suggested that the transgenic *F3′,5′H* caused the rapid decolorization of petals. However, flowering time and the number of petals did not differ significantly between the WT and transgenic plants.

It is difficult to analyze and measure anthocyanins because of their ability to undergo structural transformations and the complexity of their reaction kinetics. Thin-layer chromatography and UV-Vis spectroscopy have traditionally been used for identifying such pigments (Mazza et al., 2004). For example, the blue or red sepals of hydrangeas may be distinguished by the absorption spectra of UV-Vis spectroscopy (Yoshida et al., 2003)

Here, we evaluated the absorption spectra for each anthocyanidin from the chrysanthemum tissues. One, pelargonidin, showed a high peak at 520 nm, whereas λ_{max} of

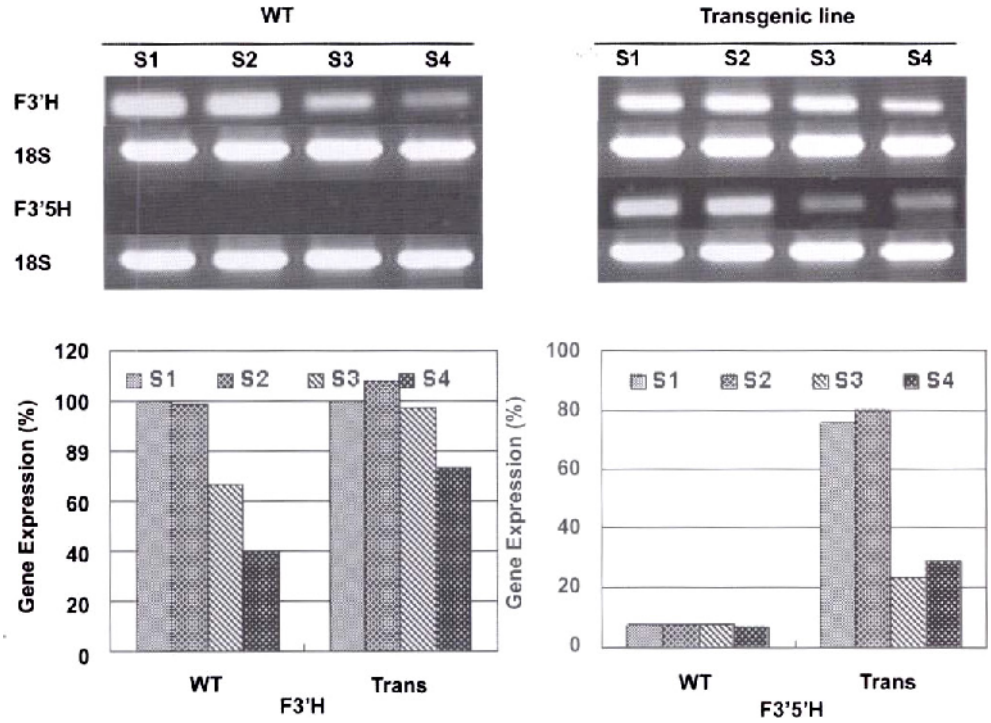

Figure 4. Expression of *F3'H* and *F3',5'H* in petals of wild-type and transgenic chrysanthemum. S1, S2, S3, and S4 indicate stage of flower development. Graph presents relative levels of *F3'H* and *F3',5'H* expression.

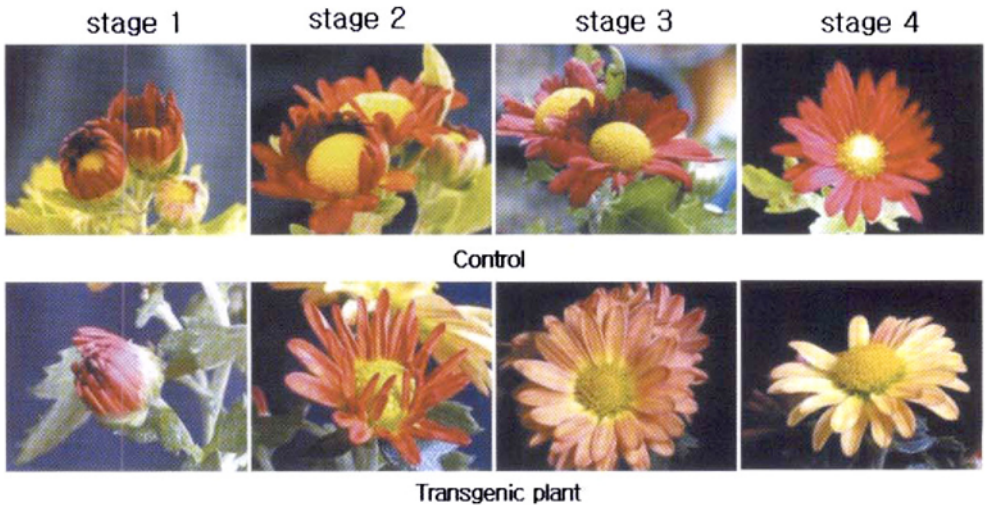

Figure 5. Petal color changes while buds were progressing to fully opened flowers, as recorded at different developmental stages in wild-type and transgenic plants: Stage 1, flower bud formation; Stage 2, early flowering; Stage 3, middle of flowering phase; and Stage 4, fully opened flower.

cyanidin and delphinidin was 535 nm and 586 nm, respectively (data not shown). To determine whether *F3'H* and/or *F3',5'H* reduced dihydrokaempferol to dihydroquercetin and/or dihydromyricetin, we used a UV-Vis spectrophotometer (range of 400 to 600 nm) to measure the extracts from flower petals of WT and transgenic plants. Extracts from both genotypes had high peaks at 535 nm (Fig. 6), suggesting that the major component was a cyanidin-type anthocyanidin rather than a delphinidin-type.

In WT petals, the cyanidin-type anthocyanidin began to be accumulated during the formation of flower buds (Stage 1, absorbance units: 0.5) and was maintained up to Stage 3 (absorbance units: 0.6). Even though the wild-type plants continued to show red flowers at Stage 4, anthocyanidin contents decreased by about 30% (Figs. 5, 6). In comparison, the transgenic plants produced only half as much anthocyanidin as the WT in Stage 1, and accumulations declined significantly from Stage 1 to Stage 4, at which time the absorbance units were almost nil (Fig. 6). Therefore, this spectrometric analysis demonstrated that transgenic petals lost anthocyanidin more quickly than did the wild type.

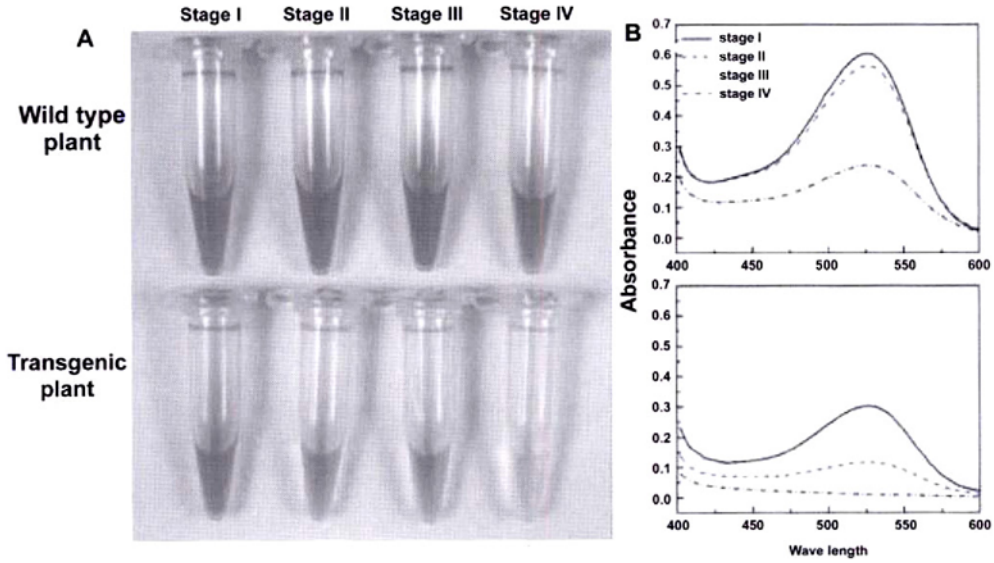

Figure 6. Spectrophotometer analysis of flower petals. **A**, Pigment extracts from wild-type and transgenic chrysanthemum. **B**, Absorption spectra of petal extracts.

DISCUSSION

Anthocyanin accumulation in the petal depends on a balance among many flavonoid biosynthesis enzymes (Forkmann, 1991; Holton et al., 1993). Synthesis of anthocyanidins is mainly determined by the enzyme activities of *F3'H* and *F3',5'H*. The latter, a member of the cytochrome P450 family, is the key enzyme in the synthesis of flavonol 3',5'-hydroxylated anthocyanins, which are generally required for the production of blue or purple flowers. *F3',5'H* competes with many enzymes for substrates, e.g., naringenin, DHK, eriodictyol, and DHQ (Forkmann and Martens, 2001; Shimada et al., 1999; Winkel-Shirley, 2001). However, our study showed that, although that enzyme was active in the transformants, it was not effective in changing the petal color of chrysanthemum. It is interesting to note the shift in petal colors in our transgenic plants (Fig. 5). A number of explanations are possible for why they did not accumulate delphinidin, but did accelerate their decolorization. Because both *F3'H* and *F3',5'H* utilize the same substrate in the flavonoid biosynthetic pathway (Forkmann and Martens, 2001; Winkel-Shirley, 2001), this may lead to similar demands between the two (Baysdorfer and Robinson, 1985). Likewise, co-expression of these genes in transgenic chrysanthemums may have resulted in strong competition for their enzymatic functioning, perhaps exhausting the substrate pools for anthocyanin accumulation. This ultimately would stimulate decolorization and cause anthocyanin contents to be lower in the transgenic plants.

Shimada et al. (1999) also have observed that transformed petunia plants show a dramatic shift in flower color, from pink to magenta, when the content of 3',5'-hydroxylated anthocyanins is high. However, the lack of petal color change in transgenic tobacco, compared with our chrysanthemum, suggests that the balance of anthocyanin biosynthetic enzymes is a mechanism that explains low delphinidin contents in the former (Shimada et al., 1999).

Johnson et al. (2001) have reported that petunia does not produce orange flowers because of the substrate specificity of *dihydroflavonol 4-reductase* (*DFR*) for the dihydro-flavonols. Not only petunia *DFR* but other *DFRs* from different plant species are thought to have an amino acid region that dictates substrate preference. Therefore, in addition to the hydroxylation of DHK by *F3'H* or *F3',5'H*, the reduction of dihydroflavonols by DFR can be an important enzymatic step toward flower color determination (Holton et al., 1993; Brugliera et al., 1999; Johnson et al., 2001). Two corporations -- Florigen Ltd. (Melbourne, Australia) and Suntory Ltd. (Osaka Japan) -- are now producing violet carnations by introducing the petunia *F3',5'H* and *DFR* genes into standard white carnations.

In conclusion, we have shown here that chrysanthemum transformed with *F3',5'H* can accumulate DHM, but cannot efficiently catalyze the reduction of colorless DHM because of substrate specificity by DFR. Moreover, the decolorization of flower petals from chrysanthemum transformed with petunia *F3',5'H* is caused by competition between *F3'H* and *F3',5'H*. Future studies that focus on the transformation of *F3',5'H* and substrate-specific *DFR* for DHM will be helpful in generating transgenic chrysanthemums with petal color that rely on the delphinidin-type anthocyanidin.

ACKNOWLEDGEMENTS

This work was supported by a grant (No. ABM0030211) to JRL from the BioGreen 21 Program, funded by the Korean Rural Development Agency; a grant to JRL from the Crop Functional Genomics Center of the 21st Century Frontier Research Program; and a grant to JRL from the Korea Science and Engineering Foundation through the Plant Metabolism Research Center of Kyung Hee University, funded by the Korean Ministry of Science and Technology.

Received September 13, 2007; accepted October 4, 2007.

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