Original Paper

Molecular characterization of the flavonoid biosynthetic pathway and flower color modification of *Nierembergia* sp

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Abstract *Nierembergia* sp., a popular floricultural species, only has violet and white flower color and lacks pink to red. To elucidate the reason, we analyzed its flavonoids. Its major anthocyanin was determined to be delphinidin 3-*O*-(6-*O*-(4-*O*-(4-*O*-(6-*O*-caffeoyl-*β*-D-glucopyranosyl)-*p*-coumaroyl)-*α*-L-rhamnosyl)-*β*-D-glucopyranosyl)-5-*O*-*β*-D-glucopyranoside. The petals rarely contained cyanidin and pelargonidin, and they contained more flavonols than anthocyanins. We also characterized the biosynthetic pathway by cloning the cDNAs encoding enzymes involved in the flavonoid biosynthesis pathway: chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase (F3′5′H), dihydroflavonol 4-reductase (DFR), flavonol synthase (FLS), and UDP-rhamnose: anthocyanidin 3-glucoside rhamnosyltransferase (3RT). Northern blot analysis revealed that the expressions of *CHS*, *F3′5′H*, *DFR*, and *3RT* genes were coordinately regulated in parallel with anthocyanin accumulation in the petals, indicating that anthocyanin biosynthesis is transcriptionally regulated; on the other hand, the transcripts of the *F3′H* gene were rarely detected. Antisense suppression of the *F3′5′H* gene decreased the amount of *F3′5′H* transcripts and that of delphinidin. It was noteworthy that the color of the transgenic flower changed from violet to white rather than to reddish, which was the expectation.

Key words: Anthocyanin, flavonoids, flower color, metabolic engineering, *Nierembergia*.

Flower color is one of the most important characteristics of floricultural crops. Flower color originates primarily from flavonoids and their colored class compounds, anthocyanins (Harborne and Williams 2000). In addition to the anthocyanin structures, co-existing co-pigments such as flavonols and flavones, metal ions and vacuolar pH give infinite array of flower colors from orange to blue (Harborne and Williams 2000, Tanaka et al. 2005). Anthocyanin color and, thus, flower color tend to be bluer when the number of hydroxy groups on the B-ring and/or attached aromatic acyl groups increases. Blue flowers usually contain delphinidin-based anthocyanins modified with plural aromatic acyl groups (Honda and Saito 2002). Co-pigments, such as flavonols, also contribute to bluing by forming a complex with anthocyanins (Goto and Kondo 1991). The flavonoid biosynthetic pathway leading to aglycons is well established and conserved among plant species (Figure 1), and most structural genes leading to their synthesis have been obtained (Tanaka et al. 2005).

Hybridization breeding has contributed to increasing the variety of available colors. However, it is still uncommon for a plant species to have a wide variety of colors because the factors influencing flower color are, as a rule, genetically regulated. It is difficult to increase the variety of flower color by using hybridization breeding alone because of the limited gene source within a species (Tanaka et al. 2005). For example, carnations and roses do not have violet/blue varieties due to lack of the flavonoid 3′,5′-hydroxylase (*F3′5′H*) gene, which is necessary to synthesize delphinidin (Holton et al. 1993a). Petunia and tobacco do not accumulate pelargonidin due to the substrate specificity of their dihydroflavonol 4-reductase (DFR); as a result, they lack
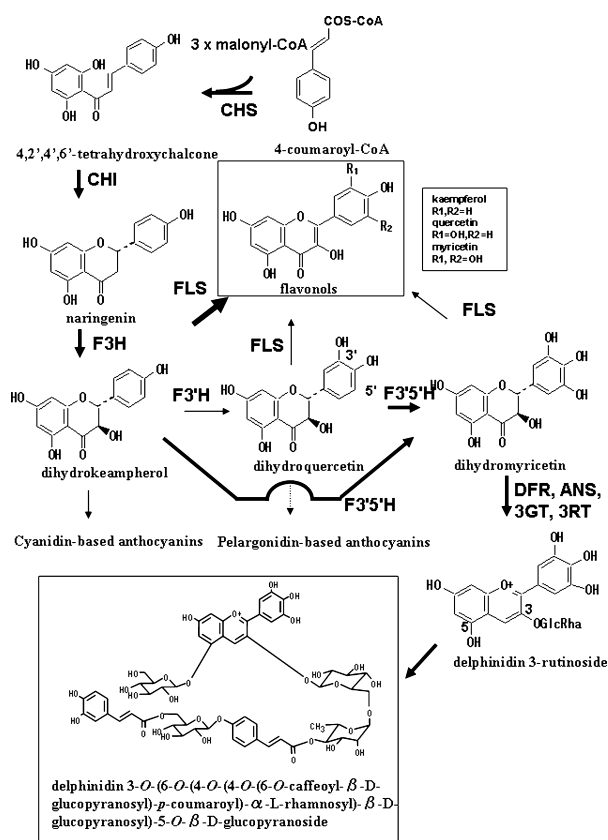
Figure 1. Anthocyanin biosynthesis pathways in *Nierembergia* sp. The dominance of delphinidin-based anthocyanins implies that the metabolic flux to delphinidin is a major pathway in *Nierembergia*. Presumably weak and absent pathways in *Nierembergia* are shown by thin and dotted arrows, respectively. See the text for details. The structure of the most abundant anthocyanin in NPLB is also shown. CHS, chalcone synthase; F3H, flavanone 3?-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, anthocyanidin 3-glucosyltransferase; 3RT, anthocyanin 3-glucoside-rhamnosyl transferase; Glc, glucose; Rha, rhamonose; FLS, flavonol synthase.

brick-red varieties (Forkmann and Ruhnau 1987). On the other hand, genetic engineering of the flavonoid pathway has successfully added novel flower color varieties in those cases in which efficient transformation systems for the target species and molecular tools for flower colour modification are available (Tanaka et al. 2005; Forkmann and Martens 2001). Violet/blue carnations and roses (Holton 1996; Tanaka et al. 2005) and brick-red petunias (Meyer et al. 1987; Helariutta et al. 1993; Tanaka et al. 1995; Tsuda et al. 2004) have been obtained, and such carnations are on the market. *Nierembergia* sp., a popular bedding and potting ornamental plant belonging to the family *Solanaceae*, only has violet and white flower varieties. Hybridization breeding has so far failed to generate pink, red, and orange flower colors, although such color varieties would command high market values.

In order to clarify the reason that pink/red varieties are lacking in this genus from the viewpoint of flavonoid

chemistry and molecular biology, we analyzed the petal flavonoids, determined the structure of their major anthocyanin, and cloned the flavonoid biosynthetic genes from the petals. Such characterization is essential to develop a strategy to increase the number of flower color varieties. We also established the transformation system of *Nierembergia* and modified its flower color by down-regulating the expression of the *F3′5′H* gene.

Materials and methods

Plant materials

Nierembergia sp. cultivar Fairy Bells Patio Light Blue (NPLB, Suntory Flowers, Ltd.) and cultivar Fairy Bells Patio Blue (NPB, Suntory Flowers, Ltd.) were grown in contained glasshouses for genetically modified plants under a 16 h day^{-1} light condition at 25°C. NPLB flowers were classified into five developmental stages: stage 1, small-closed buds with white petals (about 9 mm length); stage 2, large closed buds with white petals (13 mm); stage 3, large closed buds with pale blue petals (17 mm); stage 4, large closed buds with blue petals (22 mm); stage 5, opened flowers (28 mm) (Figure 2A).

Flavonoid analysis and structure determination

Anthocyanidins and flavonols were analyzed as previously described (Murakami et al. 2004). The anthocyanin purification and structural determination were essentially as previously described (Fukui et al. 1998). Anthocyanins were extracted from NPLB flowers (27 g, wet weight) with 50% aqueous acetonitrile containing 0.1% TFA and partially fractionated by HP-20 column chromatography. The crude pigment fraction was repeatedly purified by reverse-phase HPLC to obtain 20 mg of the most abundant anthocyanin. ^{1}H NMR, ^{13}C NMR, DQF-COSY, TOCSY, ^{1}H{^{13}C}-HSQC, and ^{1}H{^{13}C}-HMBC spectra were obtained using a DMX-750 spectrometer (Bruker Biospin, Germany). The anthocyanin was dissolved in 10% TFA-d/CD3OD. FAB-MS was recorded on a JMS-HX110/HX110A tandem mass spectrometer (JEOL, Japan) in the positive mode.

Cloning of the genes involved in the flavonoid biosynthesis of Nierembergia sp.

The molecular biological procedures have been described previously (Fukuchi-Mizutani et al. 2003), except that all DNA labeling and detection procedures were carried out with a DIG system (Roche) following the manufacturer's protocols. A petal cDNA library derived from NPLB was constructed using the directional λZAP-cDNA synthesis kit (Stratagene). The library was screened with petunia flavanone 3-hydroxylase (*F3H*, Holton, unpublished results; the sequence was the same as that reported by Britsch et al. 1992), flavonol synthase (*FLS*, Holton et al. 1993b),

F3′5′H (Holton et al. 1993a), flavonoid 3′-hydroxylase (*F3′H*, Brugliera et al. 1999), dihydroflavonol 4-hydroxylase (*DFR*, Holton 1996) and anthocyanidin 3-glucoside rhamnosyltransferase (*3RT*, Brugliera et al. 1994), and rose *CHS* (Tanaka et al. 2003) cDNAs.

Construction of binary vectors

El235S, an enhanced cauliflower mosaic virus 35S promoter from pBE2113-GUS (Mitsuhara et al. 1996), was used to transcribe the *F3′5′H* gene in NPB. An *Xho*I linker was inserted into the *Sna*BI site of pBE2113-GUS. This plasmid was digested with *Sac*I, blunted, and ligated with a *Bam*HI linker. The resultant plasmid was digested with *Eco*RI-*Hin*dIII, and this fragment was inserted into the *Eco*RI-*Hin*dIII site of pBinPLUS (van Engelen et al. 1995) to yield pSPB505. The 1.4 kb *Bam*HI-*Xho*I fragment of pSPB705 containing the coding region of NPLB F3′5′H and a 12 kb *Bam*HI-*Xho*I fragment from pSPB505 were ligated.

Transformation of Nierembergia *sp.*

The shoot apex of the plants grown in the greenhouse was surface-sterilized for 5 min with a 1% sodium hypochlorite solution containing a drop of Triton X100 and rinsed three times with sterile deionized water. The rooting was carried out in an MS (Murashige and Skoog 1962) medium (including $30 \mathrm{~g~l}^{-1}$ sucrose, $3 \mathrm{~g}^{-1}$ AgarGel, and 0.1 mg/l BA), and the cultures were kept at 25°C. The shoots were subcultured in an MS medium containing $30 \mathrm{~g}^{-1}$ sucrose and $5 \mathrm{~g}^{-1}$ AgarGel at one-month intervals (Shizukawa and Mii 1997). The stem segments were cut at about 2-mm lengths and pre-cultured in an N7 medium (an MS medium containing $30 \mathrm{~g~l}^{-1}$ sucrose, $5 \mathrm{~g~l}^{-1}$ AgarGel, $0.05 \mathrm{~mg~l}^{-1}$ BA, and $0.01 \mathrm{~mg~l}^{-1}$ NAA). *A. tumefaciens* strain AGL0 (Lazo et al. 1991) harboring the binary vector plasmid pSPB711 was cultured for 24 h at 28°C, harvested, and resuspended to 5×10^8 cell ml^{-1} in an MS liquid medium containing 0.1 mM acetosyringone. The explants were soaked in an *Agrobacterium* suspension for 5 min, blotted dry, and then transferred onto a filter paper in an N7 medium in the dark at 25°C for 3 days. After co-cultivation, the explants were transferred to a regeneration medium (an N7 medium containing $200 \mathrm{~mg~l}^{-1}$ kanamycin and $400 \mathrm{~mg~l}^{-1}$ carbenicillin) and maintained by sub-culturing at 2-week intervals. Putative transformants were grown in an MS medium containing $100 \mathrm{~mg~l}^{-1}$ kanamycin and $400 \mathrm{~mg~l}^{-1}$ carbenicillin, and, after rooting, the transformants were transferred to soil.

Results and discussion

Analysis of Nierembergia *flavonoids*

The anthocyanidin analysis of NPLB petals at stage 5 revealed that they almost exclusively contained delphinidin ($0.49 \mathrm{~mg~g}^{-1}$ petals) and a small amount of cyanidin (less than 1% of delphinidin). Kaemferol is the most abundant flavonol ($0.96 \mathrm{~mg~g}^{-1}$ petals), followed by quercetin ($0.18 \mathrm{~mg~g}^{-1}$ petals) and myricetin ($0.017 \mathrm{~mg~g}^{-1}$ petals). Flavonols are common co-pigments and shift flower color toward blue (Holton et al. 1993b). The presence of flavonols is likely to contribute to the blue flower color in *Nierembergia*.

After various spectral analyses, the structure was determined to be delphinidin 3-*O*-(6-*O*-(4-*O*-(4-*O*-(6-*O*-caffeoyl-β-D-glucopyranosyl)-*p*-coumaroyl)-α-L-rhamnosyl)-β-D-glucopyranosyl)-5-*O*-β-D-glucopyranoside (Figure 1, data not shown). NPB petals contained the same anthocyanin (data not shown). Petunia, a species closely related to *Nierembergia*, is known to contain similar anthocyanins, except that its aglycon is malvidin (Fukui et al. 1998). Fukui et al. (1998) suggested that the diacylated anthocyanins in petunia contribute to its blue flower color, and this may be applicable to *Nierembergia* flower color as well. The absence of petunidin and malvidin indicates that *Nierembergia* seems to lack anthocyanin methyltransferase, which petunia has.

The dominance of diacylated delphinidin-based anthocyanin, the absence of cyanidin and pelargonidin, and the presence of flavonols can explain the lack of pink varieties in *Nierembergia*.

Developmental and spatial accumulation of anthocyanidins and flavonols

Flavonols and anthocyanidins were quantified in the petals of the five developmental stages of NPLB petals (Figure 2A, B). The accumulation of flavonols preceded that of delphinidin in petal development, as reported for many plants, including carnation and petunia (Forkmann and Heller, 1999). NPLB accumulates more flavonols than anthocyanidins, which indicates that its metabolic flux toward flavonols seems to be larger than that toward anthocyanidins, especially at the earlier stages.

Isolation of cDNA encoding structural genes involved in flavonoid biosynthesis

About 3×10^5 plaques of the cDNA library of NPLB petals were screened with petunia flavanone 3-hydroxylase (*F3H*), *F3′5′H*, flavonoid 3′-hydroxylase (*F3′H*), flavonol synthase (*FLS*), DFR, UDP-rhamnose: anthocyanin 3-glucoside-rhamnosyl transferase (*3RT*), and rose chalcone synthase (*CHS*) cDNAs. Dozens of positive plaques were identified with each of the probes. The clones having the longest cDNA and the best homology to the respective reported cDNA clones were completely sequenced. Among them, the cDNAs of *CHS* (accession number in the DNA database, AB078513), *F3H* (AB078513), *F3′5′H* (AB078514), *FLS* (AB078512), *DFR* (AB078510), and *3RT* (AB078511)
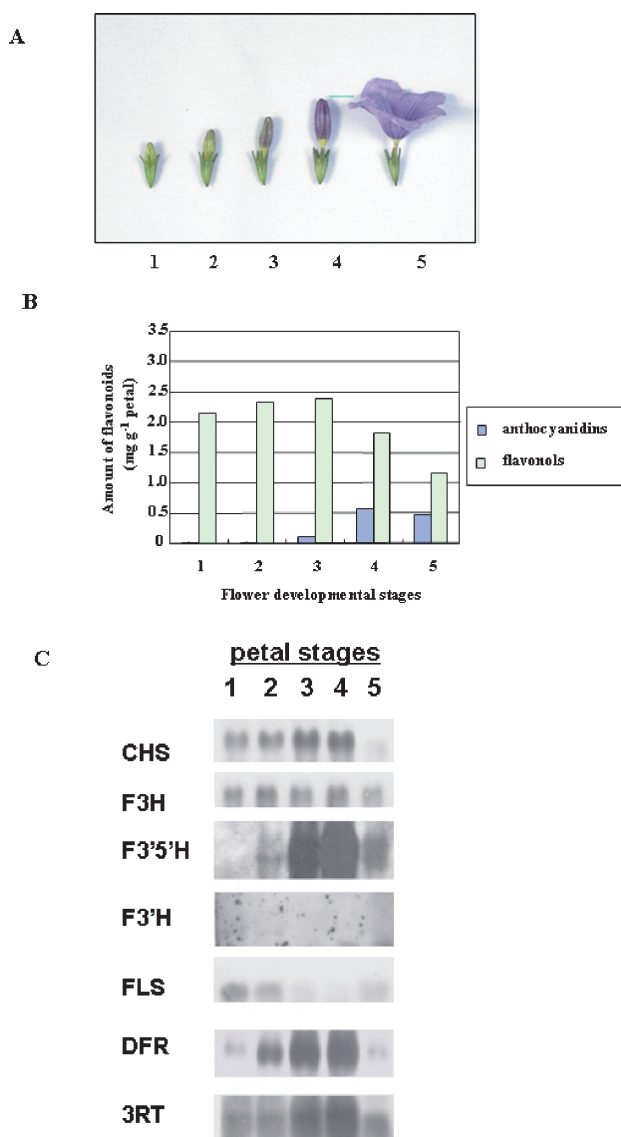
Figure 2.   Accumulation of anthocyanidins and flavonols in NPLB flowers. A: Developmental stages of NPLB flowers: stage 1, small closed buds with white petals; stage 2, closed buds with white petals and elongated tube; stage 3, closed buds with pale petals; stage 4, closed buds with purple petals; and stage 5, open flowers. B: Accumulation of anthocyanidins and flavonols at the five developmental stages of flowers. C: Total RNA was extracted from the five developmental stages of flowers and subjected to Northern analysis.

contained putative full-length open reading frames on the basis of a comparison with the reported counterpart genes. We could only obtain a partial cDNA from *F3′H*. All of these cDNAs had high identities with their counterpart petunia genes. This is reasonable because both *Nierembergia* and petunia belong to the same family, *Solanaceae*.

Petunia plants contain more than ten *CHS* molecular species. From among them, only *CHS-A* and *CHS-J* are expressed in the petals, and *CHS-A* plays predominant roles in flavonoid biosynthesis in the petals (Koes et al. 1989). Interestingly, *Nierembergia* CHS is closest to

petunia CHS-J (94.5% amino acid identity), followed by tomato CHS (92.5%), potato CHS (92.3%), and petunia CHS-A (91.3%).

The deduced amino acid sequences of *Nierembergia F3′5′H* and *F3′H* had the typical features of cytochrome P450 enzymes, including the conserved and essential heme-binding motif (Schuler and Werck-Reichhart 2003). *Nierembergia* F3′5′H, designated CYP75A11 in the P450 nomenclature (Nelson 1999), exhibited 88.1% and 87.8% amino acid identities to the two petunia F3′5′Hs (Holton et al. 1993a).

F3H and FLS belong to a 2-oxoglutarate-dependent dioxygenase family that requires 2-oxoglutarate and ferrous iron for its activities (Forkmann and Heller 1999). *Nierembergia* F3H and FLS are most homologous to petunia F3H (93.2%) and potato FLS (81.8%), respectively. The sequence identity between *Nierembergia* F3H and FLS was 41%. This is consistent with a previous study, which reported that F3H and FLS belong to different subfamilies in the 2-oxoglutarate-dependent dioxygenase family (Tanaka et al. 2003). NPLB contained only a small amount of myricetin in colored petals in spite of the high expression of the *F3′5′H* gene (Figure 2C). We speculate that this is probably because *Nierembergia* FLS does not efficiently utilize dihydromyricetin, similarly to petunia FLS, which cannot recognize dihydromyricetin as a substrate (Forkmann et al. 1985).

The substrate specificity of DFR sometimes determines the anthocyanin types accumulated in the species and, thus, flower color. Petunia, tobacco, and cymbidium DFRs have strong substrate preference and cannot reduce dihydrokaempferol (DHK). This is the reason that these species do not accumulate pelargonidin and, thus, do not produce orange flowers (Forkmann and Ruhnau 1987; Johnson et al. 2001). Johnson et al. (2001) proposed that the 134th asparagine residue of gerbera DFR is conserved in DHK-accepting DFRs, while petunia DFR has an aspartic acid residue at the corresponding position. *Nierembergia* DFR also has an aspartic acid residue at the corresponding 137th residue. *Nierembergia* belongs to the *Solanaceae* family, to which petunia and tobacco belong. These findings suggest that *Nierembergia* DFR does not accept DHK. This could be the reason that we did not detect pelargonidin in *Nierembergia* cultivars (data not shown). On the other hand, maize, gerbera, and rose DFRs can catalyze the reduction of DHK, and petunia transformed with these DFR cDNAs produced pelargonidin-derived pigments (Meyer et al. 1987; Helariutta et al. 1993; Tanaka et al. 1995).

Transcriptional profile of the flavonoid biosynthetic genes of NB

The transcripts of the cloned flavonoid biosynthetic

genes (*CHS*, *F3H*, *F3′5′H*, *F3′H*, *FLS*, *DFR*, and *RT*) were analyzed for the five stages of petals by Northern blot analysis (Figure 2C). The expression of the *FLS* gene preceded that of the *DFR* gene, which was consistent with the earlier accumulation of flavonols than anthocyanidins in petals (Figure 2B). The transcripts of the *CHS* and *F3H* genes were also detected at the earlier stages of the petals, which is reasonable because their expression is necessary to synthesize flavonols. The abundant transcripts of *F3′5′H*, *DFR*, and *RT* at stages 3 and 4 also agree with the accumulation profile of delphinidin. These results indicate that the flavonol and anthocyanin biosyntheses are transcriptionally and differently regulated, as reported for many plants, including petunia (Holton et al. 1993b). The transcripts of *F3′H* were rarely detected, which may be one reason that NB does not accumulate cyanidin.

Modification of flower color by the down-regulation of *F3′5′H* gene in Nierembergia

A binary vector, pSPB711, which contained *Nierembergia* F3′5′H cDNA in the antisense orientation under the control of the constitutive promoter, was constructed. The vector was introduced to NPB via *Agrobacterium*-mediated transformation. Sixty-three transgenic plants were obtained, and three lines, including NPB/711-9, showed a remarkable change in flower color (from blue to paler colors; Figure 3A). The level of *F3′5′H* transcripts of NPB/711-9 dramatically decreased from that of the host (Figure 3B), which confirmed that *F3′5′H* was successfully down-regulated in the transgenic plants by introducing the antisense construct. The amount of delphinidin decreased to less than one tenth in NPB/711-9 (Figure 3C). However, the amount of cyanidin did not increase in the transgenic plant, against our expectations, and only the amount of colorless kaempferol increased (Figure 3B), while the suppression of the F3′5′H gene in petunia and torenia led to cyanidin production and flower color changes (Tsuda et al. 2004; Ueyama et al. 2002, respectively). Thus, the low expression of F3′H and/or high expression FLS explain why the suppression of the *F3′5′H* gene did not increase the production of cyanidin. The low expression level of the *F3′H* gene was supported by the results of Northern blot analysis (Figure 2C). The absence of pelargonidin in NPB/711-9 may be due to the fact that *Nierembergia* DFR does not recognize DHK as a substrate. In other words, the reduction of FLS activity, the increase of F3′H activity, and/or the introduction of DFR that can reduce the DHK should lead to an increase of cyanidin/pelargonidin-based anthocyanins and, thus, to red/pink flowers, a project that is currently in progress in Suntory laboratory.

The flavonoid biosynthetic pathway and its modification have been well studied (Tanaka et al. 2005);
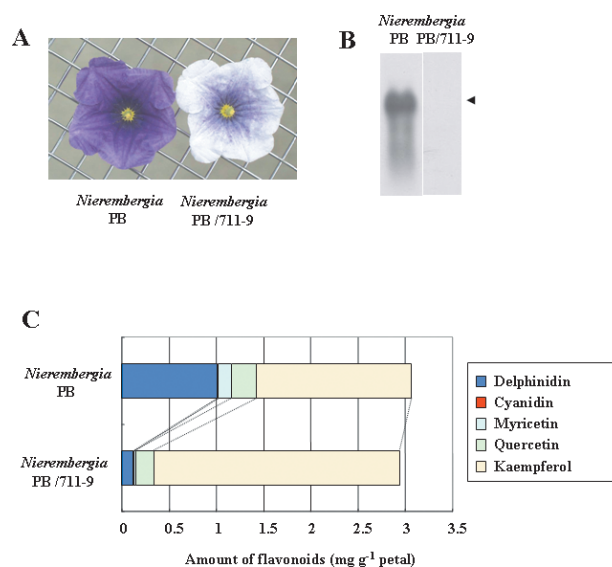

Figure 3. Transgenic plants transformed with the binary vector of the antisense *Nierembergia F3′5′H* gene. A: Phenotypic changes of flowers. Left: An NPB (host) flower. Right: A flower of the transgenic plant (NPB/711-9). B: Northern blot analysis of transgenic NPB/711-9. The arrowhead indicates the size of endogenous *F3′5′H* mRNA. The amount of F3′5′H mRNA in NPB/711-9 decreased. C: Their anthocyanidin and flavonol analysis. The amount of delphinidin decreased, and that of kaempferol increased.

however, in this study, we have been able to obtain the unexpected color changes mentioned above. The results indicate that the pathway of a target species must be characterized and that extensive engineering of the pathway is necessary to obtain desirable flower colors.

Acknowledgements

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