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Original Paper

Flower color modification of gentian plants by RNAi-mediated gene silencing

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Abstract RNA interference (RNAi) is an efficient and powerful technique for gene silencing compared with antisense and sense suppression. Here we report adaptation of RNAi technology to modify flower colors in gentian, targeted for suppression of three anthocyanin biosynthetic genes; chalcone synthase (*CHS*), anthocyanidin synthase (*ANS*) and flavonoid 3′,5′-hydroxylase (*F3′5′H*). The petals of transgenic gentian plants with a suppressed *CHS* gene exhibited pure white to pale-blue color, while those with a suppressed *ANS* gene showed only pale-blue. The suppression of the *F3′5′H* gene decreased delphinidin derivatives and increased cyanidin derivatives, and led to magenta flower colors. Northern blot analyses confirmed that all transgenic gentian plants showing typical phenotypes had strongly suppressed transcriptions of the targeted genes, corresponding with a change in anthocyanin accumulation and composition in their petals. Some rolCpro-CHSir transgenic gentians exhibited bicolor phenotypes with reduced anthocyanin accumulation along the vascular bundles. These data demonstrated that the suppression of anthocyanin biosynthetic genes by RNAi was successfully applied to gentian plants to change flower color, and this could be useful for designing novel flower color and patterns. Transgenic gentian plants produced in this study might be utilized as elite materials in the breeding of gentian plants in the near future.

Key words: Anthocyanidin synthase, chalcone synthase, flavonoid 3′,5′-hydroxylase, gentian, RNAi.

Gentians, *Gentiana triflora*, *Gentiana scabra* and their interspecific hybrid, are one of the most popular floricultural plants in Japan, and more than half of gentian production is from the Iwate prefecture. Gentians come into bloom from early summer to late autumn in Japan, and are often used as ornamental cut flowers. To further increase the demand for gentian flowers, the market requires the development of new cultivars with novel characteristics, such as flower color, shape, disease resistance and early flowering. In regard to flower color, Japanese gentians originally had unique brilliant blue flowers. Over the last decades, some gentian cultivars with white, pink and bicolor flowers have also been produced by conventional breeding. However, because the breeding of gentians began only in the 1960's and genetic variation is limited, there are few genetic resources available for breeding variations in flower color. Even if such variable gentians exist, it is difficult to use them as breeding materials, because gentian is highly heterologous and useful genotypes inherited as recessive traits do not become fixed easily. To overcome these problems, genetic engineering approaches are being applied to several ornamental plants (Forkmann and Martens 2001; Tanaka et al. 1998, 2005). For example, Florigene Ltd. and Suntory Ltd. have developed blue-flowered carnations using genetic engineering, and they are currently commercialized in North America, Australia and Japan (Tanaka et al. 2005).

We have also produced white-flowered transgenic gentians by suppressing the chalcone synthase (*CHS*) gene using antisense technology (Nishihara et al. 2006). In this case, only 3 of 17 independent transgenic lines displayed white-flowered phenotypes, but other transformants did not lead to successful suppression of *CHS* gene expression. Moreover, no transgenic gentian plants with suppressed expression of other anthocyanin biosynthetic genes, such as dihydroflavonol 4-reductase (*DFR*) and flavonoid 3′,5′-hydroxylase (F3′5′H) genes, have so far been obtained by antisense and sense suppression technology (Nishihara et al. unpublished results). The low frequency of down-regulation of the targeted genes was thought result from the use of both the cauliflower mosaic virus (CaMV) 35S promoter and antisense gene suppression technology. It was confirmed

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Abbreviations: ANS, anthocyanidin synthase; CaMV, cauliflower mosaic virus; CHS, chalcone synthase; F3′5′H, flavonoid 3′,5′-hydroxylase; HPLC, high performance liquid chromatography; RNAi, RNA interference
This article can be found at http://www.jspcmb.jp/

that the CaMV35S promoter underwent strong gene silencing by sequence-specific DNA methylation in gentian plants (Mishiba et al. 2005). By screening several promoters, we found that the *Agrobacterium rhizogenes rolC* promoter could avoid gene silencing and induce semi-constitutive gene expression in transgenic gentian plants (Mishiba et al. 2005). Sugaya and Uchimiya (1992) have reported that the activity of the *rolC* promoter was localized predominantly in vascular bundle tissues of transgenic tobacco plants. It is also known that RNA interference (RNAi) can induce more efficient and powerful gene silencing than antisense and co-suppression (Waterhouse et al. 1998; Chuang and Meyerowitz 2000). Recently, gene silencing by RNAi was also utilized to modify the flower color in some plant species, including petunia, torenia and tobacco plants (Tsuda et al. 2004; Nishihara et al. 2005; Nakamura et al. 2006; Nakatsuka et al. 2007a,b).

The deficiency of different catalytic steps in the anthocyanin biosynthetic pathway can produce various flower colors in transgenic petunia (Tsuda et al. 2004). In torenia plants the antisense suppression of *CHS* led to whiter flowers than did suppression of the dihydroflavonol 4-reductase (*DFR*) gene (Aida et al. 2000a, b). Thus, many researchers have reported white-flowered phenotypes obtained by the suppression of *CHS* or *DFR* genes using genetic engineering in floricultural plants. On the other hand, the suppression of *F3′5′H* genes, that encode a key enzyme in the biosynthesis of the blue pigment delphinidin, was reported to lead to a pink-flowered phenotype by blocking biosynthesis of the delphinidin pigments in torenia (Suzuki et al. 2000). A few examples were also reported on the suppression of other genes in anthocyanin biosynthetic pathway, such as chalcone isomerase (*CHI*), anthocyanidin synthase (*ANS*), and flavanone 3-hydroxylase (*F3H*) (Nishihara et al. 2005; Nakamura et al. 2006; Ono et al. 2006).

Here we report on the flower color modification of gentian plants by RNAi. We attempted to suppress three genes involved in anthocyanin biosynthetic pathway; *CHS*, *ANS* and *F3′5′H* (Figure 1). The flowers of transgenic gentian plants with each of the three genes suppressed by RNAi were obtained and analyzed in detail.

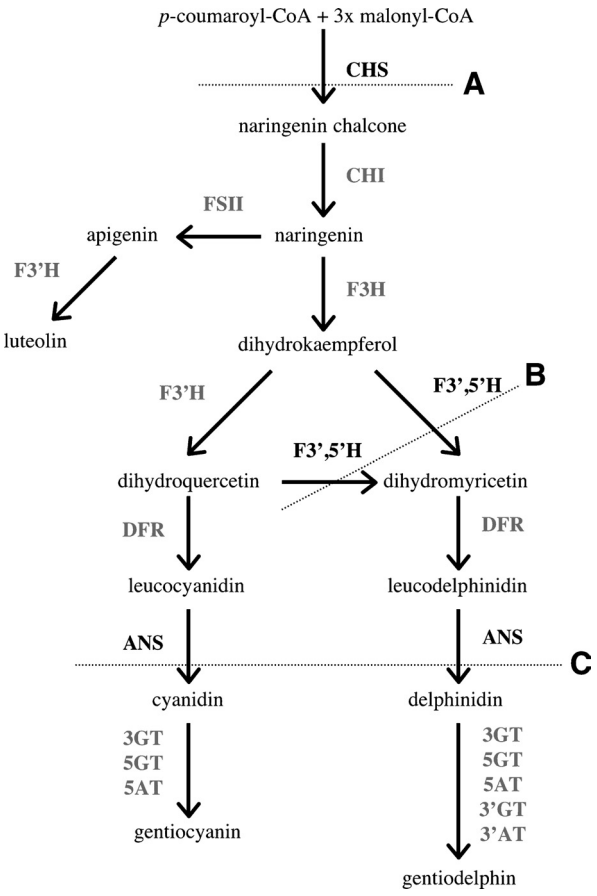

Figure 1. Illustration of flavonoid biosynthetic pathway in gentian plants. Putative anthocyanin biosynthetic pathway in gentian flowers is shown. Blue-flowered gentians accumulate mainly gentiodelphin pigment in their petals, whereas pink-flowered gentians accumulate gentiocyanin pigment. Catalytic steps of enzymes targeted for RNAi are indicated by A, B and C dashed lines. Targeted enzymes are shown in bold. Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose: anthocyanidin 3-*O*-glucosyltransferase; 5GT, UDP-glucose: anthocyanin 5-*O*-glucosyltransferase; 5AT, anthocyanin 5-aromatic acyltransferase; 3′GT, UDP-glucose: anthocyanin 3′-*O*-glucosyltransferase; 3′AT, anthocyanin 3′-aromatic acyltransferase; FSII, flavone synthase II.

Materials and methods

Construction of binary vectors for RNAi

To suppress several steps of the anthocyanin biosynthetic pathway in gentian plants, we selected three genes as targets of RNAi; *CHS* (Kobayashi et al. 1998), *F3′5′H* (Tanaka et al. 1996) and *ANS* (Nakatsuka et al. 2005a) (Figure 1). Approximately 500 bp triggers for each gene were amplified using primer sets as shown in Table 1 and then subcloned into a pCR2.1 TA cloning vector (Invitrogen, CA).

Binary vectors with RNAi-induced inverted repeat structures were constructed as described by Nishihara et al. (2005). Approximately 500 bp of each gene was connected in sense and antisense orientations with the first intron of the caster bean catalase gene (Ohta et al. 1990) as a linker. These inverted repeat structures, intCHSir, intF3′5′Hir and intANSir, were driven under the control of the *rolC* promoter derived form *A. rhizogenes* (Sugaya and Uchimiya 1992). Each expression cassette was inserted into a binary vector pSMAB704 (Nishihara et al. 2006) harboring the bialaphos herbicide-resistance (*bar*) gene (Figure 2). Each binary vector was introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by electroporation and used to transform gentian plants.

Table 1. Primers used in this study.

Gene		Sequence (5′→3′)	Additive site
CHS	Forward	5′-TCT AGA ATG CTA GGC AGG ACA TTG TG-3′	*Xba*I
	Reverse	5′-GGA TCC CCA ACC TCA CGA AGA TGA CC-3′	*Bam*HI
F3′5′H	Forward	5′-TCT AGA GGT GAT GAT TTC GGA GAT GC-3′	*Xba*I
	Reverse	5′-AGA TCT TGG TAA GTT GGG GAT GTC TG-3′	*Bgl*II
ANS	Forward	5′-TCT AGA CGT AAA ATC CGC AGG ACA AG-3′	*Xba*I
	Reverse	5′-GGA TCC GAT AAT GGA ATC AGG GAC AC-3′	*Bam*HI

Additive restriction enzyme sites are shown by underlines.

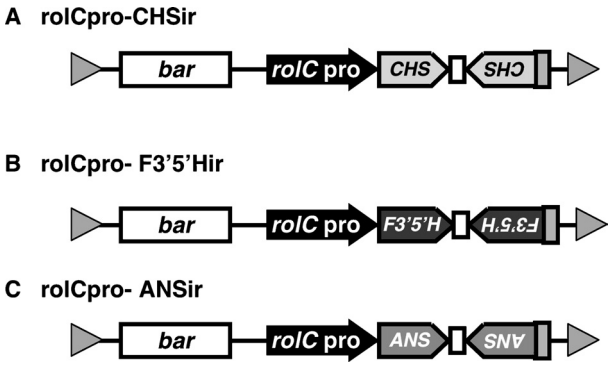

Figure 2. Vector constructs used for gentian transformation. Approximately 500 bp partial fragments of gentian *CHS*, *F3′5′H* and *ANS* genes were connected with the first intron of the caster bean catalase gene in inverted orientation and driven by the *rolC* promoter. Constructs were termed rolCpro-CHSir (A), rolCpro-F3′5′Hir (B) and rolCpro-ANSir (C), respectively. These vectors harbored the herbicide resistance (*bar*) gene as a selectable marker.

Genetic transformation of gentian

A. tumefaciens harboring each binary vector was inoculated into the interspecific hybrid gentian cultivar Albireo as described by Nishihara et al. (2006). Bialaphos-resistant shoots were transferred onto root-inducing medium. Plantlets were acclimatized and grown in a contained greenhouse. The flowers of transgenic gentian plants were collected for further analyses and stored at −80°C until use.

Expression analyses of targeted genes in transgenic gentian plants

To investigate the expression levels of flavonoid biosynthetic genes targeted for RNAi, northern blot analysis was performed as described by Nakatusuka et al. (2005a). Total RNA was isolated from the petals at flower developmental stage 3 as defined by Nakatsuka et al. (2005a), using Plant RNA Isolation Reagent (Invitrogen, CA). Total RNA of each transgenic line was separated on a 1.0% agarose gel then transferred onto a Hybond N+ membrane (GE healthcare, Sweden). Each membrane was hybridized to *CHS*, *F3′5′H* and *ANS* probes labeled with a DIG-PCR labeling Kit (Roche, Basel) then washed and detected according to DIG standard protocols.

Accumulation of anthocyanin pigments in transgenic gentian plants

To measure amounts of anthocyanin in the petals of rolCpro-CHSir and rolCpro-ANSir transgenic gentian plants, anthocyanin compounds were extracted from the petals with ethanol/water/acetic acid (10:9:1). Anthocyanin concentrations were determined by measuring the absorbance at 530 nm using a spectrophotometer. The anthocyanidin composition was further investigated in petals of rolCpro-F3′5′Hir transgenic gentian plants. Briefly, the anthocyanidin extracts were hydrolyzed with hydrochloric acid by boiling for 90 min and were then subjected to HPLC analysis. HPLC was carried out with a reverse phase column YMC-Pack Pro C18RS (4.6×150 mm, YMC) for 20 min at 40°C at a flow rate of 0.8 ml min^{-1}. The solvent system consisted of solvent A (0.1% trifluoroacetic acid [TFA]) and solvent B (acetonitrile containing 0.1% TFA) and the following elution profile; 0 min 60% A, 20 min 30% A, using linear gradients between the time points. Anthocyanidin was quantified by monitoring the peak area of absorbance at 500 nm, with pelargonidin, cyanidin and delphinidin as the standards.

Results and discussion

The flower petals of the gentian cultivar Albireo used as a transformation host showed a blue color (Figure 3A). They contained high amounts of delphinidin derivatives and smaller amounts of cyanidin derivatives (see HPLC analysis). By inoculation of *A. tumefaciens* harboring each binary vector, many resistant calli and regenerated shoots were obtained from leaf sections on selection medium containing bialaphos. Regenerated shoots were rooted on rooting medium. Acclimatized plants were transferred to a contained greenhouse and cultivated. Some transgenic gentian plants produced flowers after growing for more than 1 year in a contained greenhouse. The petals collected from 1-year-old transformants were subjected to further analyses.

Suppression of CHS gene in gentian plants by RNAi

In genetic engineering studies of petunia and torenia, the *CHS* gene, encoding the first key catalytic enzyme in the

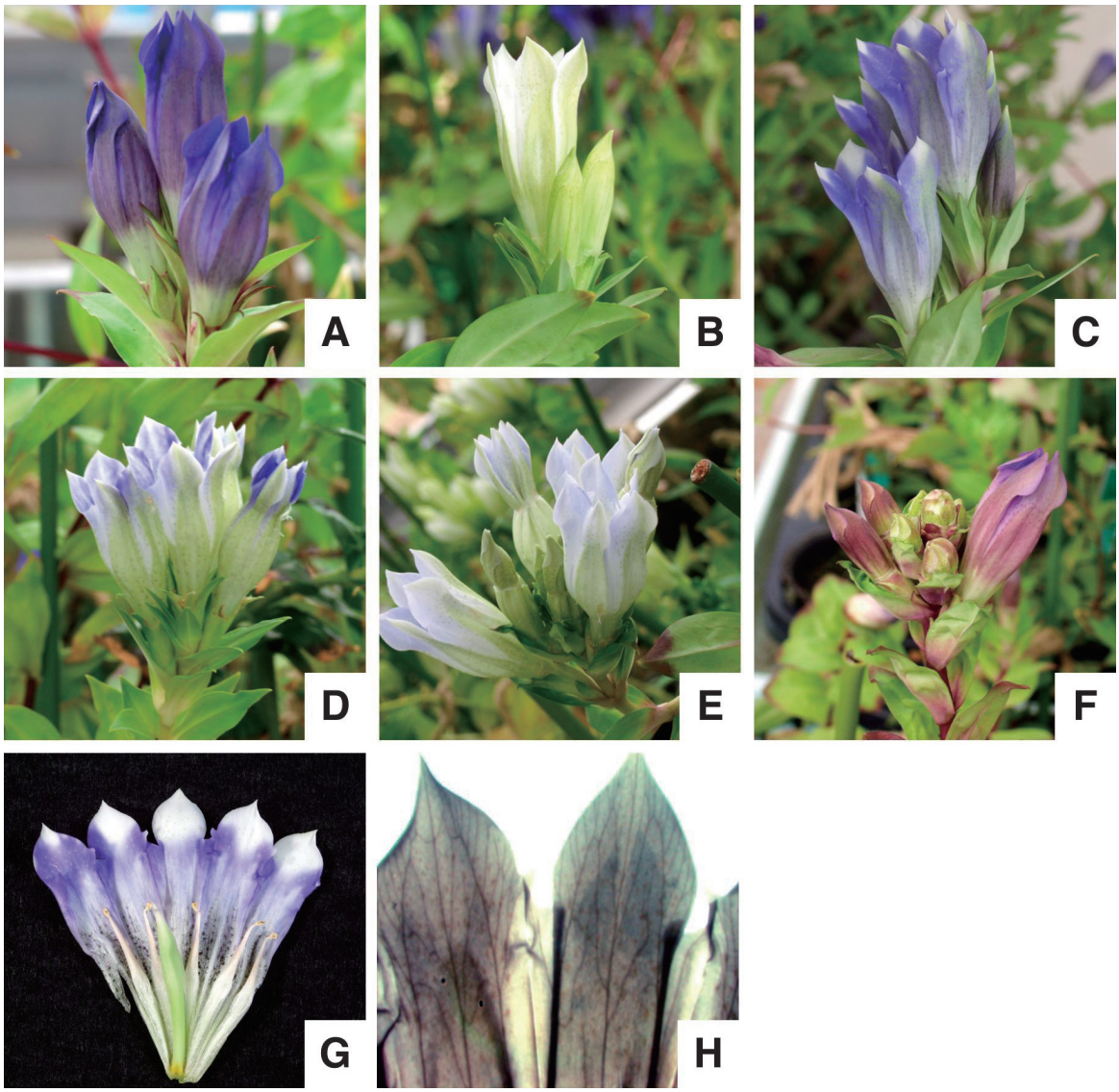

Figure 3. Typical flower phenotypes of transgenic gentian plants. A) Wild-type cv. Albireo B) rolCpro-CHSir clone no. 5 C) rolCpro-CHSir clone no. 13 D) rolCpro-ANSir clone no. 33 E) rolCpro-ANSir clone no. 102 F) rolCpro-F3′5′Hir clone no.13 G) Internal side of petals in rolCpro-CHSir clone no. 13 H) Petal tip in rolCpro-CHSir clone no. 13 G) and H) were observed by bright- and dark-field microscopy, respectively.

anthocyanin biosynthetic pathway (Figure 1), was chosen as the target for gene suppression by antisense, co-suppression or RNAi techniques (van den Korl et al. 1988; Napoli et al. 1990; Fukusaki et al. 2003). The inverted repeat structure of a partial gentian *CHS* gene under the control of the *rolC* promoter was introduced into gentian plants in this study. After selection on medium containing bialaphos, 20 lines of independent resistant shoots were obtained and grown in a contained greenhouse. Changed flower colors were observed in 17 lines of rolCpro-CHSir transgenic gentian plants. Pure-white flowers were seen in 14 out of 17 altered lines while the other 3 lines showed pale blue and bicolor flowers. Typical pictures are shown in Figures 3B, C. Anthocyanin accumulation in these transgenic flowers was significantly reduced; 0.9–86.3% compared with that of wild type (Figure 4). Northern blot analysis showed that *CHS* transcripts decreased in the petals of rolCpro-CHSir transgenic gentian plants, probably due to degradation by RNAi (Figure 5A). The reduction of anthocyanin accumulation almost corresponded with the reduction in stable levels of *CHS* transcripts in each transgenic gentian plant.

Waterhouse et al. (1998) reported that gene silencing caused by hairpin double-stranded RNA was more efficient and stable than that caused by antisense and sense suppression. We have previously produced white-flowered gentian plants by antisense suppression of *CHS* (Nishihara et al., 2006). In this case, only 3 lines of 17 transgenic plants (*ca*. 18%) showed white flowers. In contrast, our present study indicated that the suppression of *CHS* by RNAi was more efficient (85%) than that

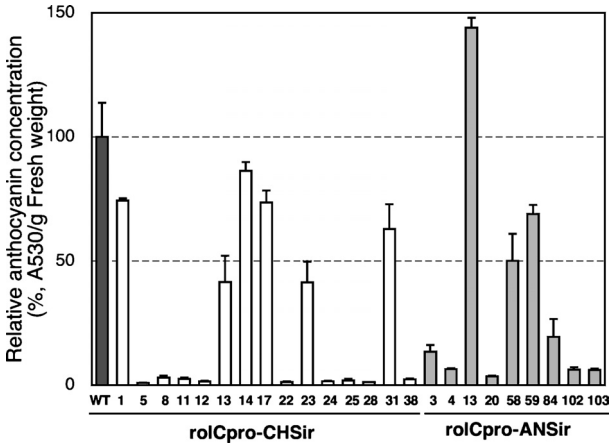

Figure 4. Anthocyanin accumulation in petals of rolCpro-CHSir- and rolCpro-ANSir-transgenic gentian flowers. Anthocyanin concentrations were determined spectrophotometrically by measuring absorbance at 530 nm. Values indicate the averages ± standard deviations of 3 to 5 flower replicates. Numerals indicate line no. of each transgenic series.

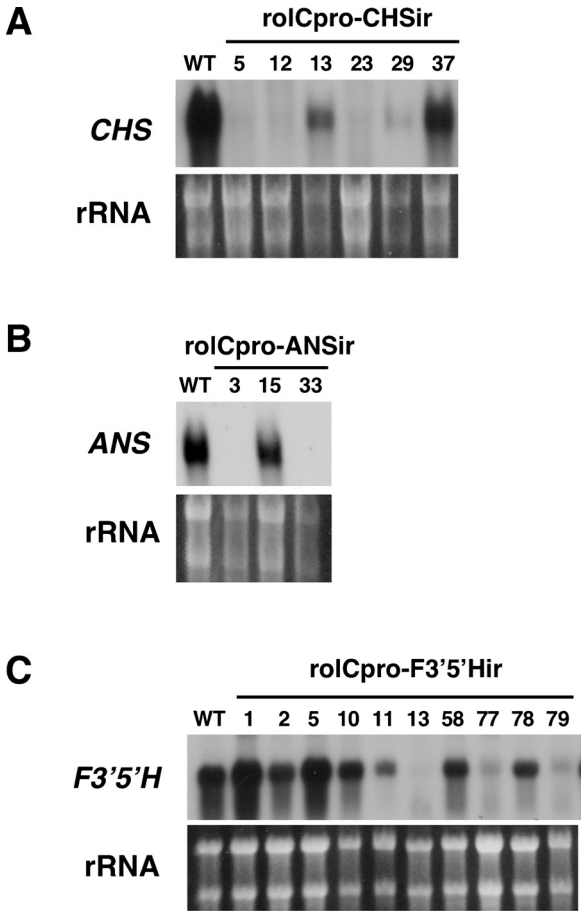

Figure 5. Expression analysis of RNAi-targeted genes in transgenic gentian flowers. Northern blot analyses were performed to examine the expression levels of *CHS*, *ANS* and *F3′5′H* genes in rolCpro-CHSir, rolCpro-ANSir and rolCpro-F3′5′Hir transformants, respectively. Membranes were hybridized using each probe as described in Materials and methods. Ethidium bromide-stained ribosomal RNA bands (rRNA) are shown as a control. Numerals indicate line no. of each transgenic series.

using the antisense strategy, although the promoters used were different. Northern blot analysis showed that the rolCpro-CHSir white-flowered transgenic gentian had low-level *CHS* transcription, even in the most suppressed line (Figure 5A), whereas no expression of the *CHS* gene was detected in the transgenic gentian plants produced by introducing antisense *CHS* (Nishihara et al. 2005). Therefore, in terms of the strength of gene silencing, the suppression using the antisense strategy was stronger, probably due to transcriptional gene silencing (TGS). That is, although suppression by RNAi was more efficient than the antisense strategy, its ability to suppress gene expression appeared to be slightly weaker in gentian plants. It is likely that if a stronger promoter was used, stronger gene silencing by RNAi could also be induced in gentian. In fact, we recently demonstrated the induction of differential flower pigmentation patterns by RNAi using three promoters with different activity in tobacco (Nakatsuka et al. 2007b).

We used the *rolC* promoter derived from *A. rhizogenes* to express the inverted repeats of *CHS* fragments in gentian plants. This promoter activity was shown to be localized predominantly in vascular bundle tissues (Sugaya and Uchimiya 1992). Some rolCpro-CHSir transgenic gentians (for example, clone no. 13) showed an interesting bicolor phenotype with whitening at the petal tips (Figures 3C, G). These bicolor-type flowers accumulated more *CHS* transcripts than did white flowers (Figure 5A). Microscopic observation demonstrated that the distribution of vascular tissues in gentian flowers was concentrated in the tips of petals but was absent in the semi-lobed petals (Figure 3H). The reduced region of anthocyanin accumulation in rolCpro-CHSir bicolor flowers was predominantly observed in the tip of petals where the localization of *rolC* promoter activity was reported. Although primary double strands derived from RNAi can move to only 10–15 of the adjacent cells, de novo synthesis of 21 nt siRNAs is necessary for them to move to more distant cells (Himber et al. 2003). Thus, it is likely that region-specific suppression by the vascular tissue-specific *rolC* promoter could yield the bicolor phenotype in gentian flowers. These results indicated that a tissue-specific suppression technique using RNAi is a useful tool not only to suppress the pigmentation but also to design new flower color patterns.

Suppression of ANS gene in gentian plants by RNAi

We also attempted to suppress the expression of the *ANS* gene in gentian. The *ANS* gene encodes a catalytic enzyme at a later step of the anthocyanin biosynthetic pathway (Figure 1). Most transgenic gentian plants with the rolCpro-ANSir construct exhibited a pale-blue flower

phenotype (Figures 3D, E). However, no pure white-flowered phenotype was observed in rolCpro-ANSir transgenic gentian plants in contrast to rolCpro-CHSir (for example, Figure 3B). Anthocyanin accumulation in the petals of rolCpro-ANSir transgenic gentian decreased 3.5 to 68.0% compared with that of wild-type plants, and was higher than levels in rolCpro-CHSir petals (Figure 4). In addition, no change of anthocyanin composition was observed in the petals of rolCpro-ANSir compared with that of wild type, nor was any change detected in flavone accumulation, which is another major flavonoid compound in gentian petals (data not shown). Northern blot analysis showed that the *ANS* transcript was remarkably reduced in some rolCpro-ANSir transgenic gentians (Figure 5B). Nakatsuka et al. (2005b) reported that the white-flowered gentian cultivar Homoi resulted from an *ANS* gene deficiency. This would imply that there was incomplete suppression of the *ANS* gene expression by RNAi, and the remaining enzyme activity was responsible for the slight pigmentation. One possible reason for incomplete suppression is that the catalytic ability of ANS is higher than that of CHS. Alternatively, because ANS is located at a later step of anthocyanin biosynthesis, the leaked pigments might be easily catalyzed to colored anthocyanin compared with CHS. Similar results have been reported in torenia, where the suppression of *ANS* by RNAi driven under a constitutive promoter yielded white flowers with various intensity and pattern (Nakamura et al. 2006).

Other than flower pigmentation, flavonoid compounds have been known to be responsible for various biological functions such as pollen viability, plant-microbe interactions, and protection from UV radiation (reviewed in Mol et al. 1998). Therefore, the deficiency of an early catalytic step, such as *CHS*, was thought to have detrimental effects on plant growth because the reduction of not only anthocyanins, but also other flavonoid compounds, was induced. Since rolCpro-ANSir transgenic gentian plants did not influence flavone biosynthesis and accumulation, they might be better adapted to environmental conditions than rolCpro-CHSir plants. It is necessary to evaluate the difference in environmental stress tolerance between *CHS*- and *ANS*-suppressed lines in field trials in the future.

Suppression of F3′5′H gene in gentian plants by RNAi

F3′5′H is a enzyme catalyzing the hydroxylation of 3′ and 5′-residues in the B-ring of flavonoids, and belongs to the cytochrome P450 family. It is responsible for the biosynthesis of delphinidin derivatives. Some transgenic gentian plants with rolCpro-F3′5′Hir exhibited magenta flower colors (Figure 3F). HPLC analysis showed that the transgenic gentian clone no. 13 had significantly increased cyanidin derivatives and decreased delphinidin

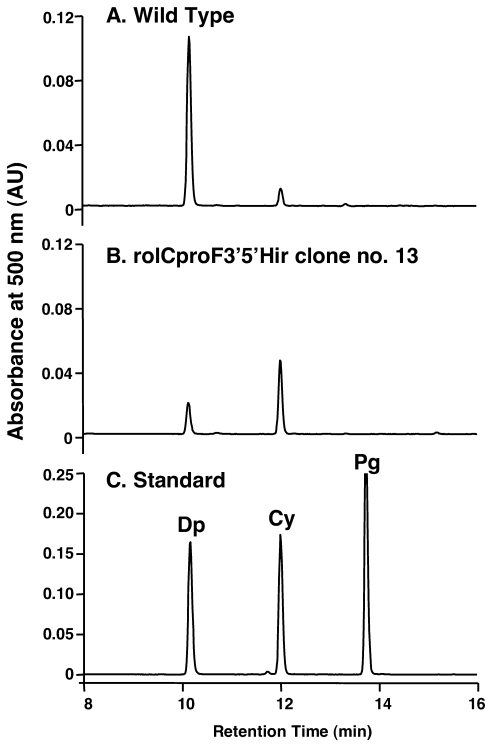

Figure 6. Chromatography of anthocyanidin composition in rolCpror-F3′5′Hir transgenic gentian flowers. Anthocyanin was extracted from the petals of wild type (A) and rolCpro-F3′5′Hir transgenic gentian clone no. 13 (B) by methanol containing 1% HCl. After hydrolysis of anthocyanin, aglycon was separated by HPLC as described in Materials and methods. Pelargonidin (Pg), cyanidin (Cy) and delphinidin (Dp) were also separated as standards (C).

derivatives compared with that of wild-type (Figure 6). Some other lines also tended to contain increased cyanidin derivatives and decreased delphinidin derivatives (Figure 7). Northern blot analysis showed that *F3′5′H* transcripts were significantly reduced using the RNAi strategy in the petals of some rolCpro-F3′5′Hir transgenic gentians (Figure 5C). However, the frequency of transgenic lines with suppressed *F3′5′H* gene expression was lower than that of the other transgenic lines rolCpro-CHSir and rolCpro-ANSir. Because F3′5′H belongs to the membrane-bound cytochrome P450 family and therefore has high enzymatic activity, the phenotype when *F3′5′H* transcripts were moderately suppressed in rolCpro-F3′5′Hir transformants might be difficult to detect. The pink-flowered gentian cultivar Momokorin and breeding line 13–98 arose from the deficiency of F3′5′H enzymatic function by the insertion of transposable elements (Nakatsuka et al. 2006). Therefore, rolCpro-F3′5′Hir transformants showed direct evidence that the deficiency of the *F3′5′H* gene led to pink flowers in gentian.

Nishihara et al. (2005) demonstrated that both white-flowered and herbicide-resistant traits were dominantly inherited by progeny in antisense *CHS* gentian plants. Transgenic gentian plants obtained in this study are also

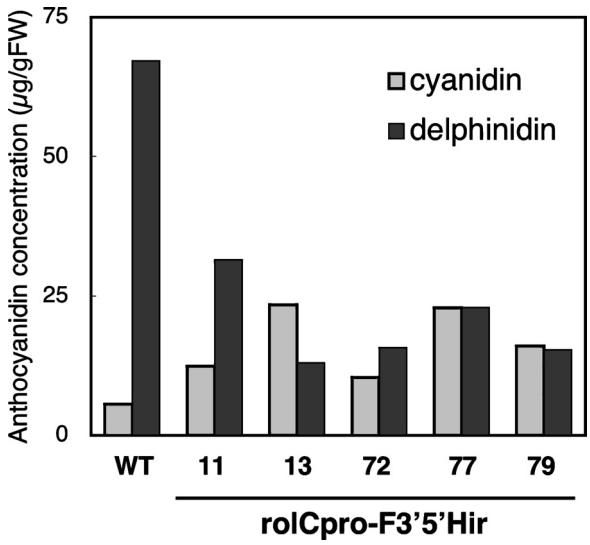

Figure 7. Anthocyanidin composition of rolCpro-F3′5′Hir transgenic gentian flowers. Anthocyanidin composition in the petals of rolCpro-F3′5′Hir transgenic gentian was measured by HPLC analysis. The HPLC analysis conditions are the same as in Figure 6. Numerals indicate line no. of the transgenic plants.

thought to have similar inheritance patterns, and might become useful genetic resources for breeding of elite gentian cultivars. Furthermore, RNAi suppression in gentian will be a useful tool to produce plant materials for studying disease resistance and morphogenesis in future studies.

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References

Aida R, Kishimoto S, Tanaka Y, Shibata M (2000a) Modification of flower color in torenia (*Torenia fournieri* Lind.) by genetic transformation. *Plant Sci* 153: 33–42

Aida R, Yoshida K, Kondo T, Kishimoto S, Shibata M (2000b) Copigmentation gives bluer flowers on transgenic torenia plants with the antisense dihydroflavonol-4-reductase gene. *Plant Sci* 160: 49–56

Chuang CF, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 97: 4985–4990

Forkmann G, Martens S (2001) Metabolic engineering and applications of flavonoids. *Curr Opin Biotechnol* 12: 155–160

Fukusaki E, Kawasaki K, Kajiyama S, An CI, Suzuki K, Tanaka Y, Kobayashi A (2004) Flower color modulations of *Torenia hybrida* by downregulation of chalcone synthase genes with RNA interference. *J Biotechnol* 111: 229–240

Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, Voinnet O (2003) Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO J* 22: 4523–4533

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Res* 2: 208–218

Kobayashi H, Oikawa Y, Koiwa H, Yamamura S (1998) Flower-specific expression directed by the promoter of a chalcone synthase gene from *Gentiana triflora* in *Petunia hybrida*. *Plant Sci* 131: 173–180

Mishiba K, Nishihara M, Nakatsuka T, Abe Y, Hirano H, Yokoi T, Kikuchi A, Yamamura S (2005) Consistent transcriptional silencing of 35S-driven transgenes in gentian. *Plant J* 44: 541–556

Mol J, Grotewold E, Koes R (1998) How genes paint flowers and seeds. *Trend Plant Sci* 3: 212–217

Nakamura N, Fukuchi-Mizutani M, Miyazaki K, Suzuki K, Tanaka Y (2006) RNAi suppression of the anthocyanidin synthase gene in *Torenia hybrida* yields white flowers with higher frequency and better stability than antisense and sense suppression. *Plant Biotechnol* 23: 13–18

Nakatsuka T, Abe Y, Kakizaki Y, Yamamura S, Nishihara M (2007a) Production of red-flowered plants by genetic engineering of multiple flavonoid biosynthetic genes. *Plant Cell Rep* 26: 1951–1959

Nakatsuka T, Pitaksutheepong C, Yamamura S, Nishihara M (2007b) Induction of differential flower pigmentation patterns by RNAi using promoters with distinct tissue-specific activity. *Plant Biotechnol Rep* 1: 251–257

Nakatsuka T, Nishihara M, Mishiba K, Hirano H, Yamamura S (2006) Two different transposable elements inserted in flavonoid 3′,5′-hydroxylase gene contribute to pink flower coloration in *Gentiana scabra*. *Mol Gen Gen* 275: 231–241

Nakatsuka T, Nishihara M, Mishiba K, Yamamura S (2005a) Temporal expression of flavonoid biosynthesis-related genes regulates flower pigmentation in gentian plants. *Plant Sci* 168: 1309–1318

Nakatsuka T, Nishihara M, Mishiba K, Yamamura S (2005b) Two different mutations are involved in the formation of white-flowered gentian plants. *Plant Sci* 169: 949–958

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* 2: 279–289

Nishihara M, Nakatsuka T, Hosokawa K, Yokoi T, Abe Y, Mishiba K, Yamamura S (2006) Dominant inheritance of white-flowered and herbicide-resistant traits in transgenic gentian plants. *Plant Biotechol* 23: 25–31

Nishihara M, Nakatsuka T, Yamamura S (2005) Flavonoid components and flower color change in transgenic tobacco plants by suppression of chalcone isomerase gene. *FEBS Lett* 579: 6074–6078

Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol* 31: 805–813

Ono E, Fukuchi-Mizutani M, Nakamura N, Fukui Y, Yonekura-Sakakibara K, Yamaguchi M, Nakayama T, Tanaka T, Kusumi T, Tanaka Y (2006) Yellow flowers generated by expression of the

aurone biosynthetic pathway. *Proc Natl Acad Sci USA* 103: 11075–11080

Sugaya S, Uchimiya H (1992) Deletion analysis of the 5'-upstream region of the *Agrobacterium rhizogenes* Ri plasmid rolC gene required for tissue-specific expression. *Plant Physiol* 99: 464–467

Suzuki K, Xue HM, Tanaka Y, Fukui Y, Fukuchi-Mizutani M, Murakami Y, Katsumoto Y, Tsuda S, Kusumi T (2000) Flower color modifications of *Torenia hybrida* by cosuppression of anthocyanin biosynthesis genes. *Mol Breed* 6: 239–246

Tanaka Y, Katsumoto Y, Brugliera F, Mason J (2005) Genetic engineering in floriculture. *Plant Cell Tiss Org Cult* 80: 1–24

Tanaka Y, Tsuda S, Kusumi T (1998) Metabolic Engineering to Modify Flower Color. *Plant Cell Physiol* 39: 1119–1126

Tanaka Y, Yonekura K, Fukuchi-Mizutani M, Fukui Y, Fujiwara H, Ashikari T, Kusumi T (1996) Molecular and biochemical characterization of three anthocyanin synthetic enzymes from *Gentiana triflora*. *Plant Cell Physiol* 37: 711–716

Tsuda S, Fukui Y, Nakamura N, Katsumoto Y, Yonekura-Sakakibara K, Fukuchi-Mizutani M, Ohira K, Ueyama Y, Ohkawa H, Holton TA, Kusumi T, Tanaka Y (2004) Flower color modification of *Petunia hybrida* commercial varieties by metabolic engineering. *Plant Biotechnol* 21: 377–386

van der Krol AR, Lentlng PJ, Veenstra JG, van der Meer IM, Koes RE, Gerats AGM, MOI JNM, Stuitje AR (1988) An antisense chalcone synthase gene in transgenic plants inhibits flower pigmentation. *Nature* 333: 866–869

Waterhouse PM, Graham MW, Wang MB (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc Natl Acad Sci USA* 95: 13959–13964