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Original Paper

Production of picotee-type flowers in Japanese gentian by CRES-T

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Abstract Chimeric repressor gene-silencing technology (CRES-T) is an efficient gene suppression system in a wide variety of dicots and monocots. In this study, we demonstrated that the CRES-T system functions in Japanese gentian. A chimeric repressor of the anthocyanin biosynthetic regulator gene *GtMYB3*, under the control of the Arabidopsis actin2 promoter, was introduced into blue-flowered gentian. Of 12 transgenic lines, 2 exhibited a picotee flower phenotype with a lack of pigmentation in the lower part of the petal. HPLC analysis showed that the petals of these lines contained less anthocyanin and more flavone than the wild-type, suggesting competitive accumulation of these two types of compounds. The expressions of 'late' flavonoid biosynthetic genes, including *F3H*, *F3′5′H*, *DFR* and *ANS*, were strongly suppressed in petals of these transgenic plants. In contrast, the 'early' flavonoid biosynthetic genes, such as *CHS* and *FNSII*, were not affected. Since *FNSII* is expressed more strongly in the lower part of petals than in the upper part, the absence of pigmentation in the lower parts might be induced by flavone synthesis. These results demonstrated that the suppression of anthocyanin biosynthetic genes by CRES-T was successfully applied to Japanese gentian to change petal color; therefore, this system could be useful for generating novel flower colors and patterns. Transgenic plants produced in this study might be utilized as elite materials in the breeding of Japanese gentian in the near future.

Key words: CRES-T, flower color, Japanese gentian, picotee, R2R3-MYB.

Japanese gentian (bred from *Gentiana triflora* and *G. scabra*) is one of the most important floricultural plants in Japan, with most plants cultivated in Iwate prefecture (Nishihara et al. 2008). Japanese gentians are perennial plants with vivid blue-pigmented flowers, and which bloom from July to October depending on the cultivar. They are mainly used as a cut flower for graves, but additional market needs such as in floral arrangements and bridal bouquets have arisen recently, leading to increased demand for Japanese gentians. Therefore, we have sought to develop novel types of gentian cultivars and breeding lines using molecular breeding technology, such as tissue culture, transgenic and molecular marker techniques (reviewed by Nishihara et al. 2008). For example, we produced dwarf gentian plants using wild-type *Agrobacterium rhizogenes* (Mishiba et al. 2006) and one of the lines was submitted for registration as an elite cultivar in Japan in 2009.

Diversification of flower color is one of the most important breeding aims, because Japanese gentians have purple, magenta, cream, and white flower colors, but no red or yellow flower colors exist. The pigmentation of gentian petals is derived from accumulation of polyacylated anthocyanins such as gentiodelphin and gentiocyanin (Goto et al. 1982; Hosokawa et al. 1995, 1997). Our own and Suntory Holdings' research groups have isolated and characterized most of the flavonoid biosynthetic genes responsible for flower pigmentation in gentian (Fujiwara et al. 1998; Fukuchi-Mizutani et al. 2003; Nakatsuka et al. 2005a, 2008a; Tanaka et al. 1996). In addition, we also revealed that the pigmentation of gentian flowers was regulated by two transcription factors, R2R3-MYB and basic Helix-Loop-Helix (bHLH), designated *GtMYB3* and *GtbHLH1*, respectively (Nakatsuka et al. 2008b). It was demonstrated that in gentian flowers the GtMYB3/GtbHLH1 transcription factor complex regulated the expressions of anthocyanin biosynthetic genes (Nakatsuka et al. 2008b).

We developed a gentian transformation system both by particle bombardment (Hosokawa et al. 2000) and by *Agrobacterium*-mediated methods (Mishiba et al. 2005). Using *Agrobacterium*-mediated transformation, the first successful transgenic gentians with modified flower color were produced by introduction of the antisense chalcone synthase (*CHS*) gene (Nishihara et al. 2006). The antisense *CHS*-transgenic lines produced completely white flowers and inheritance of the white flower trait and herbicide resistance was observed. Subsequently, RNAi suppression of anthocyanin biosynthetic genes,

Abbreviations: AtACT2pro, Arabidopsis actin2 promoter; CaMV, Cauliflower mosaic virus; CRES-T, chimeric repressor gene-silencing technology; HPLC, high performance liquid chromatography; *LUC*, firefly luciferase gene; *NOS*, nopaline synthase gene; ORF, open reading frame; *RLUC*, *Renilla* luciferase gene; RT-PCR, reverse-transcription polymerase chain reaction; TFA, trifluoroacetic acid
This article can be found at http://www.jspcmb.jp/

including *CHS*, flavonoid 3′,5′-hydroxylase (*F3′5′H*) and anthocyanidin synthase (*ANS*), was achieved and resulted in various flower colors, such as white, pink and pale blue, in transgenic gentian plants (Nakatsuka et al. 2008c). More recently, suppression of *F3′5′H* and anthocyanin 5,3′-alomatic acyltransferase (5/3′AT), involved in the modification of anthocyanin gentiodelphin, resulted in novel lilac to pale blue colored flowers (Nakatsuka et al. 2010). Thus, the silencing of flavonoid biosynthetic genes in gentian flowers was demonstrated to increase the diversity of flower colors by changing the composition and quantity of anthocyanin pigments in the petals (Nakatsuka et al. 2008c, 2010; Nishihara et al. 2006).

In addition to antisense and RNAi technology, chimeric repressor gene-silencing technology (CRES-T) has been developed as an efficient silencing system. In this system, a transcription factor is converted from activator into a repressor by fusion to the EAR motif (SRDX), and this repressor dominantly suppresses the expression of the target genes of the transcription factor (Hiratsu et al. 2003). Suppression by CRES-T has been successful in a wide variety of monocots and dicots including Arabidopsis, torenia, morning glory, cyclamen, and rice (Mitsuda et al. 2006; Narumi et al. 2008; some articles in this special issue). Chimeric repressors have been applied to various transcription factors, including those that regulate morphological development: *CUP SHAPED COTYLEDON1* (Hiratsu et al. 2003), *TCPs* (Koyama et al. 2007), *AGAMOUS* (Mitsuda et al. 2006; Narumi et al. 2008; Xu et al. 2006) and *WUSCHEL* (Ikeda et al. 2009); anther development: *MYB26* (Mitsuda et al. 2006); ethylene response: *EIN3* (Hiratsu et al. 2003); and secondary wall formation: *NST*s (Mitsuda et al. 2007, 2008). In secondary metabolic biosynthesis, a chimeric repressor for *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*), which is a positive regulator of phenylpropanoid biosynthesis in Arabidopsis, suppressed the accumulation of anthocyanin in transgenic Arabidopsis seedlings (Hiratsu et al. 2003; Matsui et al. 2004). However, to our knowledge, suppression of flower pigmentation using the CRES-T system has not been reported.

In this study, we attempted to transform the chimeric repressor of the anthocyanin biosynthetic regulator gene *GtMYB3* in gentian. The results clearly showed that a CRES-T system was successful for production of a picotee-flowered phenotype in gentian plants. This is the first report that describes application of a CRES-T system in gentian plants.

Materials and methods

Transient expression assay using mesophyll cell protoplasts in gentian

For transient expression assays, we used the reporter plasmid p35S-TATA-GAL4-LUC-NOS, which consists of the Cauliflower mosaic virus (CaMV) 35S enhancer (-800 to -46), a TATA box, five GAL4 binding sites, the Ω translation enhancer sequence, the firefly luciferase (*LUC*) gene, and the nopaline synthase (NOS) terminator. We also used the effector plasmids p35S-GAL4DB-SRDX and p35S-GAL4DB; the former contains the coding region for the GAL4 DNA binding domain (GAL4DB) fused to the SRDX domain driven by the CaMV 35S promoter, and the latter contains the same components except for the SRDX domain. pBI221 (Clontech, CA, USA) was also used as a negative control effector.

Protoplasts were prepared from immature leaves of field-grown Japanese gentian plants as described by Nakano et al. (1995). A 100-μl volume of the protoplast suspension (containing 1×10^6 gentian mesophyll cell protoplasts) was added to a reaction tube containing 4 μg reporter, 3 μg effector and 1 μg p35S-RLUC (*Renilla LUC* gene) as a transformation control. Then, 100 μl PEG solution (25% polyethylene glycol 6000, 100 mM $Ca(NO_3)_2$, 450 mM mannitol, pH 9.0) was added to the tube, and the mixture was incubated at 25°C for 12 h. After centrifugation, the protoplasts were resuspended in passive lysis buffer. Firefly and *Renilla* LUC activity were measured with the Dual-Luciferase Reporter Assay system (Promega, WI, USA) and Luminescencer JNR II (ATTO, Tokyo, Japan) according to the manufacturer's instructions. The relative LUC activities were calculated by comparison with the value of the vector control after normalization with RLUC activities.

Binary vector construct for production of transgenic gentian plants

The open reading frame (ORF) of *GtMYB3* (GenBank/EMBL/DDBJ accession no. AB289445; Nakatsuka et al. 2008b) without the stop codon was amplified using the primer set listed in Table 1. Amplified fragments were purified with the Geneclean II kit (Qbiogene, CA, USA), phosphorylated by T4 polynucleotide kinase (Takara Bio, Shiga, Japan), and cloned into the *Sma* I site of pAtACT2pro-SRDXG vector in-frame. The pAtACT2pro-SRDXG vector contains the Arabidopsis actin2 promoter (AtACT2pro; An et al. 1996), the SRDX repression domain, and the *att*L1 and *att*L2 Gateway recombination sites (Invitrogen, CA, USA) outside the regions of the AtACT2 promoter and the NOS terminator. The AtACT2pro-GtMYB3-SRDX region was transferred into the destination vector pSMABR-GW derived from pSMAB704 (Igasaki et al. 2002) using the Gateway LR clonase reaction (Invitrogen). The binary vector pSMABR-AtACT2pro-GtMYB3-SRDX was transformed into *Agrobacterium tumefaciens* EHA101 (Hood et al. 1986) by electroporation and used for the following experiments.

Production of transgenic gentian plants

Agrobacterium tumefaciens harboring the binary vector

Table 1. Primer list used in this study

		Sequences (5′→3′)
Vector constructs		
GtMYB3 ORF	Forward	ATGAACTCAGGGTTGAAGAG
	Reverse	TGGAGACCATCTATTAGGTT
RT-PCR analysis		
GtMYB3SRDX	Forward	ACGCCTTCATCGCCTCCTTG
	Reverse	TCGTCGACTTAAGCGAAACCCAAAC
endogeneous *GtMYB3*	Forward	GAGATATGGGTTCAACGAGCCTTCG
	Reverse	GGTGGTGTCCGTAACACTCGTGACT
CHS	Forward	TATGGCACCTTCCCTTGATG
	Reverse	CTATCCGGAAGAAGGGTTTGGGCTGCTGAA
CHI	Forward	ACCGCAGCAACCACATAACC
	Reverse	TTCCGGCAGCTTTCCATTGG
FNSII	Forward	GACGAGCAACATCATTTCAC
	Reverse	ACGTCATATCCAGCCACTTG
F3′H	Forward	TGGAGATTATGGTGTTAGCC
	Reverse	TCAACATTAGGCTTCTCTCC
F3H	Forward	AATGGCTCCACCACCACCACCTTC
	Reverse	TTCTGACAGAACTTCAAGCA
F3′5′H	Forward	TGCCACATGTTACTTTTGCT
	Reverse	AAAGAGCCTTGATGTTGTCG
DFR	Forward	CGGGAATCTGAAGAAGGTTC
	Reverse	GTGATGAATGGACCAACGAC
ANS	Forward	TCCCCATGATTACATACCAG
	Reverse	GATAATGGAATCAGGGACAC
3GT	Forward	CCTGTATGGACGGCTGCTTC
	Reverse	CCCGGCAAAAGATACTCTCC
5GT	Forward	CCTCTGCTGTTGCTTCCATC
	Reverse	AGTGCTTCCAGGTGCTCTTT
5/3′AT	Forward	TAAAGTGATCCCGCTCGTAG
	Reverse	GGCAATCCGCTGTAAAACTG
Actin	Forward	CTAAGCAAAGCCAGCAAGTCCT
	Reverse	CACCAGAATCCAGCACAATACC

pSMABR-AtACT2pro-GtMYB3-SRDX was inoculated into the interspecific hybrid gentian cultivar 'Albireo' as described by Nishihara et al. (2006). Regenerated bialaphos-resistant shoots were transferred onto root-inducing medium. Transgenic gentian plants were acclimatized and grown in a closed greenhouse. The flowers of transgenic gentian plants were collected for further analyses and stored at −80°C until use.

Southern blot analysis

Genomic DNA was isolated from 1 g leaves of transgenic and untransformed control gentian plants with the Nucleon PhytoPure Genomic DNA Extraction Kit (GE Healthcare, Uppsala, Sweden). Genomic DNA was digested with *Hin*d III (Takara Bio) and separated on a 0.7% agarose gel, then transferred onto Nytran N membranes (Whatman Schleicher and Schuell, Brentford, UK). Hybridization with the probe for the *bar* gene and detection were performed as described by Mishiba et al. (2005).

Pigment analysis of transgenic gentian flowers

Flavonoid compounds were extracted from the petals with ethanol/water/acetic acid (EAA, 10 : 9 : 1). HPLC analysis was carried out with a reversed-phase column (YMC-Pack Pro C18RS, 4.6×150 mm, YMC, Tokyo, Japan) 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. Anthocyanins and flavones were detected at 520 nm and 340 nm, respectively.

Gene expression analysis by semi-quantitative RT-PCR analysis

For semi-quantitative reverse transcription (RT)-PCR analysis, total RNA was isolated from the petals at flower development stage 3, as defined by Nakatsuka et al. (2005a), using the Fruit-mate for RNA Purification reagent (Takara Bio). cDNAs were synthesized from total RNA after removing the genomic DNA

using QuantiTect Reverse Transcription (Qiagen, Frankfurt, Germany) according to the manufacturer's instructions. Reaction mixtures (100 μl) consisted of 1×Ex *Taq* buffer, 200 μM dNTPs, 0.5 μM each primer, 5 U Ex *Taq* polymerase (Takara Bio) and 1 μl cDNA template. Primer sets used for semi-quantitative RT-PCR analysis are listed in Table 1. Cycle conditions were 94°C for 90 s, and 26–38 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 40 s, and extension at 72°C for 1 min. The amplified products were separated on a 2% agarose gel in TBE buffer and stained with ethidium bromide.

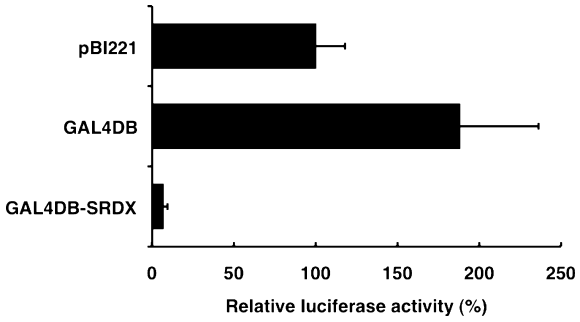

Figure 1. Suppression efficiency of CRES-T in gentian plants. Luciferase activities were measured after cotransfection of gentian protoplasts with the 35S-GAL4-TATA-LUC-NOS reporter plasmid and the GAL4DB fusion effector plasmids as described by Hiratsu et al. (2004). pBI221 and GAL4DB were used as controls, and GAL4DB-SRDX was GAL4DB fused with the ERF repressor domain. The *RLUC* gene was used as an internal standard to monitor transfection efficiency. The relative LUC activities are indicated as a percentage of pBI221. Values are the means of five measurements and error bars represent the standard deviation.

Results and discussion

Repression activity of SRDX domain in transient-expression assay in gentian

We first investigated whether the CRES-T system was functional in gentian, because the SRDX domain sequence (LDLDLELRLGFA) was originally derived from *A. thaliana* (Hiratsu et al. 2003). Transient expression analysis was performed using gentian mesophyll protoplasts with the CRES-T evaluation vector system (Hiratsu et al. 2004). The 35S-GAL4DB-SRDX effector showed strong gene repression activity and reduced LUC reporter activity to 6.9% and 3.7% of that induced by pBI221 and p35S-GAL4DB effectors, respectively (Figure 1). This result was similar to a previous study in Arabidopsis where more than 90% suppression of the reporter gene was achieved (Hiratsu et al. 2004). Recently, CRES-T was successfully applied to several horticultural plants including torenia (Narumi et al. 2008; Sasaki et al. in this issue), morning glory (Ono et al. in this issue), and cyclamen (Tanaka et al. in this issue). The EAR motif is conserved among a wide range of higher plant species, including Arabidopsis, rice, tobacco, petunia, and wheat (Ohta et al. 2001). Here, we showed that CRES-T is also applicable to gentian. Moreover, CRES-T is proposed to be suitable for plants of high ploidy (i.e. *4n* and higher), because chimeric repressors can function dominantly to overcome the activity of functionally redundant transcription factors (Hiratsu et al. 2003; Mitsuda et al. 2006). Gentians are highly heterozygous plants and transformation efficiency is not high, so an effective silencing system is desirable. Considering the results of our transient expression assay and the advantages of a CRES-T system, this system appears to be useful for gene silencing in transgenic gentian plants.

Production of GtMYB3-SRDX-transgenic gentian plants

Our previous studies demonstrated that the transcription factor *GtMYB3* regulated the pigmentation of gentian flowers (Nakatsuka et al. 2005a, 2008b). Therefore, we chose *GtMYB3* as the first target gene for CRES-T. Previously, we found that the CaMV 35S promoter could not induce transgene expression in gentian because of consistent transcriptional gene silencing (Mishiba et al. 2005, 2010). Instead of the CaMV 35S promoter, the Arabidopsis actin2 promoter was selected to control *GtMYB3-SRDX* (AtACT2pro; An et al. 1996) for gentian transformation, because AtACT2pro successfully induces transgene expression in floral organs and leaves of gentian (data not shown). A total of 1,320 leaf segments of the blue-flowered gentian cv. 'Albireo' were infected with *A. tumefaciens* harboring the binary vector pSMABR-AtACT2pro-GtMYB3-SRDX (Figure 2A). After selection on regeneration medium containing bialaphos, 49 bialaphos-resistant calli were obtained. Adventitious shoots were regenerated from the calli, and then transferred to rooting medium. Finally, 12 independent transgenic lines were obtained in this study. The transformation frequency was similar to our previous gentian transformation studies using different constructs to CRES-T (data not shown). After acclimatization, the transformants were transferred to a closed greenhouse and cultivated until flowering. Among them, two lines (clone nos. 7 and 11) showed significant changes of flower color (Figure 2C, D). The flowers of both transgenic lines exhibited a white-blue picotee phenotype with decreased pigmentation in the lower part of their petals. No phenotypic change other than flower color was observed in either transgenic line. The other transgenic lines did not show flower color changes compared with the untransformed control plants cultivated under the same conditions in the closed greenhouse. Southern blot analysis demonstrated that clone nos. 7 and 11 were independent transgenic plants with more than two copies of integration of the T-DNA region in their genome (data not shown).

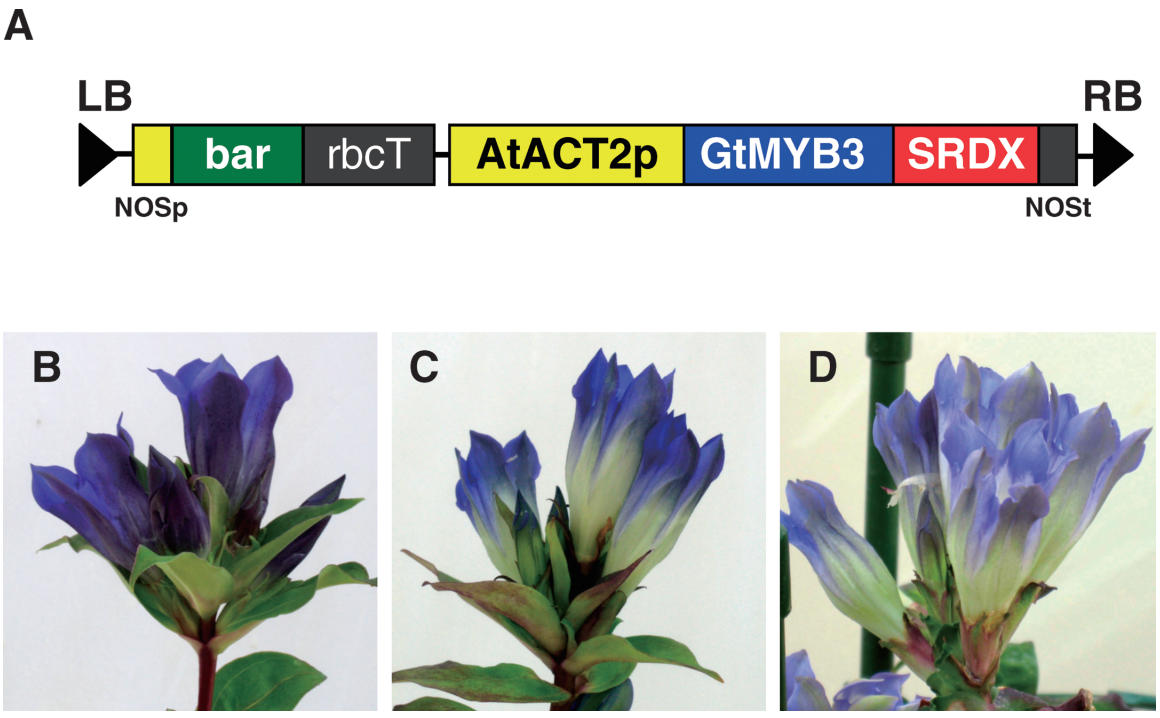

Figure 2. Construction of binary vector and flower phenotype in transgenic gentian plants. (A) Schematic representation of pSMABR-AtACT2pro-GtMYB3-SRDX. *bar*, herbicide bialaphos resistance gene as a selectable marker; NOSp, promoter of nopaline synthase (*NOS*) gene from *A. tumefaciens*; rbcSt, terminator of RuBisCO small subunit 2B gene from Arabidopsis; NOSt, terminator of *NOS* gene; LB, left border; RB, right border. (B–D) The typical flower of wild-type gentian cv. 'Albireo' (B), *GtMYB3-SRDX*-expressed transgenic gentian clones no. 7 (C) and no. 11 (D).

Flavonoid analysis of GtMYB3-SRDX gentian plants

The flavonoid composition and quantities in petals of the transgenic gentian plants were analyzed by HPLC. No difference in anthocyanin components was observed between the wild-type and two *GtMYB3-SRDX*-expressed transgenic gentians, but both transformants accumulated significantly less anthocyanin in their petals than the wild-type (Figure 3A–C). In contrast, flavone accumulation in both transgenic plants slightly increased (Figure 3D–F). The decreased anthocyanin and increased flavone content is probably because flavone and anthocyanin biosynthetic pathways both branch from the flavonoid biosynthetic pathway, and the two groups of compounds show a competitive relationship. Similarly, the flavonol and anthocyanin biosynthesis pathways are competitive, and the antisense flavonol synthase (*FLS*) construct induced decreased flavonol accumulation and increased anthocyanin accumulation simultaneously in transgenic petunia and lisianthus (Davis et al. 2003; Nielsen et al. 2002).

Expression analysis of flavonoid biosynthetic genes in transgenic gentian plants

To investigate expression of the introduced *GtMYB3-SRDX* gene and endogenous flavonoid biosynthetic genes in transgenic gentian flowers, we performed a semi-quantitative RT-PCR analysis using the divided upper and lower parts of transgenic gentian petals. Expression of *GtMYB3-SRDX* driven by the AtACT2 promoter was detected in both transgenic lines nos. 7 and 11. The petals of clone no. 11 showed stronger expression than those of clone no. 7, and the *GtMYB3-SRDX* transgene was expressed at the same levels in the upper and lower parts of the petal. The AtACT2 promoter is reportedly a constitutive promoter in Arabidopsis (An et al. 1996), and *AtACT2pro-GUS* transgenic gentian plants also showed uniform expression in floral organs and leaves (data not shown). Expression of *F3H*, *F3′5′H*, *DFR*, *ANS*, and *3GT* genes in clone nos. 7 and 11 decreased significantly compared with those of the wild-type. Clone no. 11, in which *GtMYB3-SRDX* was strongly expressed, showed stronger suppression of transcription of these genes than clone no. 7. The white-flowered gentian cv. 'Polarno-White' resulted from mutation of *GtMYB3* by insertion of a transposable element (Nakatsuka et al. 2008b). The petals of this white-flowered cultivar also showed decreased transcriptions of *F3H*, *F3′5′H*, *DFR*, *ANS* and *3GT* (Nakatsuka et al. 2005b). The gene sets suppressed by *GtMYB3-SRDX* completely corresponded with those genes downregulated in the *GtMYB3*-deficient mutant cultivar (Figure 4). In *PAP1-SRDX*-expressed Arabidopsis, expression of *CHS*, *DFR*, *LDOX* and *BAN* was also suppressed, whereas *TT2*, *PAP1* and *PAP2*, genes encoding proanthocyanin biosynthetic transcription

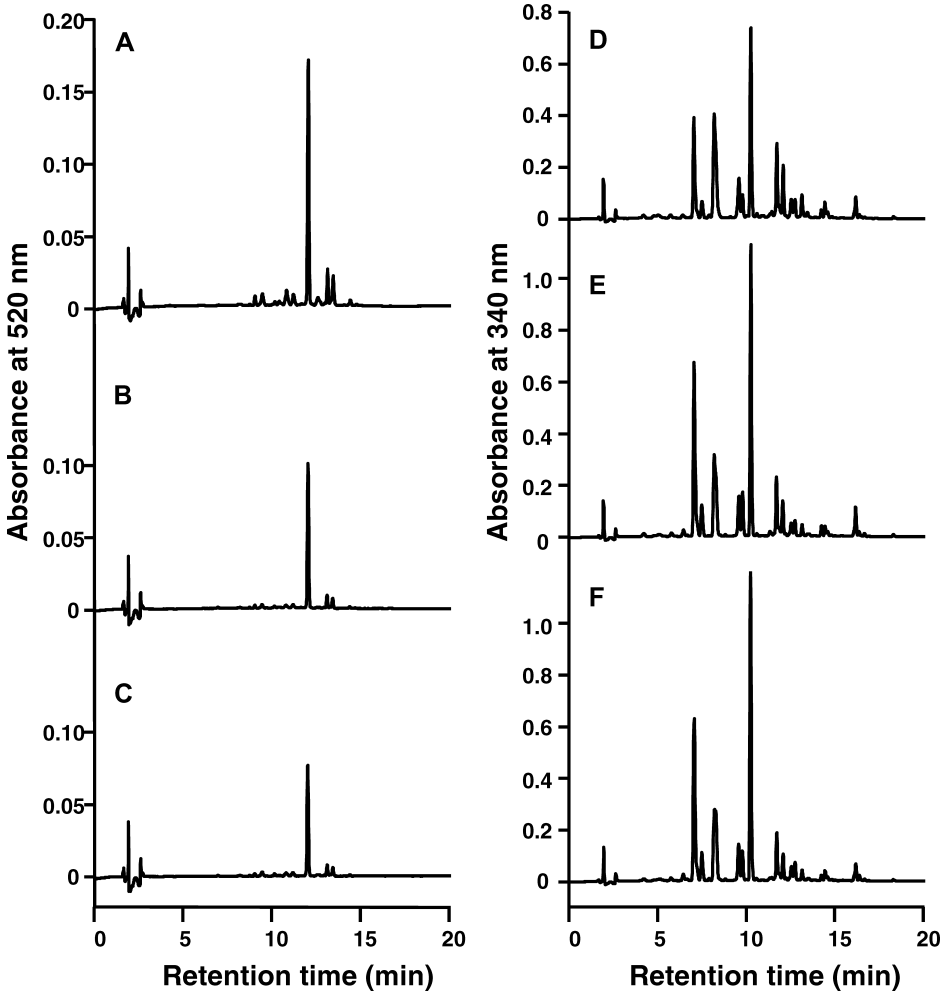

Figure 3. Flavonoid analysis in the flowers of transgenic gentian plants. Flavonoids were extracted from the petals of each gentian plant with EAA, and analyzed by HPLC as described in the Materials and Methods. Anthocyanins and flavones were detected at 520 nm (A–C) and 340 nm (D–F), respectively. The chromatographs of wild-type (A, D), and *GtMYB3-SRDX*-expressed transgenic gentian clones no. 7 (B, E) and no. 11 (C, F), are shown.

factors, were less affected (Matsui et al. 2004). In transgenic gentian plants, the expression of endogenous *GtMYB3* and *GtbHLH1* was not affected by introduction of *GtMYB3-SRDX* (Figure 4, data not shown). These results suggested that the suppression of the late flavonoid biosynthetic genes was due to the effect of *GtMYB3-SRDX* but not to cosuppression of *GtMYB3*.

One interesting property in *GtMYB3-SRDX*-expressed transgenic gentians is induction of the picotee flower phenotype, in which the lower part of the petal lacks pigmentation. Picotee and bicolor phenotypes are extremely desirable, and are some of the main targets in ornamental flower breeding. Our transgenic gentian plants showed significantly suppressed expression of late flavonoid biosynthetic genes, especially *F3H*, in the lower part of petals; therefore, the accumulation of flavanones, which are the precursors of flavones, would occur in the entire petal. In addition, early flavonoid biosynthetic genes were hardly affected by *GtMYB3-SRDX* and stronger expression was detected in the lower part of petals than in the upper part (Figure 4). Therefore, it is likely that the FNSII enzyme might convert the accumulated flavanones to flavones more effectively in the lower part of transgenic gentian petals. Pigmentation owing to anthocyanin accumulation in the lower part of petals was suppressed by competition with flavone biosynthesis, and therefore might result in the picotee phenotype in *GtMYB3-SRDX*-expressing gentian plants. That is, the picotee phenotypes of transgenic gentians are likely to result from both incomplete suppression of the anthocyanin biosynthetic pathway by *GtMYB3-SRDX* and non-uniform distribution of FNSII activity in petals. In petunia, two types of marginal picotee formation, white margin and white center, were characterized at the molecular level (Saito et al. 2006, 2007). In the unpigmented margin of white-margin cultivars, the expression of *CHS* was reduced, leading to repression of the biosynthesis of flavonols and

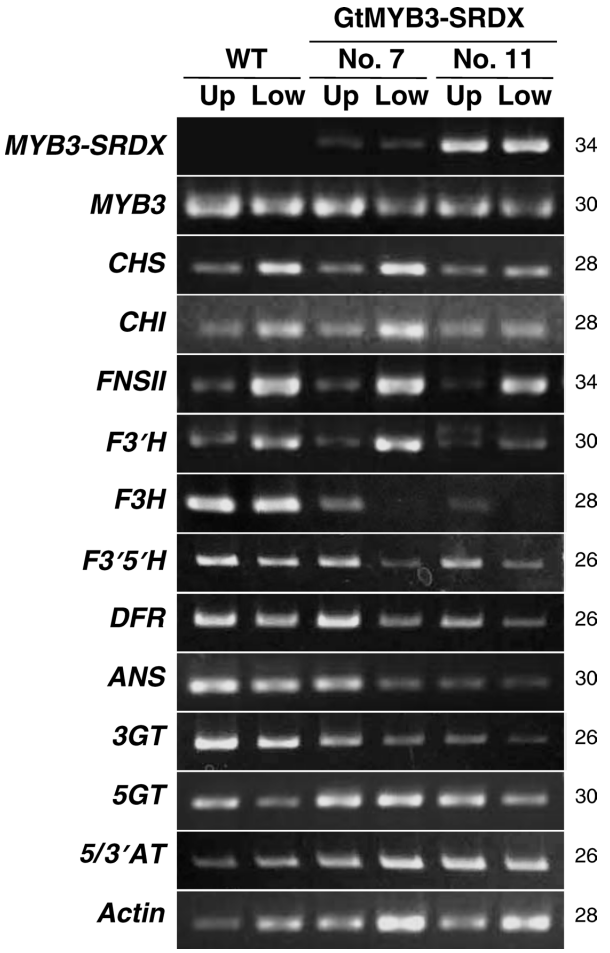

Figure 4. Expression of flavonoid biosynthetic genes in transgenic gentian plants. The expression levels of *GtMYB3-SRDX* and endogenous flavonoid biosynthetic genes were determined by semi-quantitative RT-PCR analysis in wild-type and *GtMYB3-SRDX*-expressed transgenic gentian clone nos. 7 and 11. Total RNAs were isolated from the upper (Up) and lower parts (Low) of petals of each transgenic plant. cDNAs were synthesized and subjected to semi-quantitative RT-PCR analysis using the primer sets listed in Table 1. Analyzed gene names are shown to the left of each panel. PCR cycle numbers are indicated to the right of each panel.

anthocyanins. In contrast, reduction of anthocyanin biosynthesis by enhanced expression of *FLS* is suggested to be involved in formation of the unpigmented central area of white-centered cultivars (Saito et al. 2006). *GtMYB3-SRDX*-expressing gentian plants might also exhibit a similar mechanism to that of white-centered petunia flowers, although further studies are necessary to confirm this hypothesis.

In conclusion, this is the first report of the use of the CRES-T system to silence target genes of a transcription factor in Japanese gentians. The chimeric repressor of anthocyanin biosynthetic regulator genes, *GtMYB3*, induced the picotee flower phenotype with pigmentation absent in the lower part of the petal. This phenotype was never observed in silencing of anthocyanin biosynthetic structural genes by RNAi and antisense strategies; therefore, this system has potential to generate additional novel flower colors in gentian. Furthermore, CRES-T is a useful molecular tool to produce plant materials for future studies of morphogenesis and flowering of gentian.

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