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Original Paper

RNAi suppression of the anthocyanidin synthase gene in *Torenia hybrida* yields white flowers with higher frequency and better stability than antisense and sense suppression

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Abstract Post-transcriptional gene silencing, such as antisense suppression, sense suppression (or cosuppression), and RNAi, is often used to down-regulate a target gene in transgenic plants. Novel flower color is industrially important; furthermore, flower color is a convenient tool to monitor the stability of such silencing. Previously, we obtained white torenia through sense suppression of chalcone synthase or dihydroflavonol 4-reductase (Suzuki et al. 2000). However, their phenotypes were not stable. In this study, we suppressed the anthocyanidin synthase gene using three methods in transgenic torenia. About half of the transgenic torenia plants gave white flowers by RNAi suppression of the gene, while antisense and sense suppression yielded a few and no white flowers, respectively. The white flower color obtained by RNAi has been stable for three years in a greenhouse. This study shows the usefulness of RNAi to suppress a target gene.

Key words: Anthocyanin, flavonoids, flower color, metabolic engineering, torenia.

Down-regulation of a target gene in a target crop is fundamentally important to generate useful transgenic plants through genetic engineering. The knockout of a gene by homologous recombination has been reported for a few plant species, including rice (Terada et al. 2002), but it is not yet a practical choice for most plant species. Post-transcriptional gene silencing (PTGS) is a widely used technique to suppress a target gene. Antisense and sense suppression, which are types of PTGS, have been reported for many plant species. The frequency of phenotypic changes by antisense and sense suppression in transgenic plants is less than 1% to 40%, respectively (Tanaka and Mason 2003). More recently, the transcription of double-strand RNA was shown to effectively down-regulate a gene (up to 96%; Hamilton et al. 1998; Waterhouse et al. 1998).

Flower color comes mainly from anthocyanins, a color class of flavonoids. All structural genes leading to anthocyanidins in the pathway have been cloned from many plants (Tanaka et al. 2005). Some of these genes have been used to generate white flowers from color flowers through antisense or sense suppression. Antisense and sense suppression were described in petunia using the chalcone synthase (*CHS*) gene (van der Krol 1988 and Napoli et al. 1990, respectively). Since then, flower color biosynthesis has been down-regulated through antisense or sense suppression in many ornamental plants, such as rose, carnation, and chrysanthemum (Gutterson 1995; Tanaka and Mason 2003). However, none of these plants has been commercialized. The suppression is not always consistent, and achieving such suppression is more challenging than over-expression of a gene in transgenic plants (Tanaka and Mason 2003). For example, transgenic petunia plants whose endogenous genes had been suppressed through sense or antisense suppression lost their original phenotypes after a few years (Tsuda et al. 2005).

Torenia is a popular ornamental pot and garden plant with four-petal flowers of various colors. Torenia is a model plant to study PTGS (Aida and Shibata 2001) because its efficient transformation system has been established (Aida and Shibata et al. 1995) and its flavonoid biosynthetic genes have been cloned (Suzuki et al. 2000; Ueyama et al. 2002, Figure 1). Aida et al. (2000) generated transgenic *T. fourunieri* plants that had lighter flower colors than those of the host through sense and antisense suppression of the CHS or dihydroflavonol 4-reductase (DFR) gene and reported that sense and antisense genes gave different phenotypes. Fukuzaki et al. (2004) reported that *T. hybrida* cv. Summerwave Blue

Abbreviations: ANS, anthocyanidin synthase; DFR, dihydroflavonol 4-reductase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
This article can be found at http://www.jspcmb.jp/

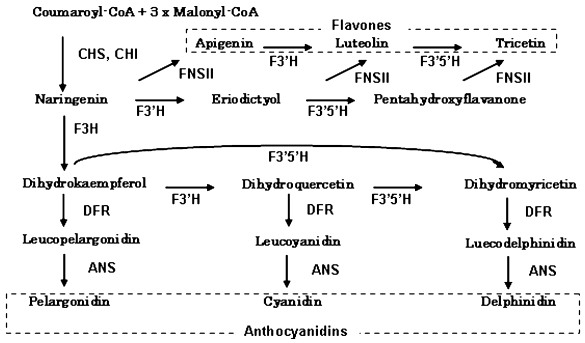

Figure 1. The anthocyanidin biosynthetic pathways in *Torenia hybrida.* CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3′-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; FNSII, flavone synthase II; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase.

has two CHS genes and that the transcription of the double-strand RNA of the 3′-non-coding sequence of one of them could specifically down-regulate that molecular species.

We reported that the sense suppression of *CHS* or *DFR* genes in *Torenia hybrida* cv. Summerwave Blue yielded white and partly white flowers (Suzuki et al. 2000) and carried out a small-scale field trial of the two selected lines exhibiting stable flower color phenotypes (Tanaka and Mason 2003). Unfortunately, their flower color was not always stable, and the blue color often reappeared in the white petals (unpublished results). Furthermore, transgenic torenia whose CHS gene had been suppressed seemed to lose its vigor (unpublished results), probably because the suppression of flavonoid biosynthesis is detrimental to the plant. Flavonoids play various physiologically important roles, such as protection from UV and various stresses, in addition to producing flower color (Harborne and Williams 2000).

In this study, we compared the efficacy of sense, antisense, and RNAi suppression in transgenic torenia using the anthocyanidin synthase (*ANS*) gene in order to obtain white torenia because ANS catalyzed the last step of coloration, *i.e.*, the conversion of colorless luecoanthocyanidins to colored anthocyanidin (Nakajima et al. 2001). The down-regulation of ANS was expected to have a less detrimental effect on transgenic plants than that of CHS.

T. hybrida cv. Summerwave Blue (Suntoryflowers, Ltd.) is an interspecies hybrid with a cascade-type morphology and vigorous flowering (Suzuki et al. 2000). Its male and female sterility makes molecular breeding the preferred way to increase its flower color varieties. The plant and its transgenic plants were grown in containment greenhouses that are suitable to grow genetically modified plants. The torenia transformation was carried out as described previously (Aida and Shibata 1995) via *Agrobacterium tumefaciens* AGL0

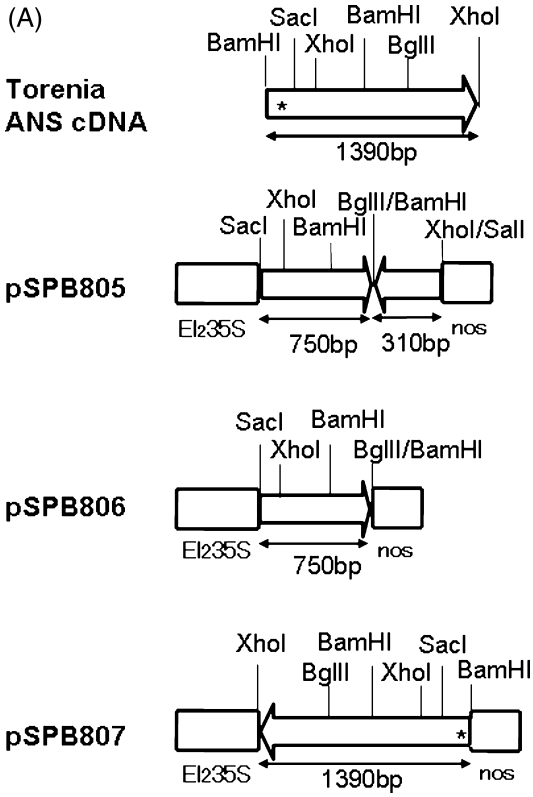

Figure 2.

(Lazo et al. 1990). The molecular biological procedures have also been reported in previous studies (Fukuchi-Mizutani et al. 2003). The binary vectors constructed in this study (pSPB805, 806, and 807) for the RNAi, sense, and antisense suppression of the torenia *ANS* gene (Nakajima et al. 2000) are shown in Figure 2A. The backbone of the binary vectors was from pBinPLUS (van Engelen et al. 1995). An enhanced cauliflower mosaic virus 35S promoter (Mitsuhara et al. 1996) was used to transcribe the ANS gene.

Quantitative RT-PCR was carried out with an ABI Prism ABI7000 sequence detection system (Applied Biosystems Inc.). The primer pairs that were used to quantify ANS, dihydroflavonol 4-reductase (DFR), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts are summarized in Table 1. Anthocyanidins and flavonoids were analyzed as previously described (Murakami et al. 2004).

The results of the transformation of torenia with pSPB805, 806, and 807 are summarized in Table 2. Only a small number of transgenic plants derived from pSPB806 (sense suppression) and pSPB807 (antisense suppression) exhibited paler flower color than the host. Only one white flower plant was obtained from pSPB807. On the other hand, about 90% of the transgenic plants by pSPB805 (RNAi suppression) gave a paler flower color, and more than 50% produced white flowers. Interestingly, one transgenic plant (Line 805–36)

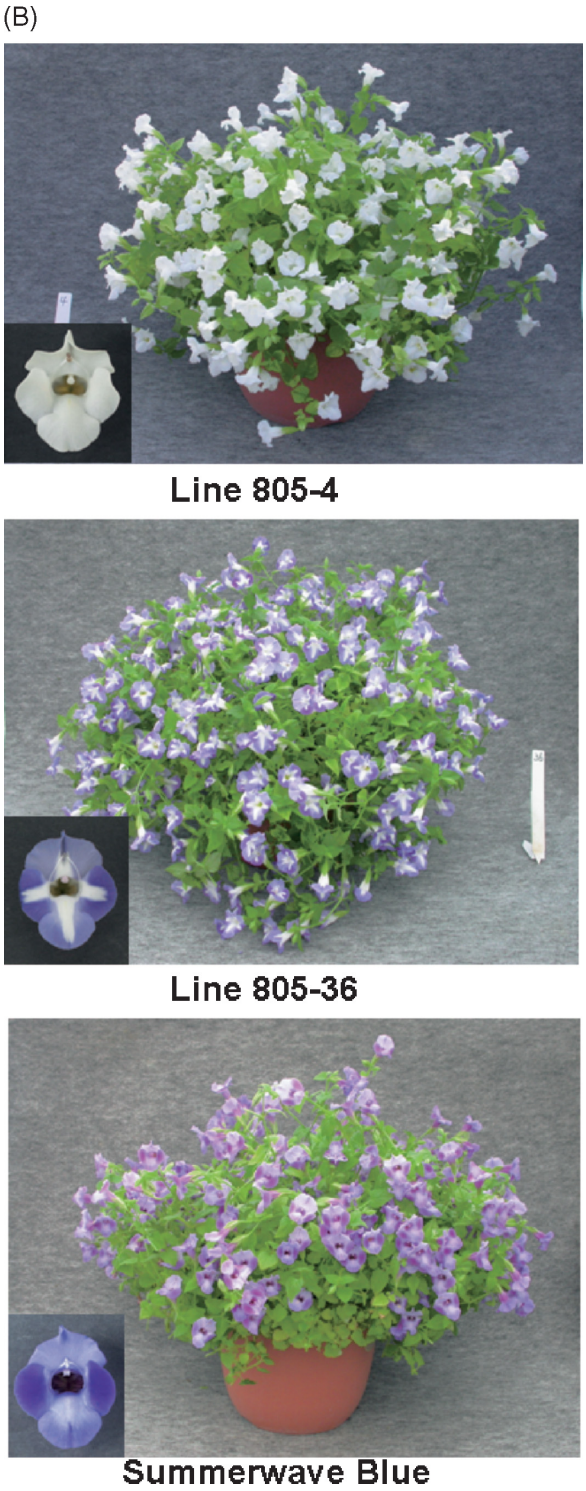

Figure 2.

exhibited a novel flower color phenotype (Figure 2B) that is different from the wavy coloration of petals previously reported (Aida et al. 2000).

Transgenic torenia with white flowers was kept in a greenhouse for about one year, and Lines 805–4, 805–25, 805–27, and 805–85 were selected for further study on the basis of flower color stability and growth

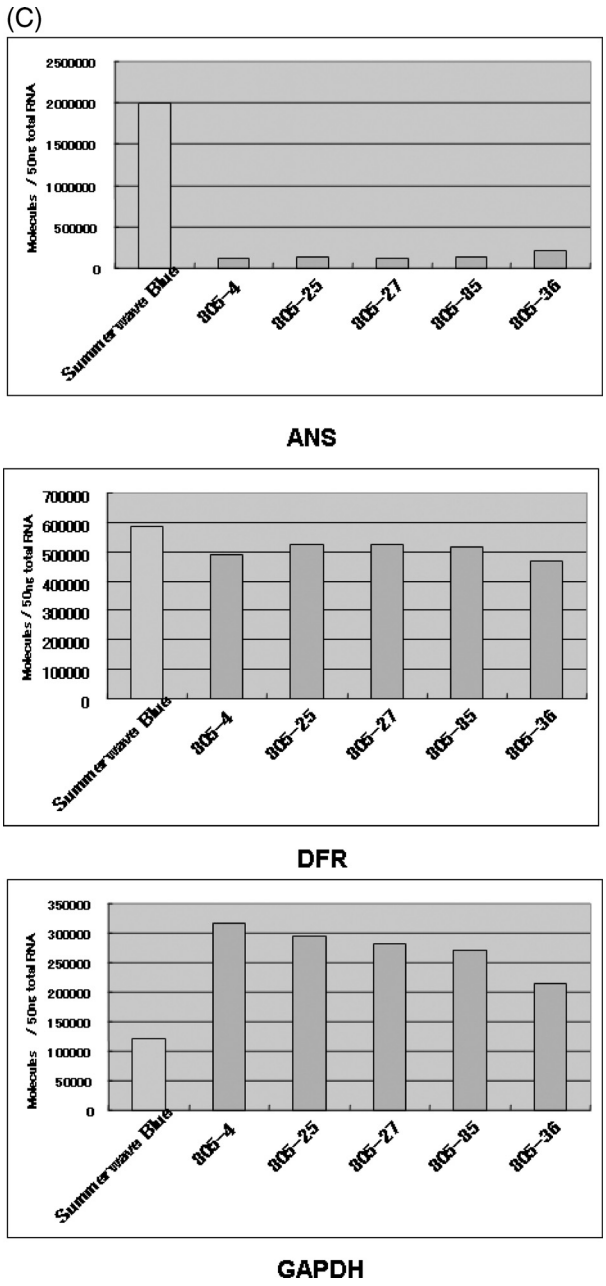

Figure 2. (A) Torenia *ANS* cDNA and binary vectors used in this study. The expression cassettes of pSPB805 (RNAi), 806 (sense), and 807 (antisense) are shown. The 310-bp inverted repeat and 440-bp loop sequences in pSPB805 are derived from the *Xho*I-*BamH*I and *BamH*I-*Bgl*II portions of the torenia *ANS* cDNA (Nakajima et al. 2000), respectively. The plasmid pSPB807 contains a full-length cDNA of the torenia *ANS* gene in the antisense orientation. The cDNA in pSPB806 lacked the 5′ untranslated sequence and amino terminal coding sequence. Some restriction enzyme sites are shown. The stars and arrows indicate the initiation codon and direction of ANS cDNA, respectively. The length is not in scale. El235S, enhanced cauliflower mosaic virus 35S promoter (Mitsuhara et al. 1996); nos, nopaline synthase terminator. (B) Flowers and plants of Line 805–4, Line 805–36, and Summerwave Blue. (C) Quantification of the *ANS*, *DFR*, and *GAPDH* transcripts. Only the *ANS* transcripts were down-regulated.

Table 1. Primers used for the quantitative RT-PCR analysis of torenia genes in this study.

Gene	Primer	Sequence
ANS	Forward	5′-GGC CTC CAA GTG CTC TAC AAA-3′
	Reverse	5′-CCC CAA CAT GCA AGA TTA TGG-3′
	TaqMan MGB Probe	5′-AGT GGG TCA CCG CTT-3′
DFR	Forward	5′-AAT GGG ATG CTT CCG ACT TCT-3′
	Reverse	5′-CAG TGG TTT CTG CCA TTG CTT-3′
	TaqMan MGB Probe	5′-AGG AAA AAA CAG GCT GAA AA-3′
GAPDH	Forward	5′-GCA TTG AGC AAG ACG TTT GTG-3′
	Reverse	5′-ACG GGA ACT GTA ACC CCA TTC-3′
	TaqMan MGB Probe	5′-AGC TTG TGT CGT GGT ACG-3′

ANS and *DFR* gene sequences were based on Nakajima et al. 2000 and Suzuki et al. 2000, respectively. The torenia *GAPDH* gene sequence was obtained in this study.

Table 2. Summary of obtained transgenic torenia plants.

Constructs	Number of the obtained transgenic plants	Number of white flower lines (%)	Number of paler flower lines (%)
RNAi (pSPB805)	98	50 (51%)	37 (38%)
Sense (pSPB806)	94	0	6 (6.4%)
Antisense (pSPB807)	100	1 (1%)	1 (1%)

Table 3. Flavonoid analysis of the flowers of the selected plants.

	Anthocyanidins (mg g^{-1} petal)	Flavones (mg g^{-1}petal)
Line 805–4	0.014	3.131
Line 805–25	0.012	2.698
Line 805–27	0.010	2.860
Line 805–85	0.013	3.077
Line 805–36	0.524	2.534
Summerwave Blue	0.818	5.799

characteristics. Line 805–36 was also studied. These selected plants have exhibited phenotypic stability for three years in a greenhouse, and vegetatively propagated plants have exhibited the same phenotypes as the mother plants. These results reveal that RNAi suppression achieved down-regulation of the target gene more frequently and consistently than antisense and sense suppression and that RNAi technology is a powerful tool to obtain transgenic plants with aimed phenotypes. The selected plants in this study will be subjected to a field trial to examine the stability of the suppression by RNAi and phenotypes in the outside environment.

The results of flavonoid (anthocyanidin and flavone) analysis, shown in Table 3, confirm the dramatic decrease in anthocyanidins. The amount of flavones also decreased, which is curious because the biosynthesis of flavones stems from that of anthocyanins at the step of flavanones. Down-regulation of the *FNSII* gene in torenia resulted in a decrease in both flavones and anthocyanins (Ueyama et al. 2002). Flavones and anthocyanins might stabilize each other, or there might be unknown cross talk between flavone and anthocyanin biosynthesis/accumulation. Further analysis is in progress. The results of the quantitative RT-PCR of *ANS*, *DFR*, and *GAPDH* transcripts in transgenic torenia are shown in Figure 2C. The *ANS* transcripts were down-regulated, while *DFR* and *GAPDH* transcripts were not affected. The results indicate that the suppression is specific to the *ANS* gene.

This study also demonstrates that the *ANS* gene can be used to block anthocyanin biosynthesis. Unlike CHS, which is often used to block anthocyanin biosynthesis, blockage of ANS, the final enzyme to synthesize color, should have fewer side effects on transgenic plants. The flower color obtained in this study should command high market value because white varieties are always popular in the flower industry. One concern with the release of genetically modified plants is gene dispersal, which can be avoided due to the sterility of the torenia used in this study. Release of such plants should contribute to the public acceptance of genetically modified plants.

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