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Altering Flower Color in Transgenic Plants by RNAi-Mediated Engineering of Flavonoid Biosynthetic Pathway

Yoshikazu Tanaka, Noriko Nakamura, and Junichi Togami

Summary

Flower color is mainly determined by the structure of flavonoids, a group of secondary metabolites of plants. The biosynthetic pathway and the genes involved in the pathway are well characterized such that it is possible to change flower color by engineering the pathway by overexpression of heterologous genes and/or suppression of endogenous genes in transgenic plants. Trimming an unnecessary pathway by suppression of endogenous genes is often essential to achieve successful engineering of the pathway and the resultant accumulation of desirable compounds. RNAi by transcription of double-stranded RNA (dsRNA) is a powerful and efficient method to command such suppression and is widely used for artificial gene suppression in transgenic plants.

Key Words: anthocyanidin; flavonoid; double-stranded RNA; RNAi; transgenic plant.

1. Introduction

Suppression of a gene is an important technique to obtain transgenic plants with desirable phenotypes and to analyze the function of the gene in plants. Recent rapid progress in plant biology has clarified many metabolic pathways, and engineering these pathways has been found feasible in transgenic plants. Since overexpression of a heterologous gene alone is not enough to compete against an endogenous pathway, downregulation of the competing endogenous gene expression is often required to obtain a desired phenotype, i.e., efficient accumulation of a desired metabolite.

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In theory, the suppression of gene expression can be achieved by replacing (knocking out) the gene in plant genomes, much as in a knockout mouse. The knockout of a gene by homologous recombination has been reported for only limited plant species ***(1)*** and is not yet a practical choice for most plant species because of the very low frequency of the knockout. Furthermore, plant species often have high ploidy (for example, cultivated wheat and chrysanthemums have six sets of chromosomes) and often have plural genes encoding the same enzymes. Clearly, it would not be practical to knock out all relevant alleles and loci in these plants.

Posttranscriptional gene silencing (PTGS) is a technique to suppress a target gene in transgenic plants. Antisense and sense suppression, which are types of PTGS, used to be a common way to achieve suppression. However, the frequency of phenotypic changes by antisense and sense suppression in transgenic plants is modest and variable ***(2)***. Low-frequency and unstable gene suppression of antisense and sense suppression often hampered the analysis of the gene function and the commercialization of transgenic plants. More recently, the transcription of dsRNA in transgenic plants was shown to effectively downregulate a gene by generating small interfering RNA ***(3,4)***. RNAi is now the preferred method for downregulating gene expression in transgenic plants.

Red, violet, and blue flower colors are usually from a colored and glycosylated class of flavonoids called anthocyanins. The flavonoid and anthocyanin biosynthetic pathway has been well characterized (**Fig. 1**) ***(5,6)***, and the modification of flower color has been achieved in many species by engineering the pathway in a transgenic plant ***(6,7)***. Suppression of one of the genes such as chalcone synthase (CHS), flavanone 3-hydroxylase, and dihydroflavonol 4-reductase (DFR) in the pathway leads to white flowers, which has been reported in many species including petunia, carnation, chrysanthemum, and rose with the use of sense and antisense suppression ***(6,8)***. We also obtained white flowers in petunia and torenia with the use of sense and antisense suppression of CHS and DFR genes, but the frequency of suppression and the phenotypic stability were unsatisfactory ***(9,10)***. More recently, we succeeded in downregulating the anthocyanidin synthase (an essential enzyme to synthesize colored compounds, anthocyanidins; **Fig. 1**) gene in transgenic torenia plants with the use of RNAi ***(11)***. RNAi can be used to obtain floricultural crops with novel flower colors, which may command high market value.

Agrobacterium tumefaciens-mediated gene transfer is the best method to transform plant cells ***(12)***. *A. tumefaciens* can transfer the T (transfer)-DNA region of a binary vector (**Fig. 2**). By inserting a desired gene in the T-DNA region, the gene can be introduced into plant cells. A binary vector containing a

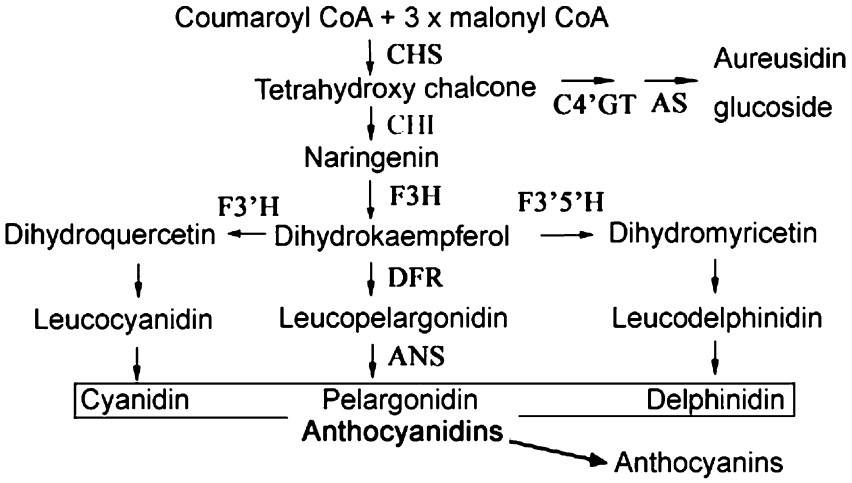

Fig. 1. The flavonoid biosynthetic pathways in *Torenia hybrida*. The flower color is mainly determined by the flavonoid compounds that the flower accumulates. A flower accumulating aureusidin glucoside (a kind of aurone) exhibits yellow color. Pelargonidin, cyanidin, and delphinidin (anthocyanidins) tend to yield orange/red, red/magenta, and violet/blue flower color depending on other factors ***(6,8)***. Anthocyanidins are usually glucosyalted (anthocyanins) and stored in vacuoles of plant cells. It is possible to change flower color by engineering this pathway in transgenic plants ***(6–8)***. 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.

selection marker and a replicon for both *E. coli* and *A. tumefaciens* is a shuttle vector between them and can be handled as a standard *E. coli* plasmid.

Plant transformation protocols depend on plant species and cultivars to a large degree. Only the torenia transformation protocol is provided in this chapter as a model plant species.

2. Materials

2.1. Binary Vector Construction

1. The plasmid pBE2113 containing an enhanced cauliflower mosaic virus 35S (CaMV35S) promoter ***(13)*** was kindly provided by Dr. Mistuhara (National Institute of Agrobiological Sciences, Tsukuba, Japan). CaMV35S is a strong and constitutive plant promoter that is universally distributed among plant molecular biologists. The CaMV35S promoter generally works well to transcribe dsRNA

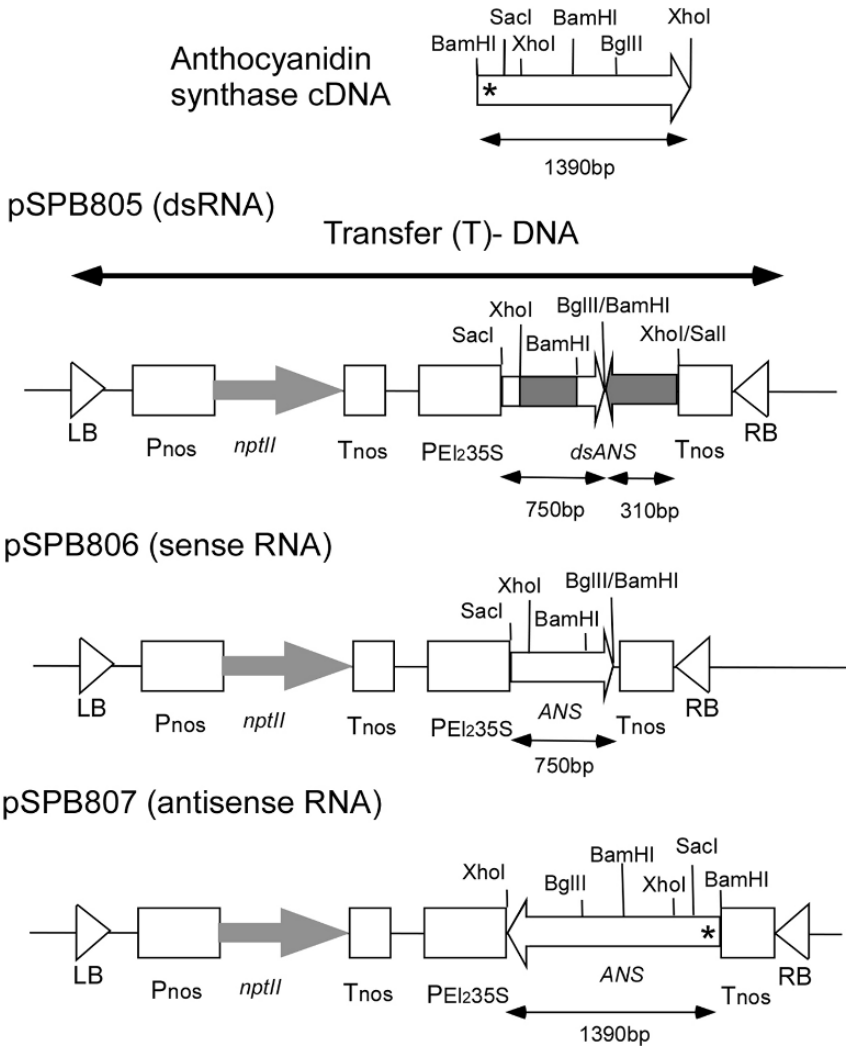

Fig. 2. Schematic presentation of torenia anthocyanidin synthase (ANS) cDNA and binary vectors used in this study. The transfer (T)-DNA regions of pSPB805 (RNAi) and a part of 806 (sense) and 807 (antisense) are shown. Only the T-DNA on the binary vector surrounded by the right border sequence (RB) and the left border sequence (LB) is transferred to plant cells and integrated into the chromosome. The plasmid pSPB805 contains the inverted repeat of 310 bp (shown with gray color) derived from the *Xho*I-*Bam*HI-digested fragment of the cDNA of the torenia ANS gene and a 440-bp loop-out sequence derived from the *Bam*HI-*Bgl*II digested fragment. The cDNA in pSPB806 lacked the 5′-untranslated sequence and the amino terminal coding sequence. The plasmid pSPB807 contains a full-length cDNA of the torenia ANS gene in the antisense orientation. Some restriction enzyme sites are shown. The arrows indicate

to cause RNAi. The plasmid is derived from a common binary vector, pBI121 (previously sold by Clontech).
2. The plasmid pBINPLUS ***(14)*** is available from Plant Research International (Wageningen, The Netherlands) (http://www.pri.wur.nl/UK/products/ImpactVector/Technology/). This plasmid is more convenient than pBI121 because it has a higher copy number in *E. coli* and contains more restriction sites, including those for 8-base recognition enzymes. An enhanced CaMV35S promoter is also available from Plant Research International (*see* **Notes 1** and **2**).
3. The phosphorylated *Sac*I linker and *Xho*I linker can be obtained from some manufacturers, such as Takara Bio, Inc. (Otsu, Shiga, Japan).
4. Restriction enzymes, T4 DNA ligase, and competent *E. coli* cells are available from many vendors such as Takara Bio and Promega (Madison, WI) with proper reaction mixtures and protocols. The DNA Blunting Kit is from Takara Bio. T4 DNA polymerase can be used instead.
5. Agarose (Takara Bio) and TAE buffer (40 m*M* of Tris, 40 m*M* of acetic acid, 1 m*M* of EDTA).
6. Geneclean (MP Biomedicals Inc., Irvine, CA).

2.2. Agrobacterium Growth

1. YEP medium: Difco Yeast Extract (BD Bioscience, San Jose, CA) (1% w/v), Difco Peptone (BD Bioscience) 1% (w/v), NaCl (0.5% w/v), pH 7.0.
2. YEP semisolid medium: YEP medium containing 1.5% (w/v) Difco agar (BD Bioscience) in a Petri dish of ~90-mm diameter.
3. Kanamycin stock solution: Kanamycin monosulfate (Kan, Sigma-Aldrich Co., St. Louis, MO) was dissolved at 50 mg/mL (w/v) in water. For example, dissolve 500 mg of kanamycin in 10 mL of sterile water and filtrate with a Millex-GV Syringe Driven Filter Unit (0.22 μm, Millipore, Co., Bedford, MA) and a sterile syringe. It was then stored in aliquots at –20 ºC.
4. YEP liquid and semisolid medium containing Kan (50 μg/mL).
5. 20 m*M* of $CaCl_2$.
6. *A. tumefaciens* LBA4404 competent-cells can be purchased from Invitrogen (Carlsbad, CA). The strain AGL0 ***(15)*** used in this study was kindly provided by Dr. Robert Ludwig (University of California, Santa Cruz, CA) (*see* **Note 3**).
7. Falcon conical centrifuge tubes (50 mL) (BD; Franklin Lakes, NJ).

Fig. 2. (*Continued*) the direction of ANS cDNA. The length is not to scale. PEl235S = the enhanced cauliflower mosaic virus 35S promoter; Pnos = the nopaline synthase promoter; Tnos = the nopaline synthase terminator; nptII = neomycin phosphor transferase II gene that confers kanamycin resistance to plant cells; * = initiation codon.

2.3. Torenia Transformation

1. *Torenia hybrida* cultivar Summerwave Blue (Suntory Flowers, Ltd., Tokyo, Japan) plants can be purchased from nurseries in the United States, Europe, Japan, and Australia. Alternatively, *Torenia frunieri* seeds can be purchased (for example, from Sakata Seed, Yokohama, Japan).
2. 1% (w/v) liquid sodium hypochlorite.
3. A paper towel that has been autoclaved and dried.
4. Murashige and Skoog (MS) Basal Medium including vitamins (4.4 g/L; Duchefa Biochemie, Haarlem, The Netherlands) containing 3 g/L of gellan gum for plant tissue culture (Kanto Chemical Co., Inc., Tokyo, Japan).
5. TA medium: 4.4 g/L of MS basal medium, 3 g/L of gellan gum, 30 g/L of sucrose, 1 mg/L of N-benzyl-9-(2-tetrahydropyranyl)adenine (BA, Sigma-Aldrich Co.), 0.1 m*M* of acetosyringone (3′,5′-Dimethoxy-4′-hydroxyacetophenone) (Sigma-Aldrich Co.), pH 5.8. Acetosyringone should be added after the media is autoclaved. Acetosyringone increases *Agrobacterium* infection.
6. TKC3 medium: 4.4 g/L of MS basal medium, 3 g/L of gellan gum, 30 g/L of sucrose, 1 mg/L of BA, 300 mg/L of Carbenicillin disodium salt (Cb, Sigma-Aldrich Co.), 300 mg/L of Kan, pH 5.8. Here and in all other media described below, Cb and Kan should be added after autoclaving of the media. Cb is to remove *Agrobacterium* and Kan is for selection of transgenic cells.
7. TKC2: 4.4 g/L of MS basal medium, 3 g/L of gellan gum, 30 g/L of sucrose, 1 mg/L of BA, 200 mg/L of Cb, 300 mg/L of Kan, pH 5.8.
8. TKC1: 4.4 g/L of MS basal medium, 3 g/L of gellan gum, 30 g/L of sucrose, 1 mg/L of BA, 100 mg/L of Cb, 300 mg/L of Kan, pH 5.8.
9. TKC0.5: 4.4 g/L of MS basal medium, 3 g/L of gellan gum, 30 g/L of sucrose, 50 mg/L of Cb, 300 mg/L of Kan, pH 5.8.
10. Filter paper (Advantec Filter Paper 2, 70-mm diameter, Toyo Roshi Kaisha Ltd., Tokyo, Japan).
11. A disposable Petri dish.
12. Phytotray II containers (Sigma-Aldrich Co.).
13. Surgical tape (Micropore 1530-0, 3M Health Ltd., Tokyo, Japan), Parafilm laboratory film (Pechiney Plastic Packaging, Menasha, WI).
14. Surgical blade (No. 10, No. 19, Futaba Co. Ltd., Tokyo, Japan).

3. Methods

RNAi can be triggered by transcribing the dsRNA of a gene of interest. The first step is the construction of a binary vector plasmid containing a transcriptional cassette to transcribe the dsRNA of the gene to be downregulated. A transcriptional cassette in the T-DNA region consists of a promoter, the gene, and a terminator (**Fig. 2**). It is convenient to have multi-cloning sites on the 3′ side of the promoter and the 5′ side of the terminator; some binary vectors containing such sites may be available. However, it is often necessary to modify

the restriction sites for convenience. A constitutive promoter including the CaMV35S promoter and its derivatives is widely used for the transcription. Organ-specific promoters can be used to knock down the gene expression in specific organs ***(16)***.

For the transcription of dsRNA to trigger RNAi, we typically construct a transcriptional cassette containing a 300–1,000-bp inverted repeat sequence and a 100–300-bp loop sequence in T-DNA. We usually determine these lengths for practical reasons, such as the availability of restriction enzyme recognition sites. Although nonfunctional sequences, such as part of GUS, are sometimes utilized for the loop sequence, the integration of an additional sequence may be a matter of concern in deregulated genetically modified (GM) organisms; thus, we only use the sequence of the gene of interest. Intron sequences may increase the frequency of suppression ***(2)***, but we have not used them in our laboratory, because a reasonable frequency of the suppression can be obtained without them, and moreover, obtaining a genomic clone is not always easy.

Anthocyanidin synthase (ANS) catalyzes the conversion of colorless leucoanthocyanidins to colored anthocyanidin (**Fig. 1**). Suppression of ANS in plants is expected to block anthocyanin biosynthesis and thus white flower color.

3.1. Binary Vector Construction for RNAi Suppression of the Anthocyanidin Synthase Gene of Torenia hybrida

1. Introduce *Sac*I and *Xho*I restriction sites on the 3′ side of the plant promoter and the 5′ side of the plant terminator, respectively. For example, digest the plasmid pBE2113 with *Sac*I, and ligate with appropriate phosphorylated *Xho*I linker. Transform *E. coli* cells with the reaction mixture. Digest the resultant plasmid with *Sna*BI and ligate with the phosphorylated *Sac*I linker to yield pBE2113′.
2. Digest pBE2113′ with *Hin*dIII and *Eco*RI and resolve by electrophoresis in a 0.7% agarose gel. Recover ~2.5-kb DNA fragment using Geneclean. Ligate the fragment with pBINPLUS that was digested with *Hin*dIII and *Eco*RI to yield pBE7.
3. Digest the plasmid pTAN1 containing torenia ANS cDNA (**Fig. 2**) with *Sac*I and *Bgl*II. Separate through 1% agarose gel electrophoresis and recover the 750-bp fragment containing the 5′ side portion of torenia ANS cDNA. Digest the same plasmid with *Xho*I and *Bam*HI and recover the 310-bp fragment containing the 5′ side portion of the cDNA. Digest the plasmid pBE7 with *Sac*I and *Xho*I, and ligate with the two DNA fragments recovered above to yield the binary vector pSPB805. Note that the *Bam*HI and *Bgl*II ends are compatible for ligation, and so are *Xho*I and *Sal*I. The 310-bp inverted repeat and 440-bp loop sequences are in pSPB805 (**Fig. 2**). When cohesive restriction sites are not available, blunt-end restriction sites, such as *Sma*I and *Pvu*II, can also be used, or cohesive restriction sites can be added by PCR amplification using primers with the recognition sequences added.

3.2. Transformation of Agrobacterium tumefaciens

1. Inoculate a colony of *A. tumefaciens* Agl0 that was streaked on YEP agar (semisolid) medium into 3 mL of YEP medium. Grow at 28 °C overnight with shaking. *A. tumefaciens* can be stored at –70 °C after adding glycerol to a final concentration of 15% (w/v).
2. Two mL of the culture are transferred to 50 mL of a YEP medium in a 250-mL flask and cultured at 28 °C for several hours with shaking until the optical density (O.D.) at 600 nm reaches approximately 1.0.
3. Chill the culture on ice and harvest cells by centrifugation at 5,000 rpm at 4 °C for 10 min. A 50-mL Falcon tube is convenient for this purpose. Suspend the collected cells in 20 mL of sterile pure water and harvest by centrifugation at 5,000 rpm at 4 °C for 10 min. Suspend the cells in 1 mL of ice-chilled 20 m*M* $CaCl_2$ solution. These competent cells can be stored in aliquots in Eppendorf tubes at –70 °C after adding glycerol to a final concentration of 15% (w/v).
4. Add ~1 μg of a binary vector plasmid pSPB805 (up to 20 μL) to 100 μL of competent cells in an Eppendorf tube, and place in ice for 30 min.
5. Freeze the tube in liquid nitrogen.
6. Thaw at 37 °C for 5 min.
7. Add 1 mL of YEP medium to the tube, and incubate at 28 °C for 2–4 h.
8. Collect the cells by centrifugation at 8,000 rpm for 5 min and suspend in 100 μL of YEP. Plate on a YEP agar plate containing 50 μg/mL of Kan (or another antibiotic depending on the binary vector used). Dozens to hundreds of colonies will appear after incubation at 28 °C for 2–3 days.
9. Deletion or the rearrangement in binary vectors sometimes occurs in *A. tumefaciens.* Binary vectors having an inverted repeat seem to result in deletion occasionally. It is recommended to check the plasmid contained in *A. tumefaciens*. For this purpose, grow a few colonies separately in 5 mL of YEP medium containing Kan at 28 °C for 2 days with shaking. Recover the plasmid using the protocol for *E. coli* plasmid preparation. PCR amplification of the entire or partial T-DNA region of the recovered plasmid will tell if the region is intact. Alternatively, transform *E. coli* with the recovered plasmid to amplify the plasmid and then compare its restriction fragment pattern with that of the original plasmid, pSPB805.
10. Streak the colony containing pSPB805 or the culture derived from the colony on YEP agar medium with Kan. A fresh plate culture (within approximately 2 weeks) should be used for plant transformation. The transformed *Agrobacterium* culture can be stored at –70 °C after 15% (w/v) glycerol is added.

3.3. Transformation of Torenia (17)

1. Establishment of torenia in a tissue culture (**Fig. 3**; *see* **Note 3**): Suspend the shoot tips of a plant of *T. hybrida* var. Summerwave Blue or the seeds of *T. furunieri* in liquid sodium hypochlorite at room temperature for 15 min for

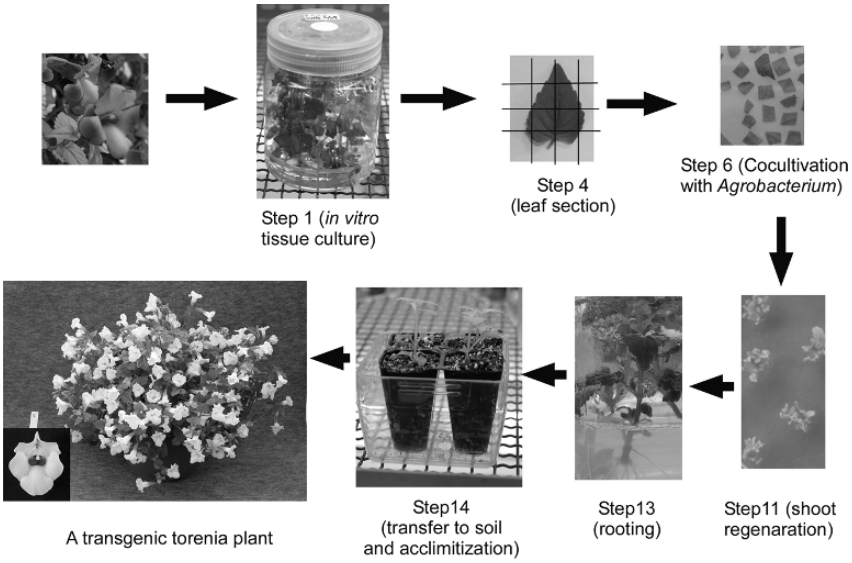

Fig. 3. Torenia transformation procedures and color change by RNAi suppression of the anthocyanidin synthase gene. The original blue flower color is modified to white by RNAi-mediated suppression of ANS gene. Some of the steps of torenia transformation are shown.

sterilization. Wash twice with sterile distilled water and transfer to an MS agar medium in a large test tube, a jar, or Phytotray II. Keep at 25 °C under fluorescent light tubes (approximately 3,500 lux). Once a culture plant is established, it can be propagated in tissue culture.

2. Culture a colony of *A. tumefaciens* containing pSBP805 in 5 mL of YEP medium containing antibiotic at 28 °C overnight with shaking. Transfer 3 mL of the culture to 30 mL of a YEP medium in a 200-mL flask containing antibiotic at 28 °C for approximately 3 h with shaking.
3. The bacterial cells are collected with centrifugation at 5,000 rpm for 10 min. The cells are suspended in an MS liquid medium (pH 5.8) at the concentration of $O.D._{550} = 0.1$.
4. Preparation of the leaf section and infection by *A. tumefaciens* (**Fig. 3**): Wet the sterile filter paper in a Petri dish with sterile water. Collect young torenia leaves from the three uppermost stems and place on the filter paper. Remove the edges of the leaves with a blade. Make three to four nicks (about 2 mm in length) parallel to the main vein and transfer to the suspended *A. tumefaciens* solution (prepared in step 3 above) in an empty Petri dish. Cut the leaves into sections of about 2 × 2 mm that are nicked at right angles to the main vein in the solution.
5. Transfer the leaf sections to a sterile paper towel, and remove the excess solution.

6. Cultivation with *A. tumefaciens* (**Fig. 3**): Place a filter paper on TA medium. Transfer the leaf sections, backside up, to this plate. Seal the plate with parafilm and store in darkness at 23 °C for 1 week.
7. Transfer the leaf sections after turning over to a plate with TKC3 medium. Wrap the plate with surgical tape and place under light at 23 °C for 2 weeks.
8. Transfer the leaf sections to a new TKC3 medium plate and maintain under light at 23 °C for 3 weeks.
9. Transfer to a TKC2 medium plate and maintain under light at 23 °C for 3 weeks.
10. Transfer to a TKC1 medium plate and maintain under light at 23 °C for 2–3 weeks. Keep transferring to a new TKC1 medium plate every 2–3 weeks until deep green calli are obtained.
11. Transfer the callus part to a new TKC1 medium plate in order to induce the shoot primordia. Once small shoots are regenerated, transfer to a TKC0.5 medium plate, and continue to grow the shoots to approximately 5 mm under light at 23 °C (**Fig. 3**).
12. Separate the shoots from the callus with a blade and transfer to a TKC0.5 medium in a Phytotray for rooting (**Fig. 3**). Only transgenic shoots form roots in this medium under light at 23 °C. Usually, rooting takes 1–2 weeks.
13. When the roots grow to approximately 5 mm, gently retrieve the plants from the medium with sterile forceps. Place the transgenic plant in appropriate soil such as expanded vermiculite and allow to grow under high humidity for approximately 3 weeks for acclimatization (**Fig. 3**).
14. Transfer to a greenhouse to grow and flower (***see*** **Note 5**). It will take ~2 months to bloom (***see*** **Notes 6–10**).

4. Notes

1. Various binary vectors (pCAMBIA series) are available to nonprofit institutions (http://www.cambia.org/daisy/cambia/585.html).
2. Binary vectors that are designed for the easy construction of dsRNA transcription have been reported and are available upon request from the authors. For example, a pHNNIBAL vector ***(2)*** allows a PCR product from a gene of interest to be converted to double-stranded transcripts, and a pPANDA vector ***(18)*** utilizes the Gateway system (Invitrogen) for efficient vector construction.
3. Import of *A. tumefaciens* from a foreign country may require quarantine permission because the bacterium is potentially pathogenic to plants.
4. All procedures for plant tissue culture should be carried out in a clean bench.
5. A greenhouse with a proper containment level that meets legal requirements is necessary to grow GM plants. The requirements may vary by country.
6. The frequency of flower color change by RNAi is much higher than that by antisense (pSPB807; **Fig. 3**) or sense suppression (pSPB806; **Fig. 3**), as shown in **Table 1** ***(11)***. The effectiveness of RNAi has also been reported ***(2)***.
7. Knockdown of a target gene can be confirmed with Northern blot analysis or quantitative RT-PCR of transcripts of transgenic plants ***(11)***. A target gene and

Table 1
Summary of the Transgenic Torenia Plants

Constructs	Number of Transgenic Plants Obtained	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%)

its close homologues including its alleles can be knocked down when the coding region is used to make the dsRNA. Since the 3′-noncoding sequence is more variable than the coding region, only one allele can be knocked down when the 3′-noncoding region is used to make the dsRNA ***(19)***.

8. The stability of gene suppression depends considerably on the transgenic events. In order to obtain a transgenic plant with consistent suppression, it is necessary to obtain as many transgenic plants as possible and select a suitable line.
9. It is possible to knock down more than one gene in transgenic plants by constructing plural cassettes to transcribe the double-stranded RNA. It is also possible to overexpress one or more heterologous genes and knock down an endogenous gene by making the corresponding number of transcriptional cassettes. For example, RNAi suppression of the flavanone 3-hydroxylase gene by RNAi and overexpression of the chalcone 4′-glucosyltransferase and aureusidin synthase genes in transgenic torenia yield yellow torenia that accumulates aureusidin glucoside ***(20)*** (**Fig. 1**). RNAi suppression of flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase in blue torenia resulted in pelargonidin-accumulating pink torenia (unpublished results).
10. Chalcone isomerase in the flavonoid pathway (**Fig. 1**) has a very high turnover number, and complete downregulation is necessary to block the pathway. This has been achieved in tobacco with the RNAi suppression of the gene, vindicating the efficacy of RNAi ***(21)***.

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