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RESEARCH PAPER

A chimeric repressor of petunia PH4 R2R3-MYB family transcription factor generates margined flowers in torenia

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ABSTRACT

The development of new phenotypes is key to the commercial development of the main floricultural species and cultivars. Important new phenotypes include features such as multiple-flowers, color variations, increased flower size, new petal shapes, variegation and distinctive petal margin colourations. Although their commercial use is not yet common, the transgenic technologies provide a potentially rapid means of generating interesting new phenotypes. In this report, we construct 5 vectors which we expected to change the color of the flower anthocyanins, from purple to blue, regulating vacuolar pH. When these constructs were transformed into purple torenia, we unexpectedly recovered some genotypes having slightly margined petals. These transgenic lines expressed a chimeric repressor of the petunia *PhPH4* gene under the control of *Cauliflower mosaic virus* 35 S RNA promoter. PhPH4 is an R2R3-type MYB transcription factor. The transgenic lines lacked pigmentation in the petal margin cells both on the adaxial and abaxial surfaces. Expressions of *Flavanone 3-hydroxylase* (*F3H*), *Flavonoid 3′-hydroxylase* (*F3′H*) and *Flavonoid 3′5′-hydroxylase* (*F3′5′H*) genes were reduced in the margins of these transgenic lines, suggesting an inhibitory effect of PhPH4 repressor on anthocyanin synthesis.

Abbreviations: ANS, Anthocyanin synthase; CHI, Chalcone isomerase; CHS, Chalcone synthase; DFR, Dihydroflavonol 4-reductase; F3H, Flavanone 3-hydroxylase; F3′H, Flavonoid 3′-hydroxylase; F3′5′H, Flavonoid 3′5′-hydroxylase; 3GT, UDP glucose flavonoid 3-O-glucosyltransferase; HPT, Hygromycin B phosphotransferase; HSP, Heat-shock protein; MYB, Myeloblastosis; RD, repression domain; TCP, Teosinte branched 1, Cycloidea, and PCF

Introduction

Our research institute is using a number of strategies to modify flower phenotype in torenia (*Torenia fournieri* Lind. ex Fourn. and related species). As well as being a suitable model flower for transformation, torenia is also popular in summertime as a domestic pot plant. The first round of trials with torenia included modification of the expressions of genes associated with anthocyanin synthesis. The purple anthocyanin of the cultivar 'Crown Violet' becomes reddish when expression of the *Chalcone synthase* (*CHS*) gene is repressed by an antisense construct, whereas the anthocyanin becomes bluish when expression of the *Dihydroflavonol 4-reductase* (*DFR*) gene is repressed by an antisense construct.[1,2] These transgenic flowers with altered colouration were also subjected to ion beam treatment to induce additional mutations and thus broaden the spectrum of phenotypes.[3]

As well as altering flower color, a new strategy was tested which modified flower shape and pigmentation pattern. 'Chimeric repressor' is a synthetic gene sequence, combining transcription factors and a small peptide called 'repression domain' (RD). Attachment of RD changes a transcription activator into a transcription repressor. As the repressor (or 'chimeric repressor') inhibits functions of endogenous homologues, chimeric repressor acts as a dominant-negative genetic factor.[4] AtTCP3 repressor (TCP: Teosinte branched 1, Cycloidea, and PCF)

derived from the *TCP3* gene of Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) is a typical chimeric repressor which has been used previously to modify flower phenotype in torenia. AtTCP3 repressor reduces petal pigmentation, generates undulated petals with rough edges, and shortens stems. AtTCP3 repressor also changes torenia leaves from dentate to crenate. Leaves are somewhat undulated in transformants.[5,6] Many other chimeric repressors were also tested by bulk transformation. In a way similar to AtTCP3 repressor, other repressors also have pleiotropic effects on flower and leaf phenotypes, including the generation of bizarre flowers.[7]

In this study with torenia, we sought new constructs that might affect vacuolar pH and so change flower color - especially toward blue. Against expectation, anthocyanin color did not change to blue with these constructs. Instead, one of these generated flowers having petals with pale margins. Possible reasons why this construct generated such margins are examined and discussed.

Results

Five genes affecting anthocyanin color

There is a large body of research on the genes affecting anthocyanin color in flowers, including antisense *CHS* and antisense *DFR*. Fig. 1A shows photographs of petunia and morning glory
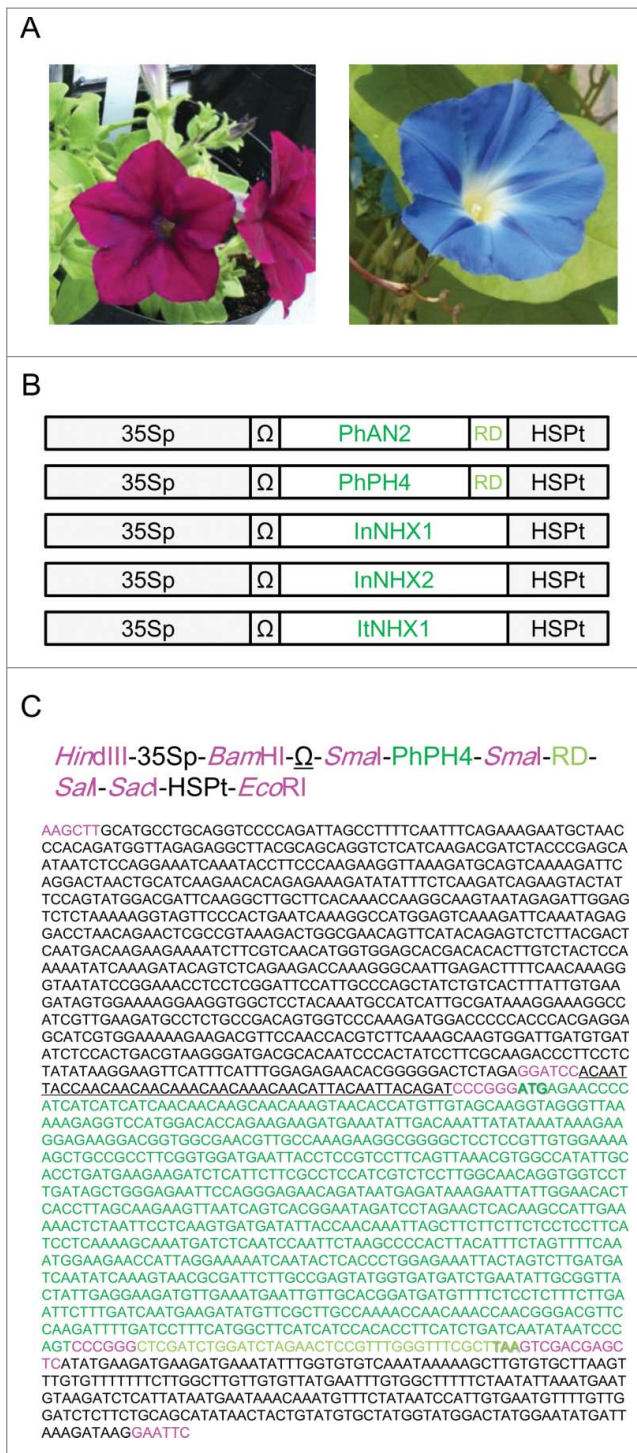
Figure 1. Genetic constructs for transformation. (A) Petunia (left) and morning glory (right). (B) Illustration of the constructs, not to scale. 35Sp, *Cauliflower mosaic virus* 35 S RNA promoter; Ω, translation enhancer sequence from *Tobacco mosaic virus*; RD, repression domain (SRDX sequence); HSPt, HSP terminator. (C) Precise sequence information for a representative construct (*PhPH4-RD*). Sequence profiles are written, up to DNA sequence. The same colors and underlining represent the same parts of the construct. All restriction sites are colored magenta.

PhAN2 and *PhPH4* are among these genes and both are MYB (Myeloblastosis) family transcription factors. Vacuolar pH is elevated by any dysfunction in these genes. In morning glory, a more direct method for increasing vacuolar pH (to make anthocyanins bluish) is by expression of Na^+/H^+ antiporters. A high vacuolar pH is responsible for the blue color of European morning glory,[14] and a Na^+/H^+ antiporter gene *InNHX1* has been identified from a mutant of blue Japanese morning glory, being responsible for vacuolar alkalisation.[15] Japanese morning glory also has a homolog *InNHX2*, whereas European morning glory has a counterpart *ItNHX1*.[16,17] Expression of these *NHX* genes in petals is expected to increase vacuolar pH by pumping H^+ ions out of the vacuole. Therefore, we prepared 5 constructs in torenia to express 3 *NHX* genes and chimeric repressors of *PhAN2* and *PhPH4* under the control of *Cauliflower mosaic virus* 35 S RNA promoter (Fig. 1B, C). Repression domains were attached to the 3′ termini of cloned *PhAN2* and *PhPH4* sequences to generate chimeric repressors.
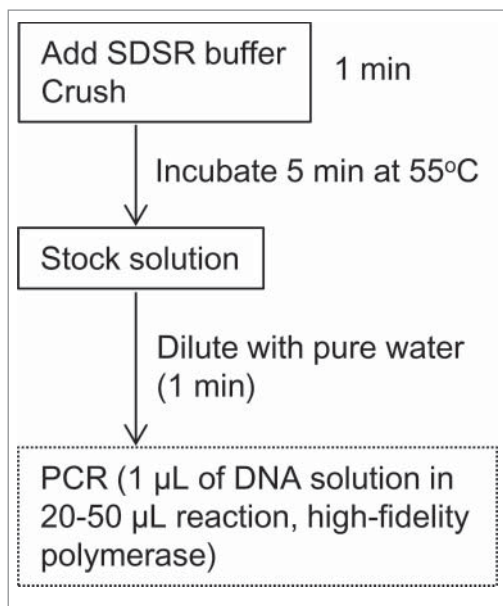
Development of a high-throughput method for DNA extraction

During the transformation processes, we developed a high-throughput method for DNA extraction from torenia. In contrast to the more popular model plant, Arabidopsis,[18,19] torenia research has previously lacked such a high-throughput method. With this newly developed method, T-DNA insertion and the construct in T-DNA can be determined by PCR immediately. Fig. 2 illustrates the procedure for DNA extraction. Leaf discs (typically 5 × 5 mm) are crushed in 200 μL of extraction buffer (we call this 'SDSR buffer': Tris-HCl pH 7.5 (200 mM), EDTA (25 mM), NaCl (250 mM), SDS (sodium dodecyl sulfate, 0.5%), RNase A (5 μg mL^{-1})) in a 1.5-mL plastic tube with a plastic rod, then heated to 55°C for 5 min. This stock solution can be preserved at −20°C. Stock solutions are diluted 10 times with sterilised, purified water. A volume of 1 μL of the diluted solution is used for one PCR reaction (20–50 μL). PCR reactions were successful with a high-fidelity polymerase (KOD Plus Neo, Toyobo, Osaka, Japan) but were not successful with a standard polymerase (Ex-Taq HS, Takara, Kyoto, Japan). Leaf discs taken from potted plants were also found to be better than those from sterilized plant culture when around 3-kb fragments of T-DNA sequences were amplified. Both types of leaf disc served well when fragments of around 500-bp were amplified.

PCR analysis was performed to determine T-DNA insertion in isolated lines. Four primers were combined to amplify 2 target sequences at a time. A 336-bp fragment of *Hygromycin B phosphotransferase* (*HPT*) gene was amplified to detect T-DNA inserts. A 573-bp fragment of endogenous *DFR* gene promoter sequence was amplified as an indicator of the success of the PCR reaction.

Generation of transgenic torenia lines

Five vectors were transformed as a bulk (mixture), into the torenia cultivar 'Crown Violet'. Mixtures of equal concentrations of plasmids were transformed into agrobacterium. After growing transformed colonies of agrobacterium, colonies were combined in sterile water and inoculated onto leaf discs of torenia.

flowers. *PhAN1*, *PhAN2* and *PhAN11* are genes controlling both pigmentation and vacuolar pH in petunia petals.[8,9,10] Petunia also has a series of *PhPH* genes regulating vacuolar pH in the petals.[11,12,13] Because anthocyanins are accumulated in the vacuole and their colors change with pH (reddish at lower pH and bluish at higher pH), the *PhPH* genes also regulate petal color.

Figure 2. High-throughput DNA extraction from torenia. Illustration of the extraction procedure.

After inoculation on co-existence media and incubations on selection/regeneration media, transgenic shoots were grown in plastic containers and later transferred to pots. Flowers where observed after plants had set at least 5 flowers. We observed 16 independent transgenic lines (Fig. 3A). Contrary to expectation, none of the lines set blue flowers. Flower colors were quite similar to those of non-transgenic (NT) plants. Our constructs do not seem to be able to generate blue flowers. In Fig. 3A, 4 lines (marked with red frames) shared the same distinct phenotypic change. This new phenotype is more clearly shown in Fig. 3B. The petals of these lines have narrow, pale margins. Genomic PCR analyses revealed T-DNA insertion of PhPH4 repressor (PhPH4-RD) in all 4 of these lines. We named these lines PhPH4-RD-1, PhPH4-RD-2, PhPH4-RD-3 and PhPH4-RD-4. A new composition of agar was developed, modified for slicing torenia petals. Using this, cross-sections of the ventral limbs were prepared from NT and PhPH4-RD-2, and were observed under a light microscope (Fig. 3C). Anthocyanin accumulation was observed in both adaxial and abaxial epidermal cells in NT. The adaxial epidermal cells (upside in Fig. 3C) were found to accumulate pigment more densely than the abaxial epidermal cells. All epidermal cells of the NT limbs accumulated anthocyanins, including those along the very edge. In contrast, the epidermal cells along the edge of the PhPH4-RD-2 limbs lacked anthocyanins both in the adaxial and the abaxial surfaces. Fig. 3D shows whole plants of NT and PhPH4-RD-4. The chimeric repressor of PhPH4 does not have any further distinguishable effects on plant structure or appearance.

Expression analysis of genes involved in anthocyanin synthesis

How does the PhPH4 repressor function in torenia? The amino acid sequence of PhPH4 possesses R2R3-type MYB domain,[12] harbouring homology to the MYB transcription factors regulating anthocyanin accumulations such as AtPAP1 in Arabidopsis and TfMYB1 in torenia.[20] Fig. 4 shows the amino acid alignment in these R2R3-type MYB proteins.

AtPAP1 and TfMYB1 positively regulate the expression of genes for the anthocyanin synthesis pathway which could be considered in 3 parts (Fig. 5A): The first step transforms coumaroyl-CoA and malonyl-CoA into dihydrokaempferol with Chalcone synthase (CHS), Chalcone isomerase (CHI) and Flavanone 3-hydroxylase (F3H). In the second step, dihydrokaempferol is transformed into other dihydroflavonols, dihydroquercetin and dihydromyricetin, with Flavonoid 3′-hydroxylase (F3′H) and Flavonoid 3′5′-hydroxylase (F3′5′H). Dihydroflavonols are then catalyzed into anthocyanins by Dihydroflavonol 4-reductase (DFR) and Anthocyanin synthase (ANS).[21] These anthocyanins then undergo further modification with enzymes such as UDP glucose flavonoid 3-O-glucosyltransferase (3GT). Torenia genes corresponding to the genes involved in anthocyanin synthesis above have been cloned and are the subject of an earlier report.[22]

Expressions of genes involved in anthocyanin synthesis were compared between NT and PhPH4-RD lines by semi-quantitative RT-PCR analysis (Fig. 5B). Expression levels of PhPH4-RD transgene were similar between all 4 PhPH4-RD lines in the whole petals, consistent with similar phenotypes of these lines. TfCHS expression seems to be up-regulated in all PhPH4-RD lines compared with NT in whole petals. Some other genes are also up-/down-regulated in some specific lines but, in the whole petals, no uniform up-/down-regulation of genes in PhPH4-RD lines was observed except for TfCHS. Clearer differences in gene expression were observed when flower tubes, limbs and petal margins were compared. Expression of PhPH4-RD was stronger in limbs than in tubes, and it was elevated even in the margin in the PhPH4-RD line. TfCHS expression in the tube was higher in the PhPH4-RD line than in NT, whereas TfCHS expression was much lower in the limb and margin both in the NT and PhPH4-RD lines. The expression of no other genes was significantly different in the tube between the NT and PhPH4-RD lines. Interestingly, genes in the 'second step' and 'third step' of anthocyanin synthesis were more highly expressed in the petal margins than in the whole limbs of NT flowers. The third-step genes maintained high levels of expression, similar to NT, in the petal margins in the PhPH4-RD line, whereas the expressions of the second-step genes were downregulated in PhPH4-RD line compared with NT in the margins. In addition to expression of the second-step genes expression of TfF3H, one of the first-step genes, was also down-regulated in PhPH4-RD in the limb and petal margins.

Discussion

### Significance of vacuolar pH modification for petal color modification

The original goal of this report was to generate blue flowers in torenia with a simple genetic modification. Many floricultural species accumulate red, magenta, purple or, less frequently, violet anthocyanin in the vacuoles of the petal cells. Species accumulating completely blue anthocyanins are quite rare (e.g., morning glory, hydrangea, delphinium, blue poppy). Considering the importance of color variation for sales of ornamental flowers, the
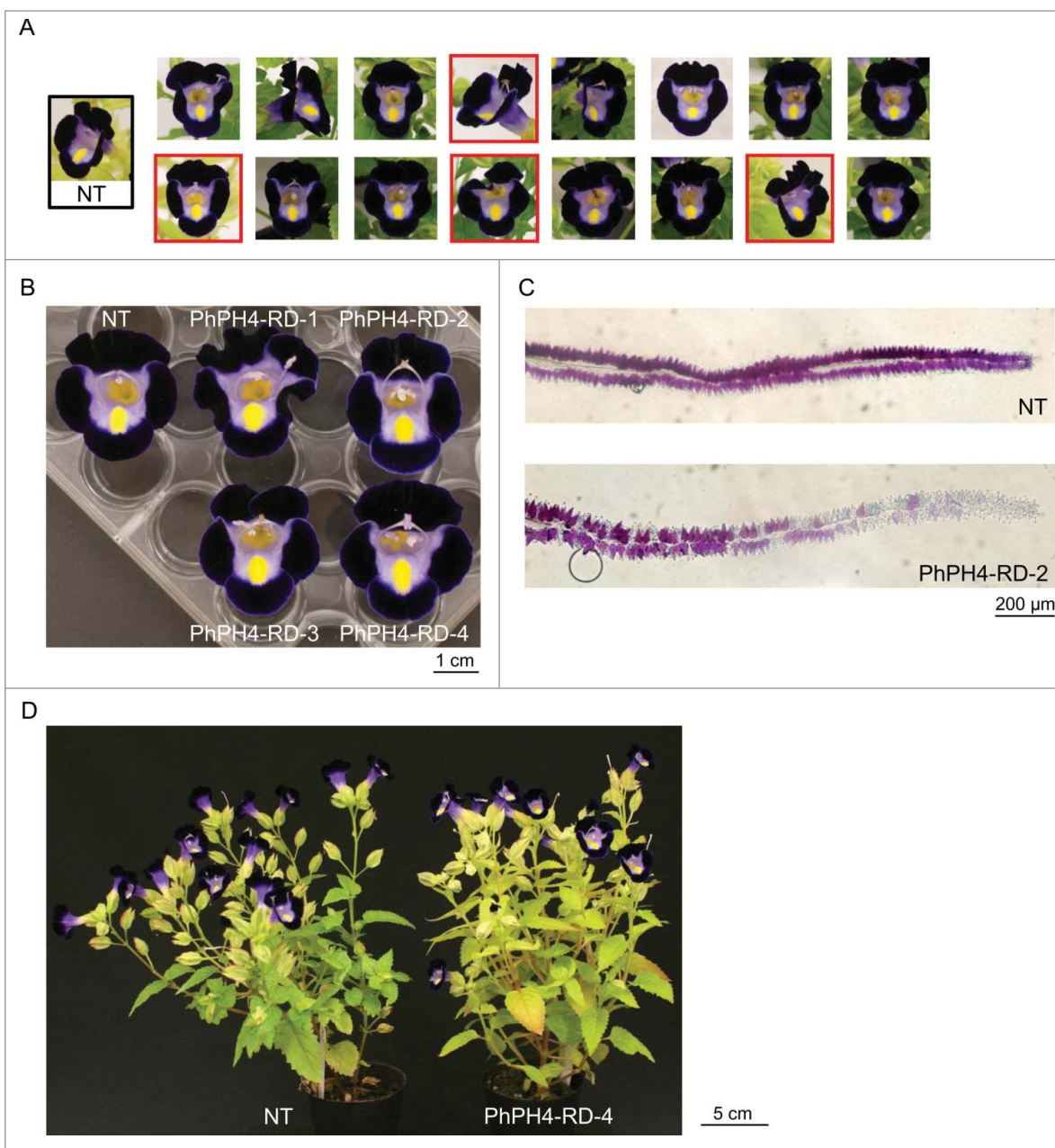
Figure 3. Phenotypes of transgenic lines. (A) Flowers of independent transformants. NT, non-transgenic line. Lines having pale margins are framed with red squares. (B) PhPH4-RD flowers photographed on a black background. (C) Cross-section of NT and PhPH4-RD-2 limbs. The adaxial petal surfaces are orientated upwards. (D) Whole plants of NT and PhPH4-RD-4.
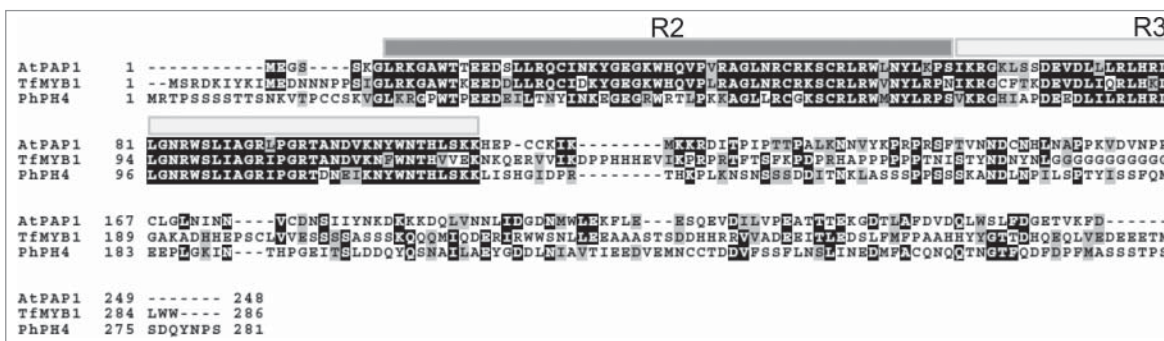
Figure 4. Sequences of R2R3 MYB transcription factors. R2 and R3 domains[26] are indicated on the amino acid sequences with gray bars.
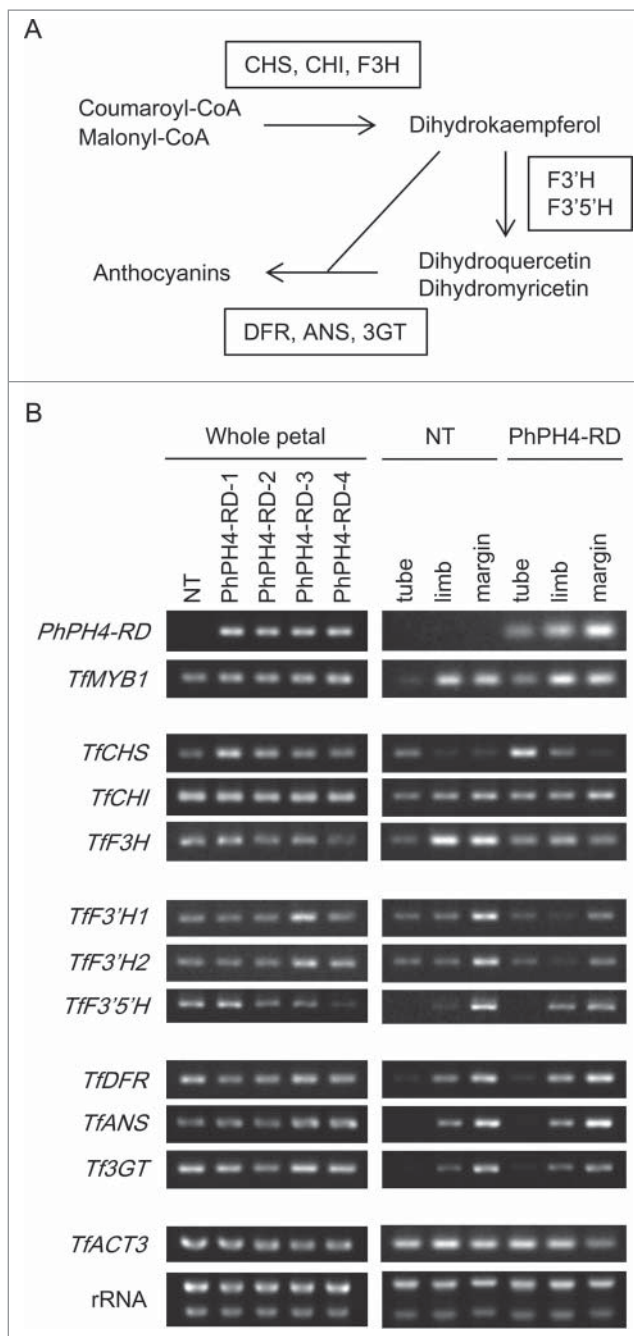
Figure 5. Expression analysis of genes involved in anthocyanin synthesis. (A) Simplified illustration of the pathway of anthocyanin synthesis. (B) Semi-quantitative RT-PCR analysis. RNA samples were examined from whole petals (left) and from parts of petals (tube, limb, petal margin: right). rRNA, rRNA bands observed after electrophoresis of extracted RNA.

generation of truly blue flowers is an important goal for many flower breeders. Modifications of anthocyanin structure by genetic transformation have been successful in generating bluer flowers from reddish ones. However, these were obtained at massive experimental effort and cost. Multiple genes had to be introduced by transformation in some cases. In addition, many genes and promoters must usually be tested to find a proper construct which successfully catalyzes anthocyanin modification.[23,24] Even after all this effort, anthocyanin color is never truly blue, but is more usually purple or violet.

Simpler strategies for modifying anthocyanin color have already been presented in an earlier study, as mentioned in the introduction. Aida et al.[1,2] introduced antisense sequences of *CHS* or *DFR* in torenia. As well as reducing the density of pigmentation, antisense *CHS* changed the color (hue) of torenia from purple to reddish purple, whereas antisense *DFR* changed the color from purple to violet. These color changes were caused by an altered balance of the concentrations of anthocyanins and flavones (co-pigments). Anthocyanin can be made bluer in this simple manner, but this strategy is not universally effective in other floricultural species. Also, the colors of the transgenic flowers are not really blue.

Some cultivars of morning glory set completely blue flowers (Fig. 1A) and the *InNHX1* gene is necessary for this color. With a mutation in this gene, flower color of blue morning glory can change to purple.[15] The *InNHX1* gene is believed to transport H$^+$ ions from the vacuolar lumen to the cytosol, and this increases vacuolar pH during flower opening. The anthocyanins of purple flowers of other species also undergo the same bluish color shift under neutral and weakly-alkaline pHs. A truly blue pigment can be generated by crushing purple torenia petals and adding the resulting paste to water. This indicates that the pigments in purple torenia flowers can be modified to blue, merely by raising the pH to about 7. In other words, anthocyanin color is a pH indicator. Unfortunately, flower color change was not observed in this study, suggesting that the 5 constructs did not raise vacuolar pH significantly. The pH values of petal exudates from the 3 transgenic lines examined (2 of them possessing the *PhPH4-RD* construct and the other the *InNHX2* construct) were substantially the same as in NT (i.e. pH 5.0 to 5.4). Further effort is required to render these genes functional in torenia petals.

Genetic factors inducing margined flowers

To our knowledge there has been only one report on a genetic factor inducing margined flowers. Morita et al.[25] examined a margined (picotee) flower of petunia. siRNA was generated from a tandem repeat of 2 *CHS* genes in this flower. For reasons unknown, gene silencing seems to occur due to siRNA accumulation only in the margins of petals. Although the *PhPH4-RD* lines generated in this study also induced color change in the petal margins, they modified gene expression by a chimeric repressor (artificial transcription repressor), not by gene silencing. The target genes (downregulated genes) of the *PhPH4* repressor were 3 hydroxylases, *F3H, F3'H* and *F3'5'H*, and not *CHS*. Our study thus revealed a new type of genetic factor inducing margined flowers. This is also the first report of a transgenic modification being successful in inducing margined flowers.

PhPH4 is an R2R3-type MYB transcription factor. R2R3 is a major family of MYB in plants. The number of MYB genes in the torenia genome is not clear at this stage, but there are certainly more than 100 MYB genes in the Arabidopsis genome.[26] The original function of *PhPH4* in petunia is vacuolar pH modification, whereas *PhPH4-RD* down-regulates anthocyanin

synthesis in torenia, and does not seem to affect the pH of the whole petal. This result is consistent with its homology to *AtPAP1* and *TfMYB1*, which regulate anthocyanin accumulation in Arabidopsis and torenia.[27,20] The *AtPAP1* repressor also cancels sucrose-responsive accumulation of anthocyanins in Arabidopsis.[4] The reasons for the differential functions of PhPH4 in petunia and torenia are not clear. For example, the torenia genome may lack some unknown endogenous target gene for PhPH4, involved in regulation of vacuolar pH. Also, PhPH4 may partially regulate other genes involved in anthocyanin synthesis in place of the actual target genes. Regulation of the expression of genes involved in anthocyanin accumulation is not restricted to R2R3-type of MYB. The chimeric repressor of *AtRL2*, a single-type MYB gene from Arabidopsis, seems to strongly down-regulate anthocyanin accumulation in torenia petals in an earlier report.[7] A contrastingly restricted activity of *PhPH4-RD* in downregulation of genes involved in anthocyanin synthesis, could lead to margined flowers.

If the function of *PhPH4-RD* is restricted, it is not yet clear why anthocyanin synthesis is stopped only in the marginal cells. Because *PhPH4-RD* expression was stronger in the petal margins, than in the tubes and whole limbs, a possible explanation may be that *PhPH4-RD* inhibits the transcription of target genes only when it is strongly expressed - that is, only in the marginal cells. Another plausible idea is that the factor leading to the induction of pale margins in the PhPH4-RD lines has to do with the nature of the torenia flower. Petal margin cells seem to be more 'susceptible' to genetic change (and so tend to lose pigmentation) than the more central cells. Pale margins appear in transgenic torenia flowers harbouring T-DNA insertions of the chimeric repressors of *AtAGL6* and *AtGNL* genes,

together with other phenotypic alterations.[7] Although transgene expressions and reproducibility of the phenotype were not examined, these chimeric repressors could be said to induce pale margins. Margined flowers also appear in the bulk of ion-beam induced torenia mutants.[3] These observations would suggest that marginal cells of torenia petals have a more fragile anthocyanin synthesis machinery and so more easily lose their ability to synthesize anthocyanin after genetic perturbation – the central region of the limb being not so susceptible to genetic perturbation.

Materials and methods

Plant materials

Petunia (*Petunia* x *hybrida*, an unnamed cultivar setting red-purple flowers, Fig. 1A) and morning glory (*Ipomoea tricolor* Cav. 'Heavenly Blue', Fig. 1A) were used for cDNA isolation. A sterilised culture of the torenia cultivar Crown Violet was incubated in transparent plastic containers ($7 \times 7 \times 10$ cm) at $25°C$ under fluorescent light (PPFD $= 150$ μmol m^{-2} s^{-1}). Media contained half-strength Murashige-Skoog salt and vitamin, 3% sucrose, 50 mg L^{-1} shiomarin and was solidified with 0.2% gellam gum. After incubation for at least 2 months, plants were transferred to soil, in pots, in a naturally lit greenhouse.

Transformation

The transformation procedure followed a previous report.[28] Briefly, leaf discs prepared from the sterilised culture were

Table 1. Primers for genomic PCR, cloning and sub-cloning.

Primer name*	Sequence	Target gene
[Genomic PCR]		
HPT-F1	5′-ATTCCGGAAGTGCTTGACAT-3′	*HPT* (T-DNA)
HPT-R3	5′-CAGTCCTCGGAAAGCATCAG-3′	
PDFR-F1	5′-TGGCGTTCCTTAAACAGCTT-3′	*TfDFR promoter*
PDFR-R2	5′-TGAACACCGTTGCTTTTTGA-3′	
35S-F1	5′-CGCACAATCCCACTATCCTT-3′	*35 S promoter*
PhAN2-R1	5′-GGTCCATGCACCTTTCCTTA-3′	*PhAN2*
PhPH4-R1	5′-TGATGATGATGGGGTTCTCA-3′	*PhPH4*
InItNHX1-R1	5′-CCGAATTTTGGAGCAAAGAA-3′	*InNHX1, ItNHX1*
InNHX2-R1	5′-AGTGTCCCAACGTCAACTCC-3′	*InNHX2*
[Cloning]		
AN2-UP-F3	5′-CTTTCTTTGTCCTTTAGTTGTCAGTTGCAGTGAG-3′	*PhAN2*
AN2-UP-R1	5′-AATTTCAAATGTCCAACGATTTCAACTGTAGTGTTT-3′	
PH4-UP-F1	5′-CTCCCCTCCCAAATTTAGTACTATGACTTTATC-3′	*PhPH4*
PH4-UP-R3	5′-AACCCTCACATACATATATATATAGCTACAACATCA-3′	
InNHX1-UP-F1	5′-CCCCACATCTCACCTTTCAAGTGATTTGTATGTTTT-3′	*ItNHX1*
InNHX1-UP-R1	5′-TGAGAAGACTTTCACCCAGCCCATTTCATAAGAG-3′	
[Subcloning]		
AN2-F1-SmaI	5′-AATAATCCCGGGATGAGTACTTCTAATGCATCAACATCAGGA-3′	*PhAN2*
AN2-R1-SmaI	5′-AATAATCCCGGGACTAACTAAATCCCATATGTCATCAATATCAACTG-3′	
PhPH4-F1-SmaI	5′-AATAATCCCGGGATGAGAACCCCATCATCATCATCAACAA-3′	*PhPH4*
PhPH4-R1-SmaI	5′-AATAATCCCGGGACTGGGATTATATTGATCAGATGAAGGTGTGGA-3′	
InNHX1-F1-SmaI	5′-AATAATCCCGGGATGGCGTTCGGGTTGTCTTCTT-3′	*InNHX1*
InNHX1-R1-SalI	5′-AATAATGTCGACTCATCTAGGGCTCTGCTCAACTGG-3′	
InNHX2-F1-DraI	5′-AATAATTTTAAAATGGGAGTTGACGTTGGGACAC-3′	*InNHX2*
InNHX2-R1-SalI	5′-AATAATGTCGACTCACTGCCATTGTGGTTCATTCTG-3′	
ItNHX1-F1-SmaI	5′-AATAATCCCGGGATGGCGTTCGGATTGTCTTCTTT-3′	*ItNHX1*
ItNHX1-R1-SalI	5′-AATAATGTCGACTCATCTAGGGCTCTGCTCAGCTG-3′	
HSPt-F-SacI	5′-AAAGAGCTCATATGAAGATGAAGATGAAA-3′	*HSP terminator*
HSPt-R-EcoRI	5′-AAAGAATTCCTTATCTTTAATCATATTCC-3′	

*'F' represents forward primer and 'R' represents reverse primer.

Table 2. Primers for semi-quantitative RT-PCR.

Primer name[*]	Sequence	Target gene	PCR Cycles
PhPH4-RD-F2	5′-TTGCCGAGTATGGTGATGAT-3′	PhPH4-RD	30
PhPH4-RD-R3	5′-CGAAACCCAAACGGAGTTCT-3′		
TfMYB1-F1	5′-ATCGGTGCAGAAAGAGTTGC-3′	TfMYB1	35
TfMYB1-R2	5′-CCACCACATGAGTGTTCCAA-3′		
TfANS-F	5′-CGCCCTCACGTTCATCCTCCAC-3′	TfANS	30
TfANS-R	5′-CTCCACCCATCACTCAACACTC-3′		
TfCHI-F	5′-TTCCTCGAAGTGTTCAAGAACG-3′	TfCHI	30
TfCHI-R	5′-TACAAATCCACGATCATTGACC-3′		
TfCHS-F1	5′-CTGAGCGAGTACGGGAACAT-3′	TfCHS1, TfCHS2	30
TfCHS-R3	5′-CACACACACACACACCACAAA-3′		
TfDFR-F1	5′-TGATGGGAATGGGATTTACC-3′	TfDFR	35
TfDFR-R1	5′-CACGACATTTCACCATTTTCC-3′		
TfF3H-F3	5′-AGGCCTGTCTGAACATGGAC-3′	TfF3H	30
TfF3H-R4	5′-GGGTACACGATAGCCTCTGG-3′		
TfF3′H1-F	5′-GCGGGTCGTAGGATCTGCGCC-3′	TfF3′H1	30
TfF3′H-R	5′-CAGCAGTACAAACTAGATTTATC-3′		
TfF3′H2-F	5′-GTCAAGGGGAACGATTTCGAAG-3′	TfF3′H2	30
TfF3′H-R	5′-CAGCAGTACAAACTAGATTTATC-3′		
TfF3′5′H-F	5′-GAGAATGTTGGAGCAGCATGCG-3′	TfF3′5′H	30
TfF3′5′H-R	5′-GAGATAATTTATTTGATAGATTGG-3′		
Tf3GT-F	5′-TCGAATGGTGCAAGATGTTTGG-3′	Tf3GT	35
Tf3GT-R	5′-GAAGACGTAGACATTTGACTCG-3′		
TfACT3-F	5′-AAATACAGTGTTTGGATCGGAGGTTC-3′	TfACT3	30
TfACT3-R	5′-GAATAGCACACAGAGAATAGCAAACC-3′		

[*]'F' represents forward primer and 'R' represents reverse primer.

inoculated with agrobacterium, incubated in the dark on co-existence media for one week, and transferred to selection/regeneration media containing 150 mg L^{-1} hygromycin and 300 mg L^{-1} carbenicillin (in this study, the hygromycin concentration was higher than that usually used for torenia viz. 20 mg L^{-1}).

cDNA isolation

RNA was extracted with the RNeasy Plant Mini-Kit (Qiagen K. K., Tokyo, Japan), from the petals of petunia and morning glory. After reverse transcription with oligo-(dT)$_{20}$ primer using ReverTra Ace α (Toyobo, Osaka, Japan), partial cDNA sequences including whole coding sequences of *PhAN2* (petunia), *PhPH4* (petunia) and *ItNHX1* (morning glory) were amplified by PCR with KOD Plus Neo (Toyobo, Osaka, Japan). Amplified DNA sequences were TA cloned and sequenced. The *InNHX1* and *InNHX2* clones (derived from Japanese morning glory, *Ipomoea nil*) were provided by the National Institute for Basic Biology. Primer sequences are listed in Table 1. Compared with the original database sequences (AF146702.1, AY973324.1, AB054979.1), the cloned *PhAN2* sequence had 8 nucleotide substitutions within the coding sequence, leading to 8 amino acid substitutions in the encoded protein. Similarly, *PhPH4* had 3 nucleotide substitutions within the coding sequences, and *ItNHX1* had 11, but these mutations did not result in amino acid substitutions.

Construction of plasmids

For construction of the *PhAN2-RD* and *PhPH4-RD* vectors, coding sequences excluding stop codons were amplified from plasmids with restriction sites (*Sma*I sites) attached on both DNA ends by primers. Amplified fragments were TA cloned

and sequenced. A new vector containing the terminator sequence of a heat shock protein (HSP) gene was prepared as follows. A DNA fragment containing the HSP terminator (HSPt)[29] was amplified by PCR, with *Sac*I and *Eco*RI sites attached to either end of the DNA. The amplified HSPt fragment was digested with *Sac*I and *Eco*RI. The *Sac*I-*Eco*RI portion of the p35SSRDXG vector[30] was replaced with this fragment, to produce a pG35S-SRDX-HSPt vector. After digestion with *Sma*I, the coding sequences were introduced into pG35S-SRDX-HSPt. SRDX, a typical repression domain encoding 12 amino acid residues,[4] was attached to the 3′ end of the coding sequences in the frame of these constructs. To construct the 3 *NHX* vectors, coding sequences including stop codons, were amplified with restriction sites (*Sma*I (or *Dra*I for *InNHX2*) and *Sal*I sites) attached by primers, TA cloned, digested with *Sma*I (or *Dra*I) and *Sal*I, and then introduced into pG35S-SRDX-HSPt. These five vectors were LR cloned into a binary vector pBCKH.[31] Binary vectors were transformed into agrobacterium strain EHA105 by electroporation. The primer sequences used for sub-cloning are listed in Table 1.

Genomic PCR

Crude DNA was extracted with 'SDSR buffer' as described in the text. Constructs were separately amplified with a forward primer binding to the 35 S promoter sequence and specific primers binding to each coding sequence. The *InNHX1* and *ItNHX1* constructs were simultaneously amplified with the same primer. PCR conditions were the same both for the detection of genomic DNA and for the detection of the constructs: PCR was performed with KOD Plus Neo in 3-step cycles. The annealing temperature was 55°C, extension was for 1 min and the number of thermal cycles was 40. The other conditions followed the manufacturer's instructions.

The primer sequences used for genomic PCR are listed in Table 1.

Cross-section of petals

The ventral flower limbs (limb, flat parts on flower tubes; ventral, the lower part) were excised. A newly-developed 'petal slicing agar' (28.4 g trehalose, 110 mg $CaCl_2$-$2H_2O$, 3 g agar and 300 μL Tween-20 per 150 mL of water) was melted in a microwave oven and cooled to approx. 60°C. After pouring the melted agar into small plastic dishes, the excised ventral limbs were embedded. The agar blocks were later sectioned (55 μm) parallel to the petal's longitudinal axes with an electric microslicer (DTK-1000, Dosaka EM Co., Ltd., Kyoto, Japan). Slices were observed with a light microscope (Provis AX70, Olympus, Tokyo, Japan).

Protein alignment

Amino acid sequences were aligned with ClustalW[32] and shades supplemented with BoxShade.

Gene accessions

PhAN2 (AB982128), *PhPH4* (AB982129), *InNHX1* (AB033989.1) , *InNHX2* (AB194065.1), *ItNHX1* (AB982130), *TfMYB1* (AB719455.1), *AtRL2* (NM_127736). The sequence of the *TfDFR* promoter will be reported elsewhere (Sasaki K *et al.*). Accessions of genes involved in anthocyanin synthesis and *TfACT3* have been described previously.[22]

Gene expression analysis

RNA was extracted from whole petals using the RNeasy Plant Mini-Kit. Flower tubes, limbs and margins of limbs (approx. 1 mm of margin) were also gathered and their RNA extracted. The width of the margin samples (1 mm) was the least that could be prepared manually from the fragile petals. The 'PhPH4-RD' sample (Fig. 5B, right) derives from the PhPH4-RD-2 line for tube and limb, and this derives from a mixture of PhPH4-RD lines for margin. RNA samples were reverse-transcribed with ReverTra Ace α by oligo-$(dT)_{20}$ primer. The resulting cDNA samples were diluted to 10% with water and used for PCR amplification. Volumes of 1 μL of the cDNA samples were used in total volumes of 20 μL for the PCR reactions with Ex-Taq HS (Takara, Kyoto, Japan). Primer sequences and PCR cycles in semi-quantitative RT-PCR analysis are presented in Table 2. The RT-PCR primers for *TfANS, TfCHI, TfF3′H1, TfF3′H2, TfF3′5′H, Tf3GT* and *TfACT3* are from Sasaki *et al.*[22] The other RT-PCR primers were designed in this study. *TfCHS1* and *TfCHS2* (cloned in Sasaki et al.[22]) shared nearly the same nucleotide sequences and seemed to be alleles. New primers were designed to amplify *TfCHS1* and *TfCHS2* simultaneously (this gene is here referred to as *TfCHS*).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Author contributions

Experiments were designed by IK and KS, and carried out by IK. The pG35S-SRDX-HSPt vector was constructed by KS. The manuscript was written by IK and checked by KS.

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