A Torenia Mutant Bearing Shrunken Reddish-purple Flower and its Potential for Breeding

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An ¹-torenia mutant, designated as “reddish purple shrunken” (RPS) because of its shrunken reddish-purple petals, was isolated from the S¹ progeny of a “flecked” mutant containing an active DNA transposon Ttf¹. Cyanidin-type anthocyanins—i.e., peonidin 3,5-diglucoside and cyanidin 3,5-diglucoside—were detected in petals of the RPS mutant, whereas delphinidin-type anthocyanins—i.e., malvidin 3,5-diglucoside, petunidin 3,5-diglucoside, and delphinidin 3,5-diglucoside—were the major anthocyanins in normal-type petals. Petal shrinkage could be attributed to this change in anthocyanin composition. A frameshift mutation in the first exon of flavonoid 3',5'-hydroxylase (TfF3'5'HΔC) inhibited biosynthesis of delphinidin-type anthocyanins in RPS. The “white” (W) mutant bore pure white petals in response to Ttf¹ insertion in the first exon of the flavanone 3-hydroxylase gene (TfF3H'Ttf¹). Some F² plants derived from the cross between RPS and W bore reddish-purple petals without any shrinkage; these were designated as “reddish purple” (RP). This RP genotype was revealed as a heterozygote of TfF3H²⁺ and TfF3H'Ttf¹ in the background of the TfF3'5'HΔC homozygote. A gene dosage effect of TfF3H reduced anthocyanin biosynthesis in RP compared with RPS, whereas this decrease caused no reduction in visual color density. Therefore, a partial decrease in anthocyanin biosynthesis may be beneficial if petal shrinkage prevents using a new-color mutant for breeding.

Key Words: anthocyanin, flower color, peonidin, petal disorder, transposon.

Introduction

Torenia (Torenia fournieri Lind. ex Fourn.) is a summer bedding plant with excellent growth characteristics. It is a compact plant with high tolerance to intense heat and humidity in the summer and is adaptable to varying light conditions from shade to full sunshine (Aida et al., 2000; Okazawa and Nishijima, 2017). Variations in the flower shape of torenia cultivars are relatively poor; only cultivars with small single flowers are known. However, variations in flower color and pattern are relatively rich. In addition to the typical violet wild-type flower, white, pink, dark-red, and pale-yellow cultivars are also common. Lateral and ventral petals are densely colored in wild-type torenia. In some cultivars, all—or only lateral—petals of flowers are densely colored. At least a part of all pattern variation is regulated by the genes responsible for dorsal–ventral asymmetry in flowers (Kondo et al., 2020; Niki et al., 2016; Su et al., 2017).

Torenia flower color is mainly expressed by anthocyanin, with exception of the yellow nectar guide present in the ventral petal (Aida et al., 2000). The increased number of hydroxyl groups in the B-ring of anthocyanin molecules renders a more blueish color, although substituting a hydroxyl group with a methoxy group turns this color slightly more reddish (Ozeki et al., 2011; Tanaka et al., 2008). Dihydrokaempferol—a precursor of anthocyanin—has a 4'-hydroxyl group in its B-ring that is metabolized into pelargonidin. Flavonoid 3'-hydroxylase (F3'3'H) and flavonoid 3',5'-hydroxylase (F3'5'H) catalyze 3'- and both 3'- and 5'-hydroxylation of dihydrokaempferol, respectively. This catalytic behavior results in the formation of dihydroquercetin and dihydromyricetin, which are then further
metabolized into cyanidin and delphinidin, respectively. Pelargonidin-type anthocyanins express an orangish-red color, cyanidin-type anthocyanins express a reddish-purple color, and delphinidin-type anthocyanins express a violet color. The expressed color varies depending on a variety of factors including co-pigmentation, metal ion complex formation, and vacuole pH. Meanwhile, the expression of the genes encoding anthocyanin biosynthesis enzymes is regulated by a complex of R2R3-MYB, bHLH, and WD40 transcription factors (Grotewold, 2006).

The “common violet” strain of torenia is almost identical to its wild-type ancestor in bearing violet flowers. The anthocyanins present are the delphinidin-type—namely, malvidin 3,5-diglucoside (Mv3,5dG), petunidin 3,5-diglucoside (Pt3,5dG), and delphinidin 3,5-diglucoside (Dp3,5dG) (Nishijima et al., 2013), with slight amounts of cyanidin-type anthocyanins present, i.e., peonidin 3,5-diglucoside (Pn3,5dG) and cyanidin 3,5-diglucoside (Cy3,5dG). Insertion of a retrotransposon in the 5'-upstream region of the flavanone 3-hydroxylase gene (TfF3'H) causes a pure white flower to be expressed in a commercial cultivar ‘Crown White’ (Nishihara et al., 2014). Mutations of TfF3'H and TfF3'5'H are also detected in ‘Crown White’; however, the contribution of such mutations to flower color is still unknown (Nishihara et al., 2014).

Another mutant variation referred to as “flecked” was isolated with an active DNA transposon Tpn1 (Nishijima et al., 2013). The presence of an active transposon greatly contributes to the expansion of ornamental variations in floricultural plants. For example, a mutable strain “flecked” of Japanese morning glory (Ipomoea nil), which contains the active DNA transposon Tpn1, appeared earlier than 1750 in the Edo-era, and many mutations in flower shape and color appeared in the late Edo-era (1806–1860) (Imagaki et al., 1994; Kawasaki and Nitasaka, 2004; Nitasaka, 2007). Transposons belonging to the Tpn1 family are major mutagens of Japanese morning glory, and most of these mutations are thought to be induced by insertions of transposons or footprints caused by transposon excisions (Iida et al., 2004; Morita and Hoshino, 2018).

Active transposons also contributed to the understanding of molecular mechanisms affecting flower color and pattern formation (Goodrich et al., 1992; Imagaki et al., 1994; Luo et al., 1991; Martin et al., 1985; Morita et al., 2006). In torenia, selfed progenies of “flecked” generated various new mutants including a “petaloid” bearing a double flower (Nishijima et al., 2016) and “begonia” bearing an apparent di-symmetric flower (Niki et al., 2016). Therefore, it is deduced that the “flecked” mutant causes conspicuous mutagenesis as shown in Japanese morning glory.

In the current study, a mutation specifically exhibiting reddish-purple and shrunken petals was isolated from the selfed progeny of the “flecked” mutant. Interestingly, crossing this mutant with another mutant bearing white petals resulted in progenies without any petal shrinkage, while also retaining the reddish-purple color in petals. The molecular and genetic mechanisms underlying this mutation phenomenon in torenia were investigated and are discussed in terms of its horticultural significance.

Materials and Methods

Plant materials and genetic analysis

Torenia plants were grown as described previously (Nishijima et al., 2013). Plants with the “flecked” mutation were self-pollinated by hand to obtain S1 plants. Eighteen S1 plants per parent plant were grown to screen new mutations. These new mutants were then self-pollinated for several generations to examine inheritance, purity, and stability of the mutations. Unstable mutants were crossed with normal-type (NT) plants, and stable progenies without an activation factor of non-autonomous Tfn1 were then obtained. The mode of inheritance of a particular mutation was examined by using reciprocal crosses with NT plants.

Analysis of petal structure, color, and flavonoids

The limbs of lateral petals were used for analysis of overall petal structure, color, and flavonoids. A stereo microscope was used to observe the surface structure of the petal epidermis. Color measurements were based on CIE L*a*b* color space and conducted on the adaxial face of the petal using a colorimeter (CD110; Yokogawa Test & Measurement Corp., Tokyo, Japan).

For flavonoid analysis, 20–30 mg fresh weight of lateral limbs was collected, frozen in liquid nitrogen, and stored at −80°C until analysis. Flavonoids were extracted with 10% aqueous acetic acid; the 10 μL of extract was then analyzed using a high-performance liquid chromatography (HPLC) system, together with a photodiode array detector (Agilent 1100 series; Agilent Technologies Inc., Wilmington, DE, USA) and an Inertsil ODS-2 analytical column (4.6 mm × 250 mm; GL Science Inc., Tokyo, Japan). A linear gradient of 20%–100% of solvent B (1.5% H3PO4, 20% acetic acid, 25% MeCN) in solvent A (1.5% H3PO4) was run over a total of 40 min with a flow rate of 0.8 mL·min⁻¹ at 40°C. Absorption spectra were monitored over 220–600 nm. The content of flavonoids was converted from peak areas detected at 530 nm for anthocyanins and 330 nm for flavonoids. These conversions were based on calibration curves observed for cyanidin 3-rutinoside and quercetin 3-rutinoside (rutin), respectively.

Structure and expression analysis of genes

Full-length cDNAs and gDNAs of TfF3'5'H and TfF3'H were isolated as described previously (Nishijima et al., 2013). Total RNA and gDNA were extracted from young petals. Target cDNAs and gDNAs were amplified by PCR using primer sets as shown in
Table S1. PCR products were cloned with the base sequence analyzed by a DNA sequencer.

Expression analysis of the genes involved in anthocyanin biosynthesis was conducted using real-time quantitative PCR (qPCR) as described previously (Nishijima et al., 2016). Primer sets shown in Table S1 were used for analysis of TjF3’5’H and TjF3H, while the other genes were analyzed using primer sets described previously (Nishijima et al., 2016).

**Results and Discussion**

*Isolated mutants and genetic analysis*

A mutation exhibiting reddish-purple and shrunken petals was detected in the S1 generation of “flecked” (Fig. 1). Stamens and pistils were observed to be normal and fertile, with no somatic reversion. All S1 plants of the mutant exhibited the same reddish-purple and shrunken petals (Table S2), indicating that the mutation was stably inherited. This mutation was assigned the name “reddish purple shrunken” (RPS).

All F1 plants obtained from the cross between RPS and NT were NT irrespective of the cross direction (Table S2). The segregation ratio of F2 plants was examined by the \( \chi^2 \) test to the theoretical segregation ratio, assuming that the mutation has a single recessive trait—i.e., the NT:RPS population ratio is 3:1. The observed \( P \)-value was 0.77 when RPS and NT were seed and pollen parents, respectively, whereas the \( P \)-value was 0.55 for the reverse cross. Therefore, the RPS phenotype was probably induced by the homozygote of a single recessive factor assigned as \( r \).

Another mutation exhibiting white petals with violet spots was detected in the S1 generation of “flecked”, generating somatic reversion and bearing entirely violet petals. From this, it was deduced that the mutation was stabilized in response to the cross with NT. The stabilized mutant exhibiting entirely white petals without any somatic reversion was segregated in the F2 generation. None of the S1 plants derived from this stabilized mutant showed reversion (Table S3); this mutant was assigned as “white” (W) (Fig. 1).

All F1 plants obtained from the cross between W and NT were NT irrespective of the cross direction (Table S3). The segregation ratio of F2 plants was examined by the \( \chi^2 \) test to the theoretical segregation ratio, assuming that the mutation is a single recessive trait—i.e., NT:W population ratio is 3:1. The \( P \)-value was 0.28 when W and NT were seed and pollen parents, respectively, whereas it was 0.79 for the reverse cross. Therefore, the W phenotype was probably expressed by the homozygote of a single recessive factor assigned as \( w \).

All F1 plants obtained from the cross between RPS and W were NT irrespective of the cross direction (Table S4). F2 plants included—in addition to NT, W, and RPS—a new phenotype exhibiting reddish-purple petals without any shrinkage. This phenotype was assigned as “reddish purple” (RP) (Fig. 1). It was estimated that RP has \( r/r \) genotype because the petal color of RP was similar to that of RPS. If it is assumed that the W trait is inherited semi-dominantly—i.e., \( w/w, W/w, \) and \( W/W \) induce W, RP, and RPS, respectively—in the background of \( r/r \), the theoretical segregation ratio of NT, W, RP, and RPS is 9:4:2:1 (Fig. S1). The real segregation ratio of F2 plants was examined by the \( \chi^2 \) test to the theoretical segregation ratio; the \( P \)-value was 0.51 when RPS and W were seed and pollen parents, respectively, whereas the \( P \)-value was 0.44 for the reverse cross. Therefore, the RP phenotype was probably induced by a combination of \( r/r \) and \( W/w \).

**Development of petal color**

Pigmentation of the adaxial face of lateral and ventral petals was obvious when observed in corolla with a length of 8 mm or more, specifically in NT, RPS, and RP, although faint pigmentation was observed in the ventral petal of a 6-mm long corolla (Fig. S2). The color of the young petal was violet in NT and purple in RP. Young RPS petals exhibited an almost identical petal color to RP. However, in corolla with a length of 12 mm or more, a brown discoloration was observed mainly in the center of the pigmented area (Fig. S2). Although the dorsal petal was also slightly pigmented in 16-mm long corolla in NT, RPS, and RP, no discoloration was observed. The young petals of W exhibited no pigmentation as well as mature petals (data not shown).

Uniform round-shaped epidermal cells were tightly arranged in the adaxial face of lateral NT petals at all testing stages (Fig. S3). Epidermal cells were consistently and evenly violet in color. The epidermal cells in the normal petal area—i.e., the area without brown discoloration—of RPS also displayed a uniform round
shape. However, the density of the purple color varied slightly in 12-mm long corolla and markedly in the opened flower, but was relatively uniform in 8-mm long corolla. In contrast, discolored petal areas of RPS in 12-mm long corolla had brown and white epidermal cells in addition to the normal purple cells. Cells were round and relatively irregular in size. The brown—and some purple—cells became much paler in the opened flowers, i.e., white or pale brown, whereas the other purple cells retained the color. The white or pale brown cells were flat, whereas the purple and pale purple cells were round. The brown and white cell colors indicated that some disorder occurred, including necrosis and inhibition of anthocyanin biosynthesis and/or accumulation.

The shape and color of RP petal epidermal cells developed similarly to those of the normal area of the RPS petals. Uniform, round cells were tightly arranged with a slight variation in the purple color density, with the variation in color density enhanced in the opened flowers.

**Color measurement and flavonoid analysis**

The L* value was the largest in RPS, followed by RP, and the smallest was in NT, indicating that the color was the brightest in RPS, followed by RP, with the darkest in NT (Table S5). The a* value was the largest in RP, followed by RPS, and the smallest in NT, indicating that reddishness was the strongest in RP, followed by RPS, and was weakest in NT. The b* value was slightly higher in RPS and slightly lower in RP than in NT. The higher b* in RPS probably indicates involvement of brown epidermal cells as described above (Fig. S3). This is because a higher b* value indicates a more yellowish color. The L* value of W was considerably higher, whereas a* and b* were close to 0, indicating that W had almost pure white petals.

Anthocyanins contained in opened NT flowers were mainly the delphinidin-type as described previously (Fig. 2; Nishijima et al., 2013). Concentrations of Mv3,5dG, Pt3,5dG, and Dp3,5dG were high, although low concentrations of cyanidin-type anthocyanins, Pn3,5dG, and Cy3,5dG were also detected. In contrast, only cyanidin-type anthocyanins, Pn3,5dG, and Cy3,5dG were detected in RPS and RP; the concentration of Pn3,5dG was much higher than NT, whereas that of Cy3,5dG was as low as NT. The total anthocyanin concentration was the highest in NT (Fig. 2), followed by RP, and the lowest in RPS. Almost no anthocyanins were detected in W.

Apigenin 7-glucuronide (Ap7G acid), luteolin 7-glucoside (Lut7G), and luteolin 7-glucuronide (Lut7G acid) were detected as flavones in NT. No distinct difference in composition of those flavones was observed among NT, RPS, RP, and W. The total flavone concentration was the highest in NT and RP, slightly lower in W, and much lower in RPS. Low concentrations of anthocyanins and flavones in RPS were probably caused by the marked occurrence of white and brown epidermal cells (Fig. S3).

Anthocyanins were not detected in the lateral petals of NT, RPS, and RP in 6-mm long corolla (Fig. S4). However, high concentrations of anthocyanins were detected in 8-mm long corolla. The composition of anthocyanins was the same as that of the opened flowers (Fig. 2). The total anthocyanin concentration was slightly higher in RPS and lower in RP than in NT (Fig. S4). In the more developed 12-mm long corolla, the total anthocyanin concentration was increased in NT and RP, but decreased in RPS when compared with the 8-mm long corolla. As a result, the total anthocyanin concentration reached the highest in NT, followed by RP, while the lowest was in RPS, which was similarly observed in the opened flowers.

The total flavone concentration was markedly increased in 8-mm long corolla when compared with 6-mm long corolla (Fig. S5). No substantial difference in flavone constituents was observed among the lines. However, the total flavone concentration of RPS was decreased in the more developed 12-mm long corolla, whereas those of NT and RP increased, fluctuating seemingly in parallel with the anthocyanin concentration.

**Fig. 2.** Concentrations of anthocyanins (left) and flavones (right) in the limb of a lateral petal. NT: normal type, RPS: reddish purple shrunken, RP: reddish purple, W: white. Vertical bars represent ± SE (n = 3). ANOVA showed significant differences (P < 0.001) among lines in terms of total concentrations of anthocyanins, cyanidin-type anthocyanins and flavones, respectively.
The simultaneous decrease in anthocyanin and flavone concentrations in the developed petals of RPS appears to be associated with the petal disorder described above. It is assumed that the biased anthocyanin composition consisting solely of cyanidin-type probably caused this disorder. Meanwhile, RP showed no apparent petal disorder irrespective of having the same anthocyanin composition as RPS (Figs. 1, S2, and S3), probably because of moderate anthocyanin accumulation. Moderate anthocyanin accumulation in RP was indicated by the lower total anthocyanin concentration than RPS in the 8-mm long corolla, which exhibited no apparent petal disorder and thus presumably exhibited “intrinsic” anthocyanin accumulation of the lines (Fig. S4).

**Structure and expression analysis of *TjF3’5’H***

Loss of delphinidin-type anthocyanins in RPS and RP indicated the possible mutation of *TjF3’5’H*. Therefore, cDNA and gDNA of *TjF3’5’H* were cloned to analyze the structure. The gDNA of *TjF3’5’H* consisted of two exons and one intron (Fig. 3A). A deletion of cytosine at +147 bp downstream from the first base of the coding region was detected in RP and RPS. This deletion caused a frameshift mutation, further resulting in an ectopic stop codon at +421 bp downstream from the first base of the coding region. The mutated *TjF3’5’H* was assigned as *TjF3’5’H*<sup>sc</sup>. This mutation may be as a result of a *Tjf1* transposition footprint, while other natural mutations caused by a mistake in DNA replication, natural radiation, chemical mutagens, etc. cannot be ruled out. The region of gDNA spanning 579 bp around the deletion of C was amplified by PCR using the primer set shown in the “genotype analysis” of Table S1, and then cloned. Each of the 12 clones per line of RPS and RP was of a mutant-type with the deletion of C, indicating that RPS and RP are homozygotes of *TjF3’5’H*<sup>sc</sup>. Therefore, the substance of *r* is *TjF3’5’H*<sup>sc</sup>, which is recessively inherited, and the homozygote of *TjF3’5’H*<sup>sc</sup> is responsible for the RPS and RP phenotypes.

The expression of *TjF3’5’H* in NT, RPS, and RP was conspicuously increased along with corolla growth from 6- to 8-mm long. This corresponds with the onset of petal coloration (Fig. 4), followed by a decreasing trend in the more developed 12-mm long corolla. The expression was conspicuously lower in both RPS and RP than in NT in 8-mm long corolla, and was attributable to the frameshift mutation described above. In 12-mm long corolla, the expression was lower in both RPS and RP than in NT.

In 8-mm long corolla, the expressions of other genes encoding anthocyanin biosynthesis enzymes—i.e., *TfCHS*, *TfCHI*, and *TfANS*—were also lower in RPS and RP than in NT, while expression of *TjF3H* was lower in RP than in NT (Figs. 4 and 5). The expression of *TfMYB1*—the transcription factor inducing genes encoding anthocyanin biosynthesis enzymes (Nishijima et al., 2013)—was also lower in RPS and RP than in NT (Fig. 4). Expressions of those genes decreased in NT and RPS in the more developed 12-mm long corolla, whereas expressions did not change greatly in RP.

In contrast, the expression of *TjUFGT* in RPS and RP...
was conspicuously higher than that in NT in 8-mm long corolla (Fig. 5). In 12-mm long corolla, the expression of *TjUFGT* increased in NT as much as in RP. The expression of *TjF3'H* was slightly higher in RPS and RP than in NT in 8-mm long corolla (Fig. 5).

Petal disorder of the 12-mm long RPS corolla may have reduced expressions of the genes encoding anthocyanin biosynthesis enzymes possibly through reduced expression of *TfMYB1* (Figs. 4 and 5).

**Structure and expression analysis of *TjF3H***

Anthocyanins were not detected in W (Fig. 2). However, *TjCHS* and *TjCHI* were probably functional, based on the concentrations of flavones, AP7G acid, Lut7G, and Lut7G acid not decreasing. Among the anthocyanin biosynthetic genes, expressions of *TjF3H*, *TjDFR*, and *TfMYB1* were almost completely suppressed. Although no mutation was found in *TjDFR* or *TfMYB1*, *Ttf1* was inserted in the first exon of *TjF3H* (Fig. 3B). This mutated allele was designated as *TjF3HΔt*. As a result of PCR performed on W gDNA using primer sets that specifically amplify *TjF3HΔt* or *TjF3H* (Table S1), only *TjF3HΔt* was detected (Fig. S6). Combining this result and that of the genetic analysis of W (Table S3), it was revealed that the causative gene of W is *TjF3HΔt*, whereas the W trait is expressed by homozygous *TjF3HΔt*. Since the insertion of *Ttf1* was in the exon on the 5' side of the gene, it was inferred that the gene function was greatly reduced.

**Control of petal shrinkage by combining *TjF3'5'H* and *TjF3H* mutations***

As described above, RP is a homozygote of *TjF3'5'H*. A genotype of *TjF3H* was examined on the five RP plants randomly selected from the F2 generation of RPS × W and their reverse crosses, respectively. All 10 plants were heterozygotes of *TjF3H* and *TjF3HΔt* (Fig. S6). Therefore, it was proven that the RP trait is expressed by a combination of a homozygote of *TjF3'5'H* and a heterozygote of *TjF3H* and *TjF3HΔt*.

This genotype of RP corresponded to observations that expressions of *TjF3'5'H* and *TjF3H* in RP are almost the same as—and tended to be slightly lower than—that of RPS, respectively (Fig. 4), when examined in 8-mm long corollas without apparent petal disorder.

As described above, suppression of the petal disorder shown in RP was probably in response to the moderate accumulation of cyanidin-type anthocyanins when compared with RPS (Fig. S4). The moderate anthocyanin accumulation was probably caused by the reduced gene dosage of *TjF3H* in the heterozygote of *TjF3H* and *TjF3HΔt*, which corresponded well to the semi-dominant inheritance of *TjF3H* (Table S4). Semi-dominant inheritance of *F3H* has also been observed in snapdragon mutants (Coen et al., 1986; Martin et al., 1991), indicating that *F3H* is a limiting factor for anthocyanin biosynthesis not only in snapdragon, but also in torenia.

In the F2 generation of RPS and W, genotypes of
some plants should have been heterozygotes of $TfF3'H'$ and $TfF3'H''$ in the background of homozygotes of $TfF3'S'H'$ or heterozygotes of $TfF3'S'H'$ and $TfF3'S'H''$, in which the delphinidin-type anthocyanin is synthesized. Variations in violet color density were observed among F$_2$ plants that may reflect genotypic differences. However, variation was slight probably because of the dense petal color hindering visual discrimination with mild reductions in anthocyanin concentration.

A few cases have been reported in which the accumulation of certain anthocyanins or related compounds caused petal shrinkage. Accumulation of delphinidin 3-glucoside causes marked petal shrinkage in Petunia × hybrid (Ando et al., 2004). The chalcone isomerase (CHI) mutation in Ipomoea nil bears flowers with shrunken petals conferred by the accumulation of 2',4',6', and 4-tetrahydorocyalone (THC) 2'-O-glucoside (Hoshino et al., 2019). This petal shrinkage is restored by the decreased THC 2'-O-glucoside concentration caused by the overexpression of both chalcone 4'-O-glucosyltransferase and aureusidin synthase genes. In torenia, accumulation of cyanidin-type anthocyanins probably caused petal shrinkage as described above. However, Accumulation of cyanidin-type anthocyanins may have accompanied enhanced production of the precursors and related byproducts, i.e., dihydroquercetin, leucocyanidin, quercetin, catechin, (-)-epicatechin, etc., which were not quantified in the current study. It cannot be ruled out that these compounds may have promoted petal shrinkage.

Several other results in the current study also indicated that cyanidin-type anthocyanins were “toxic” to torenia petal development. The expression of UFGT in RPS and RP increased from the earlier stage of petal coloration compared with the normal type (Fig. 5). This phenomenon may be an enhanced “detoxication” process, because glycosylation functions in the detoxication of various molecules by promoting compartmentation into the vacuoles. The reduced expression of $TfMYB1$, which may have reduced the expression of genes encoding anthocyanin biosynthesis enzymes in RPS and RP, implied negative feedback to biosynthesis actions of cyanidin-type anthocyanins (Fig. 5).

From an evolutionary and adaptive viewpoint, the low tolerance to cyanidin-type anthocyanins does not affect petal growth of wild T. fournieri, hence there are no negative impacts on the reproductive efficiency. This is because the delphinidin-type is the main anthocyanin in wild T. fournieri (Nishijima et al., 2013), and therefore, tolerance to cyanidin-type anthocyanins may be neutral to natural selection.

**Implications on flower color breeding**

The RPS obtained in the current study has a defect referred to as a “shrunken” flower appearance. Therefore, even if the flower color is new, it does not work as a breeding material as it is. However, the present study revealed that combining this mutation with a genotype partially suppressing anthocyanin biosynthesis—i.e., $TfF3'H'/TfF3'H''$—restored normal development of petals that retained a new flower color. As described above, the partial suppression of anthocyanin biosynthesis did not dilute the color, but rather increased the anthocyanin concentration, resulting in a darker color (Table S5; Fig. 2). This means that even when petal shrinkage occurs in response to specific compositions of anthocyanins, it can also be used as a breeding material when combined with a genotype that decreases anthocyanin biosynthesis. The present study provides a theoretical basis for the use of new-color mutations with petal disorders.

A torenia cultivar ‘Crown Violet’ generated descendants bearing dark-red shrunken petals when self-pollinated (data not shown). This phenomenon appeared somewhat similar to RPS, although the petal color was much darker than RPS. Nishihara et al. (2014) discovered a deletion mutation in $TfF3'S'H$ in ‘Crown White’, as well as a mutation in $TfF3'H$, causing a white flower color. These findings confirm the prediction that some torenia cultivars already had $TfF3'S'H$ and $TfF3'H$ mutations as RP was observed to have. Burgundy-colored—i.e., wine-red—torenia cultivars look much darker and more reddish than RP, but are also somewhat similar in tone. Future research is necessary to clarify whether the burgundy color is induced by the same mechanism as shown in RP.

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