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Review

# Recent advances in flower color variation and patterning of Japanese morning glory and petunia

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The Japanese morning glory (*Ipomoea nil*) and petunia (*Petunia hybrida*), locally called "Asagao" and "Tsukubane-asagao", respectively, are popular garden plants. They have been utilized as model plants for studying the genetic basis of floricultural traits, especially anthocyanin pigmentation in flower petals. In their long history of genetic studies, many mutations affecting flower pigmentation have been characterized, and both structural and regulatory genes for the anthocyanin biosynthesis pathway have been identified. In this review, we will summarize recent advances in the understanding of flower pigmentation in the two species with respect to flower hue and color patterning. Regarding flower hue, we will describe a novel enhancer of flavonoid production that controls the intensity of flower pigmentation, new aspects related to a flavonoid glucosyltransferase that has been known for a long time, and the regulatory mechanisms of vacuolar pH being a key determinant of red and blue coloration. On color patterning, we describe particular flower patterns regulated by epigenetic and RNA-silencing mechanisms. As high-quality whole genome sequences of the Japanese morning glory and petunia wild parents (*P. axillaris* and *P. inflata*, respectively) were published in 2016, further study on flower pigmentation will be accelerated.

**Key Words:** anthocyanin, epigenetics, floral pigmentation pattern, *Ipomoea*, petunia, RNA silencing, vacuolar pH.

## Introduction

The Japanese morning glory (*Ipomoea nil*) has been a floricultural plant in Japan since the 17th century Edo period. While its wild-type plants produce blue flowers, a number of spontaneous mutants displaying various flower colors and pigmentation patterns have been isolated and maintained to the present. Recent molecular studies on the mutants showed that *Tpn1* family transposons are the major mutagen in *I. nil* cultivars (Chopra *et al.* 2006, Iida *et al.* 2004). Most of the *I. nil* mutations are insertions of transposons or footprints caused by transposon excisions. *Tpn1* family transposons are class II DNA elements that can transpose into new locations via a cut-and-paste mechanism. Even now, they can cause new spontaneous mutations exhibiting novel flower colors and patterns.

The petunia (*Petunia hybrida*) originated from interspecific hybridization between two petunia wild parents, *P. axillaris* and *P. integrioforia* (or its related species, *P. inflata*) (Stehmann *et al.* 2009, Vandenbussche *et al.* 2016). *P. axillaris* has large white flowers, while *P. integrioforia* and *P. inflata* display small purple flowers. The first hybrids were produced by European breeders in 19th century, which is considered to have been produced from multiple crossings between different accessions of the wild parent species (Sink 1984). Recent varieties of flower traits in commercial petunias have resulted from intense breeding since the first interspecific hybridizations. Like in the case of *I. nil*, class II DNA transposons have contributed to the phenotypic diversity of petunia flowers (Vandenbussche *et al.* 2016). The transposons in *I. nil* and petunia are useful tools for isolating genes responsible for flower pigmentation.

Flavonoids are major flower pigments that accumulate in vacuoles (Tanaka *et al.* 2008, Winkel-Shirley 2001). Anthocyanins are members of flavonoids responsible for a wide range of flower colors—red, orange, blue, and purple pigmentations. In the anthocyanin biosynthesis pathway (**Fig. 1**), chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase (F3H) catalyze "early" steps required for the synthesis of all flavonoids. Dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS),

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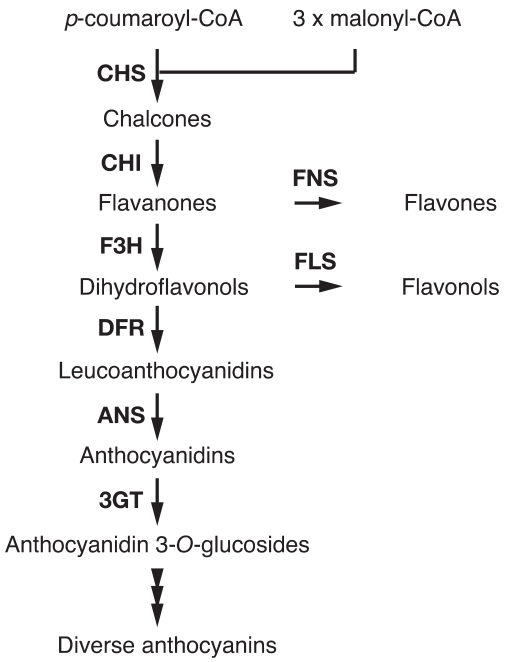


**Fig. 1.** Simplified flavonoid biosynthesis pathway. The enzymes in the pathway are: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase; FNS, flavone synthase; FLS, flavonol synthase. The arrowheads indicate modification steps of anthocyanins, which are mediated by glycosyltransferases, acyltransferases, and methyltransferases. Hypothesis 1 of EFP function: EFP enhances CHS activity. Hypothesis 2 of EFP function: EFP interacts with biosynthesis enzymes and forms a metabolon complex.

and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (3GT) mediate "late" steps that lead to anthocyanin and proanthocyanidin biosynthesis. Enzymes from CHS to ANS produce anthocyanidins that are anthocyanin aglycones and the central chromophores of anthocyanins. Anthocyanidins are generally glucosylated at the 3 position by 3GT to give anthocyanidin 3-glucosides that are the first stable anthocyanin. Further modifications, such as additional glycosylation and acylation to anthocyanidin 3-glucosides, occur in a species-specific manner. Transcriptional regulators for structural genes encoding enzymes in the pathway are known to include proteins containing an R2R3-MYB domain, an R3-MYB domain, a basic-helix-loop-helix (bHLH) domain, and WD40 repeats (WDR). The proteins constitute complexes that control the transcription of structural genes (Albert *et al.* 2014, Quattrocchio *et al.* 2006a).

The diversity of anthocyanin structures is an important factor in the wide range flower color. In addition, the pH inside the vacuole, the vacuolar lumen, largely affects flower color (Yoshida *et al.* 2009b). Anthocyanins show reversible structural transformation in their chromophores dependent on the pH shift of the solvent, and the structural transformation dramatically affects their absorption spectra. The vacuolar lumen is generally acidified by $H^+$-ATPase (V-ATPase) and $H^+$-pyrophosphatase (PPase) on the vacuolar membrane, tonoplast (Eisenach *et al.* 2014, Gaxiola *et al.* 2007). Anthocyanins normally show reddish to purplish colors in the acidic vacuoles, and change to bluish colors due to increased pH in the vacuole. Other than vacuolar pH, co-pigments and epidermal cell shapes also affect flower color.

Thanks to a number of flower color mutations, *I. nil* and petunia have been utilized as ideal model plants to study flower pigmentation (Iida *et al.* 2004, Quattrocchio *et al.* 2006a, Tornielli *et al.* 2009). These plants have contributed to the advancement of studies on flower pigmentation, e.g., discovery of the blue gene (Holton *et al.* 1993). In this review, we describe recent advances in flower colors of the two species, especially with regard to a novel enhancer of flower pigmentation, new aspects on 3GT, color patterning regulated by epigenetics and RNA silencing, and vacuolar pH regulation.

## A novel color intensifier, EFP (Enhancer of Flavonoid Production)

Classical genetic studies of *I. nil* reported several mutations resulting in alterations of flower color intensity that were probably due to changes in anthocyanin quantity (Imai 1931). To elucidate mechanisms underlying the regulation of anthocyanin quantity in flower petals, we employed a newly isolated mutation that confers pale-colored flowers (**Fig. 2A**). In the mutant, the total amount of anthocyanin was less than approximately 22% of that in the parental wild-type plant, which displays normally colored flowers. In addition, the colorless flavonoid content, flavonol (**Fig. 1**), was also decreased by 27% in the mutant. Flavonoid analysis indicated that the responsible gene plays a key role in flavonoid biosynthesis, increasing flavonoid total content by approximately 3–4 times. We named the gene *Enhancer of Flavonoid Production* (*EFP*), and isolated it by transposon tagging (Morita *et al.* 2014). The *EFP* gene encodes a CHI-like protein that is classified as a type IV CHI protein of unknown function (Ralston *et al.* 2005). EFP is not a functional CHI enzyme, because the *chi* mutant of *I. nil* accumulates chalcone derivatives rather than anthocyanins (Hoshino *et al.* 2001, Saito *et al.* 2011). The mutation named *efp-1* was an insertion of *Tpn13* into the *EFP* promoter sequence, resulting in the complete suppression of *EFP* expression (**Fig. 2B**). *EFP* and the flavonoid biosynthesis genes showed similar spatiotemporal expression patterns and were coordinately activated by the regulatory genes, *InMYB1* and *InWDR1* (Morita *et al.* 2006, 2014). We also isolated cDNA for the *EFP* in the petunia and torenia (*Torenia hybrida*) and obtained RNAi knockdown mutants of the *EFP* genes to examine whether EFP proteins in other ornamental flowers also increase flavonoid production efficiency (Morita *et al.* 2014). As expected, the transgenic RNAi knockdown petunia and torenia plants conferred pale-colored flowers. In the knockdown petunia, anthocyanins and other flavonoid (flavonols, **Fig. 1**) contents of the

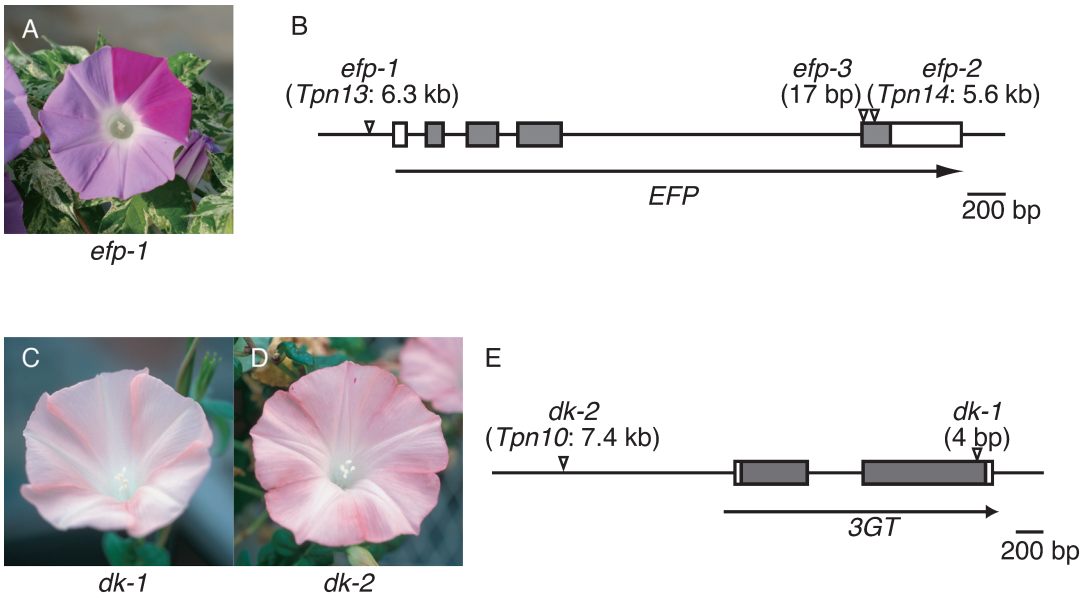


**Fig. 2.** Flower phenotypes of flower hue mutants and the genomic structure of the mutated loci. The gray and white boxes indicate the coding and the untranslated regions, respectively. Flower phenotype of *efp-1* mutant (A) and the structure of the *EFP* gene (B). The *efp-1* mutant shows pale-colored flowers with normal pigmented spots and sectors. The arrowheads indicate insertion sites of *Tpn13*, *Tpn14*, and a 17-bp sequence of the *efp-1*, *efp-2*, and *efp-3* mutations, respectively. Flower phenotypes of *dk-1* (C) and *dk-2* (D) mutants and the structure of the *3GT* gene (E). The *dk-1* and *dk-2* mutants display pale and dull-colored flowers. The flower color in the *dk-2* mutant is slightly darker than that in the *dk-1* mutant. The arrowheads show a 4-bp sequence and *Tpn10* insertion sites of the *dk-1* and *dk-2* mutations, respectively.

flower decreased to approximately 28% and 12%, respectively, of those in the control plants. Moreover, in the knockdown torenia, anthocyanins and other flavonoid (flavones, **Fig. 1**) contents of the flower also decreased to approximately 49% and 31%, respectively, of those in the control plants. These results clearly show that EFPs of *I. nil*, petunia, and torenia enhance flower pigmentation by increasing flavonoid production ability. Recently, we also reported the remarkable expression of *EFP* homologs in the flower petals of the carnation (*Dianthus caryophyllus*), chrysanthemum (*Chrysanthemum morifolium*), and snapdragon (*Antirrhinum majus*) (Morita *et al.* 2015b). The *EFP* gene may contribute to flower color intensity in these floricultural crops.

The molecular mechanisms of how EFP enhances flavonoid production remain to be elucidated. The reduction of flavone or flavonol contents in mutants of *I. nil*, petunia, and torenia suggests that EFPs activate at least the early steps of flavonoid biosynthesis (**Fig. 1**). In Arabidopsis (*Arabidopsis thaliana*), the *EFP* homologue *AtCHIL* and the genes for the enzymes that mediate the early steps are coordinately regulated (Yonekura-Sakakibara *et al.* 2008, 2012). In the moss *Physcomitrella patens*, the *EFP* homologue is coordinately expressed with *CHS* genes under a flavonoid-producing condition promoted by UV irradiation (Wolf *et al.* 2010). These observations are consistent with the idea that EFP activates the early steps of flavonoid biosynthesis.

We hypothesize two possibilities to explain the activation of the early steps by EFP: 1) EFP enhances CHS activity, 2) EFP is one component of the flavonoid biosynthesis enzyme complex (**Fig. 1**). In *I. purpurea*, an *I. nil*-related morning glory, dosage-dependent expression of the *CHS* gene led to the incomplete dominance of flower coloration (Johzuka-Hisatomi *et al.* 2011), suggesting that the reduction of CHS activity confers pale-colored flowers in *I. nil*. Incomplete dominance has not been reported in other genes in the early steps. In the *efp-1* mutant flower, the levels of flavonoid precursor-related compounds, chlorogenic acid and caffeoyl glucoside were increased remarkably. The null *chs* mutants also accumulate these compounds (Hoshino *et al.* 2009, Saito *et al.* 1994). These observations support the first hypothesis. With respect to the second hypothesis, metabolon is an enzyme complex consisting of sequential enzymes of a metabolic pathway; it enables the effective synthesis of specific products and the avoidance of metabolic interference (Jorgensen *et al.* 2005). In Arabidopsis, CHS, CHI, F3H, and DFR are thought to form a complex ensuring efficient flavonoid biosynthesis (Burbulis and Winkel-Shirley 1999, Winkel-Shirley 1999, 2001). As the secondary structure of EFP is closely similar to that of CHI (Morita *et al.* 2014, Ngaki *et al.* 2012), EFP probably constitutes a metabolon with flavonoid biosynthesis enzymes that involve the early steps and enhance flavonoid production through the metabolon. Elucidating the mechanism of the activation of the early steps by EFP will provide insight into how flavonoid synthesis is controlled as well as the evolution of the flavonoid biosynthesis pathway.

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## Mutations of the *3GT* gene conferring pale and brownish or grayish flowers in morning glories

In the anthocyanin biosynthesis pathway, anthocyanidin 3-glucosides are generally the first stable anthocyanins, and 3GT catalyzes glucosylation at the 3-position of anthocyanidins. 3GT belongs to the family 1 glycosyltransferases (UGTs) that uses UDP-sugars as the sugar donor. Although the gene for 3GT was first isolated from maize (*Zea mays*) more than 30 years ago (Fedoroff *et al.* 1984), no 3GT affecting flower petal color had been reported. Mutations in other flavonoid UGT genes for anthocyanidin 3-glucoside glucosyltransferase (3GGT) and anthocyanidin 3-glucoside rhamnosyltranferase (RT) were identified in *I. nil* and petunia, respectively (Kroon *et al.* 1994, Morita *et al.* 2005). They result in brownish or grayish flowers due to the accumulation of anthocyanins with fewer modifications of glycosylation and acylation, which are necessary for species-specific anthocyanin production and the bright coloration of flowers. The *Dusky* (*Dy*) locus encodes 3GGT in *I. nil*.

The *duskish* (*dk*) mutants of *I. nil* display brownish or grayish flowers (**Fig. 2C**, **2D**). The flower colors of *dk* and *dy* mutants are similar; however, *dk* mutants show paler pigmentation than do *dy* mutants. Recently, we found that the *Dk* gene encodes 3GT, and we identified two allelic mutations, *dk-1* and *dk-2* (**Fig. 2E**, Morita *et al.* 2015a). The *dk-1* and *dk-2* mutations are a 4-bp insertion and an insertion mutation of *Tpn1* family transposon, *Tpn10*, respectively. Pale brownish or grayish flowers are also found in cultivars of *I. purpurea*. We found a single base deletion in the *3GT* gene in the cultivars, and we named the allele *ip3gt-1* (Morita *et al.* 2015a). Among these mutations, *dk-1* and *ip3gt-1* seem to be null mutations, while *dk-2* is a leaky mutation (see below).

Anthocyanin analysis of the *dk-1* and *ip3gt-1* mutants suggested that the absence of 3GT leads to the reduction of anthocyanin accumulation and the inhibition of glucosylation and acylation found in the anthocyanins in wild-type plants (Saito *et al.* 1998, Toki *et al.* 2001). The mutants accumulate small amounts of anthocyanidin 3-glucoside and its derivatives. Since the *I. nil* genome carries the unique *3GT* gene, glucosylation at the 3 position of anthocyanidins is assumed to be mediated by glucosyltransferases other than Dk protein, the *bona fide* 3GT. *UGT78D2* is the unique *3GT* gene in Arabidopsis. Because its mutant accumulates small amounts of anthocyanidin 3-glucosides, the presence of secondary 3GT activities has also been suggested (Tohge *et al.* 2005). The formation of 3-glucosylated anthocyanins without *bona fide* 3GT may be conserved among divers plant species. In wild-type *I. nil* and *I. purpurea*, anthocyanidins are glucosylated at its 5 position, and their glucose moiety at the 3 position is decorated with glucose and caffeoyl moieties. Such glucosylation and acylation are not found in the major anthocyanins in *dk-1* and *ip3gt-1* mutants. This indicates that *bona fide* 3GT is necessary for the appropriate glucosylation and acylation of anthocyanidin 3-*O*-glucoside in the species. It can be speculated that glucosyltransferases and acyltransferases for anthocyanidin 3-*O*-glucoside form metabolon with 3GT in the cytosol to ensure effective and precise anthocyanin production. Therefore, the absence of 3GT could inhibit the appropriate modification of anthocyanidin 3-*O*-glucoside through the inhibition of metabolon formation.

## A particular flower variegation involving epigenetic control of *3GT* gene expression

Among *dk-2* mutants, the Q531 line shows genetically and epigenetically unstable expression of the *3GT* gene (**Fig. 3A–3E**, Morita *et al.* 2015a). The line's phenotype is variable, and plants with variegated flowers (**Fig. 3A**), pale grayish-purple flowers (**Fig. 3B**), and fully pigmented flowers (**Fig. 3C**) are segregated. Usually, flower variegation is due to recurrent somatic mutations caused by the excision of transposons and shows clonal chimera spots and sectors. *Tpn1* family transposons inserted into genes for anthocyanin pigmentation can cause such flower variegation (Fukada-Tanaka *et al.* 2000, Hoshino *et al.* 2001, Inagaki *et al.* 1994, Morita *et al.* 2014). The Q531 line shows both clonal and non-clonal spots and sectors. Imai (1931, 1935) called the flower variegation phenotype with non-clonal and apparently non-chimeric spots and sectors in other *dk* mutants in particular "ruled". In ruled flowers, fully pigmented areas appear within the pale grayish-purple areas and vice versa (**Fig. 3A**). The non-clonal variegation seems to be generated by reversible expression changes of the *3GT* gene between an active state and an inactive state. Such a reversible change of the expression state is hard to explain by recurrent somatic mutations caused by transposons. The *dk-2* mutation is an insertion mutation of the *Tpn1* family transposon, *Tpn10*, 1.3 kb upstream of the *3GT* start codon, leading to the gene's leaky expression (**Fig. 2E**, Morita *et al.* 2015a). No footprint sequences generated by excisions of *Tpn10* could amplify in the ruled flowers, even in fully colored sectors (**Fig. 3A**, Morita *et al.* unpublished). This indicates that reversible changes of the *3GT* expression state resulting in the ruled phenotype occur independently from somatic excisions of *Tpn10*. It is speculated that epigenetic changes in DNA methylation and/or histone modification promote reversible changes in the ruled flowers (Morita *et al.* 2015a).

Imai called the segregated plants with pale grayish-purple flowers and fully pigmented flowers "plain" (**Fig. 3B**) and "self-colored" (**Fig. 3C**), respectively. Somatic changes of the *3GT* expression state observed in ruled flowers are suppressed in these plants. Interestingly, the transposition of *Tpn10* has been observed only in plain plants; clonal and chimera sectors and spots, as well as germinal revertants, appeared in the plants (**Fig. 3D**, **3E**, Imai 1931, 1935, Morita *et al.* 2015a). In self-colored plants, the reversion of *3GT* gene expression is also unrelated to the somatic excisions of *Tpn10*. We hypothesize three epigenetic states on

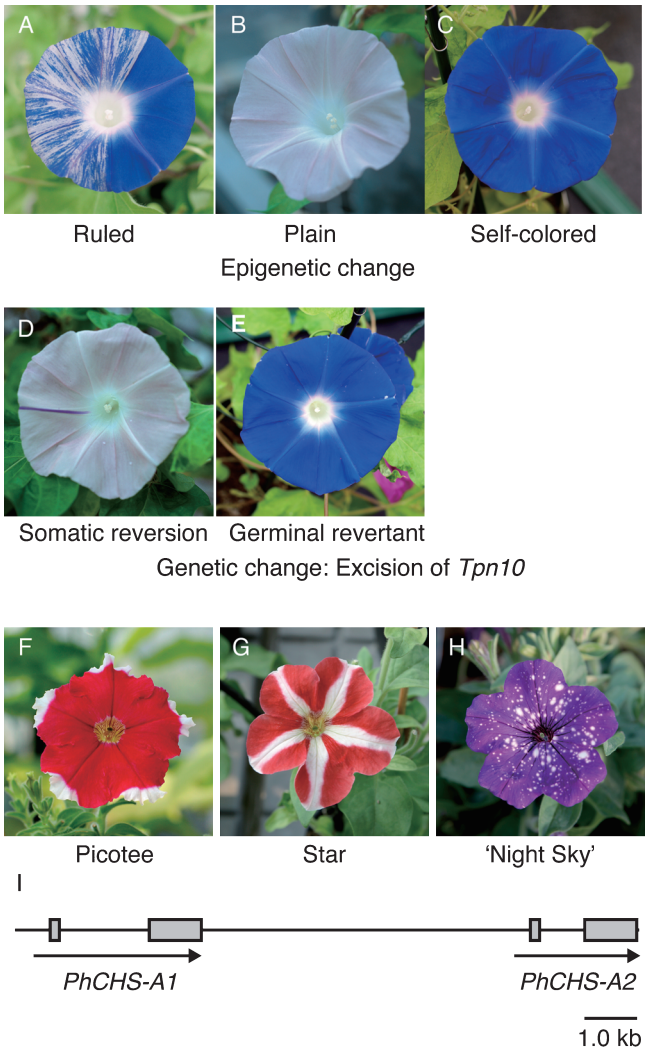


**Fig. 3.** Flower color patterns of the Japanese morning glory and petunia. A–E: Flower phenotypes of the Q531 line of the *dk2*-mutant of the Japanese morning glory. (A) Ruled plants confer non-clonal spots and sectors, *hakeme-shibori* (brush marks variegation). Apparently clonal spots and sectors are also occasionally observed in ruled plants. (B) Plain plants display pale pigmentation petals. (C) Self-colored plants display fully pigmented flowers. These plants carry an identical *dk-2* mutation, and *Tpn10* seems to be able to transpose only in plain plants. (D) Somatic reversions caused by *Tpn10* excisions are occasionally observed in plain plants. (E) Germinal revertant from a plain plant. (F–I) Naturally occurring bicolor RNAi mutants of the petunia. Flower phenotypes of Star (F), Picotee (G), and the bicolor cultivar 'Night Sky' (Ball Seed Co.) (I). (H) Genome structure of *CHS* siRNA-producing locus of the bicolor petunia. Two copies of *CHS* genes are tandemly located.

the *dk-2* locus to explain the phenotypes of the *dk-2* mutants: 1) an epigenetic state in the ruled plants can induce variable expression of the *3GT* gene, resulting in non-clonal variegation, and suppresses *Tpn10* transposition; 2) an epigenetic state in plain plants can suppress the expression of the *3GT* gene but not *Tpn10* transposition; and 3) an epigenetic state in the self-colored plants can ensure the stable expression of the *3GT* gene and might suppress *Tpn10* transposition.

In other ornamental plants, transposons sometimes generate variations in floral phenotypes. Transposons integrated within the promoter sequence of flower pigmentation genes might be involved in flower variegation, as in the *dk-2* mutants.

## Naturally occurring RNAi and flower coloration patterns

The petunia has contributed to the discovery of the RNAi phenomenon. The first molecular observations associated with RNAi occurred after the appearance of irregular bicolored or white flowers in transgenic petunias that harbored exogenous flower pigmentation genes (Jorgensen 1995, Napoli *et al.* 1990, van der Krol *et al.* 1990). Picotee and Star, naturally occurring bicolor petunia cultivars, have pigmented petals with white margins and stars, respectively (**Fig. 3F**, **3G**). These popular bicolor traits are believed to have arisen during the early impregnation events of interspecific hybridization breeding in the 19th century (Ewart 1984, Jorgensen 1995). Analyses of Star (Koes *et al.* 1987) and Picotee (Saito *et al.* 2006, 2007) petunias have indicated that the main cause of bicolor floral traits is the spatially regulated repression of *CHS* genes in the white tissue of petals (**Fig. 1**). Transcripts for both *CHS* copies, *CHS-A* and *CHS-J*, which are responsible for flower pigmentation, were down-regulated in the white tissue. Phenotypic similarities between transgenic and naturally occurring bicolor petunias suggested the involvement of an RNAi phenomenon in the expression of the Picotee and Star bicolor traits. Koseki *et al.* (2005) showed that while the *CHS-A* gene is transcribed into precursor mRNA in both white and pigmented tissues of Star petunias, mature *CHS-A* mRNA is accumulated only in the pigmented tissue. As the 21-nt *CHS-A* siRNA can be detected only in the white tissue, it was concluded that spatially expressed *CHS-A* siRNA guides the degradation of the mature *CHS-A* mRNA, resulting in the display of the Star phenotype. The spatially regulated production of *CHS-A* siRNAs was considered to be the main cause of bicolor floral traits; however, details of the molecular mechanisms, even in the genomic structure of *CHS-A* siRNA-producing locus, remained to be elucidated.

Morita *et al.* (2012) revealed the structure of the *CHS-A* siRNA-producing locus of Picotee and Star petunias. Interestingly, Picotee and Star petunias carry the same *CHS-A* siRNA-producing locus, consisting of two intact *CHS-A* copies in a tandem head-to-tail orientation (**Fig. 3I**). The *CHS-A* siRNAs were found to originate from the exon 2 region of both *CHS-A* copies, and those that restrict accumulations in the white tissues of Picotee and Star flowers are closely correlated with the spatial disappearance of the *CHS* mature mRNAs. These results suggest the existence of trans-acting factors, which regulate the spatiotemporal production of *CHS-A* siRNAs, originating from tandemly oriented *CHS-A* genes. Recent studies of naturally occurring

seed pigmentation mutants of soybeans (*Glycine max*) reported that the *CHS* siRNA production from inversely located *CHS* gene clusters is controlled by AGO5, which is one of ARUGONAUTE (AGO) proteins (Cho *et al.* 2017). AGO proteins directly bind small RNAs and constitute a component of the RNA-induced silencing complex (RISC). Mutations in the gene for AGO5 lead to the altered spatial distribution of *CHS* siRNAs in the seed coat. Genetic diversities in the genes for RISC components may also lead to the spatiotemporal production of *CHS-A* siRNAs in bicolor petunias.

Morita *et al.* (2012) also predicted that tandemly oriented *CHS-A* genes were introduced into the petunia by interspecific hybridization breeding. As each copy of the tandemly oriented *CHS-A* genes is distributed independently in the genomes of wild *Petunia* species, the tandem arrangement was formed by a chromosomal rearrangement of the *CHS-A* locus. Dedicated screening was performed using many accessions of wild *Petunia* species collected from South America; however, the same tandem structure in the *CHS-A* locus was not to detectable (Morita *et al.* 2012). Surprisingly, *P. inflata* accession S6, which was employed for whole genome sequencing (Bombarely *et al.* 2016), was found to carry a tandemly oriented *CHS-A* genes almost identical with those of Picotee and Star petunias (data not shown). It was suggested that the *CHS-A* siRNA-producing locus was introduced from a wild population of *P. inflata* (or *P. integrifolia*) into the petunia genome, whereas other loci for hypothetical trans-acting factors regulating the spatiotemporal production of *CHS-A* siRNAs were from *P. axillaris*. We consider that recent bicolor cultivar, 'Night Sky' (**Fig. 3H**), which displayed irregular white spots in the pigmented petal, also carries the tandemly oriented *CHS-A* genes as a *CHS-A* siRNA-producing locus. The identification of the regulating locus in bicolor petunia cultivars, Picotee, Star, and 'Night Sky', might hold the key to understanding mechanisms for spatiotemporal siRNA production.

## Vacuolar pH control—the proton transporters

Petunias (**Fig. 4A–4E**) and the morning glories of *I. nil* (**Fig. 4F–4H**) and *I. tricolor* are ideal model plants to investigate vacuolar pH affecting flower color. In the petunia, purplish flower buds change to reddish open flowers due to decreased pH in the vacuole. In contrast to the petunia, the vacuolar pH of morning glories increases during flower opening, and reddish buds turn to blue open flowers (**Fig. 4F**, **4G**). The pH values of petunia flowers were estimated from petal homogenates, and those of purplish buds and red flowers are around 6.3 and 5.3, respectively (Quattrocchio *et al.* 2006b). The pH values in the epidermal cells of *I. tricolor* were measured using microelectrodes, and those of reddish buds and blue flowers were 6.6 and 7.7, respectively (Yoshida *et al.* 1995). By comparing reflective spectra of the petal with absorption spectra of the homogenates in various pH solutions, the vacuolar pH of *I. nil* flowers was estimated to be around 8.0 (Yamaguchi *et al.* 2001).

The $H^+$ transporters controlling the vacuolar pH in petunia and *I. nil* flowers were uncovered by using the flower color mutants. In petunias, seven *PH* genes that affect vacuolar pH have been reported (de Vlaming *et al.* 1983, van Houwelingen *et al.* 1998). Recessive mutants of these genes show bluish flowers, due to increased vacuolar pH. Color shift due to the *ph* mutations depends on anthocyanin structure. The *ph* mutants that accumulate malvidin or petunidin derivatives show purple flowers, and those with cyanidin derivatives bloom dull gray flowers (**Fig. 4B**, Tornielli *et al.* 2009). Among the seven genes, *PH5* and *PH1* encode P-ATPases belonging to the 3A and 3B subfamilies, respectively (**Fig. 4A**, Faraco *et al.* 2014, Verweij *et al.* 2008). The P-ATPase superfamily is comprised of five subfamilies of ATP-driven membrane transporters that translocate distinct cations; each subfamily is divided into subgroups (Palmgren and Nissen 2011). The 3A subfamily ($P_{3A}$-ATPases) is comprised of $H^+$ pumps on the plasma membrane of plants, fungi, and other unicellular eukaryotes. Of the characterized $P_{3A}$-ATPases, PH5 is the only one residing on the tonoplast (all others were shown to be on the plasma membrane); it transports $H^+$ into the vacuolar lumen (Faraco *et al.* 2014, Verweij *et al.* 2008). PH1 is required for strong $H^+$ pumping activity. The 3B subfamily ($P_{3B}$-ATPase) includes $Mg^{2+}$ prokaryote pumps. However, PH1 is not considered an $Mg^{2+}$ pump, since it lacks the conserved aspartate residue that is thought to be essential for cation binding and translocation (Buch-Pedersen *et al.* 2000). PH1 can make a heteromeric complex with PH5 on the tonoplast and boosts the $H^+$ pumping activity of PH5 (**Fig. 4A**, **4C**, **4D**, Faraco *et al.* 2014). It has been proposed that the interaction of PH1 and PH5 decreases $H^+$/ATP stoichiometry hypothetically from 1.0 to 0.5 $H^+$/ATP, which enables hyperacidification (Eisenach *et al.* 2014). Recently, Faraco *et al.* (2017) showed that PH1 has a role in the fusion process of the small vacuoles and the central vacuoles in petunia petal cells. The relationship between the fusion process and vacuolar hyperacidification is still obscure; however, PH5 as well as the vacuolar hyperacidification itself is not necessary for the fusion process. *PH3*, *PH4*, and *PH6* encode transcriptional regulators that are required for the expression of *PH1* and *PH5* (Quattrocchio *et al.* 2006b, Spelt *et al.* 2002, Verweij *et al.* 2016, see below).

In *I. nil*, the *Purple* gene encoding a cation/$H^+$ exchanger, InNHX1 (**Fig. 4G**, Fukada-Tanaka *et al.* 2000), is the only known $H^+$ transporter gene controlling vacuolar pH and flower color in this species. Plant NHX proteins passively exchange $H^+$ with $K^+$ and $Na^+$, consuming the $H^+$ gradient (Bassil and Blumwald 2014). They are classified into three types: vacuolar, vesicular, and plasma membrane NHXs. InNHX1 belongs to vacuolar NHXs that mediate $H^+$ efflux from vacuolar lumen. The InNHX1-deficient *purple* (*pr*) mutant shows purple flowers due to the decreased vacuolar pH (Yamaguchi *et al.* 2001). The *pr* mutant can increase vacuolar pH partially during flower opening, and reddish-

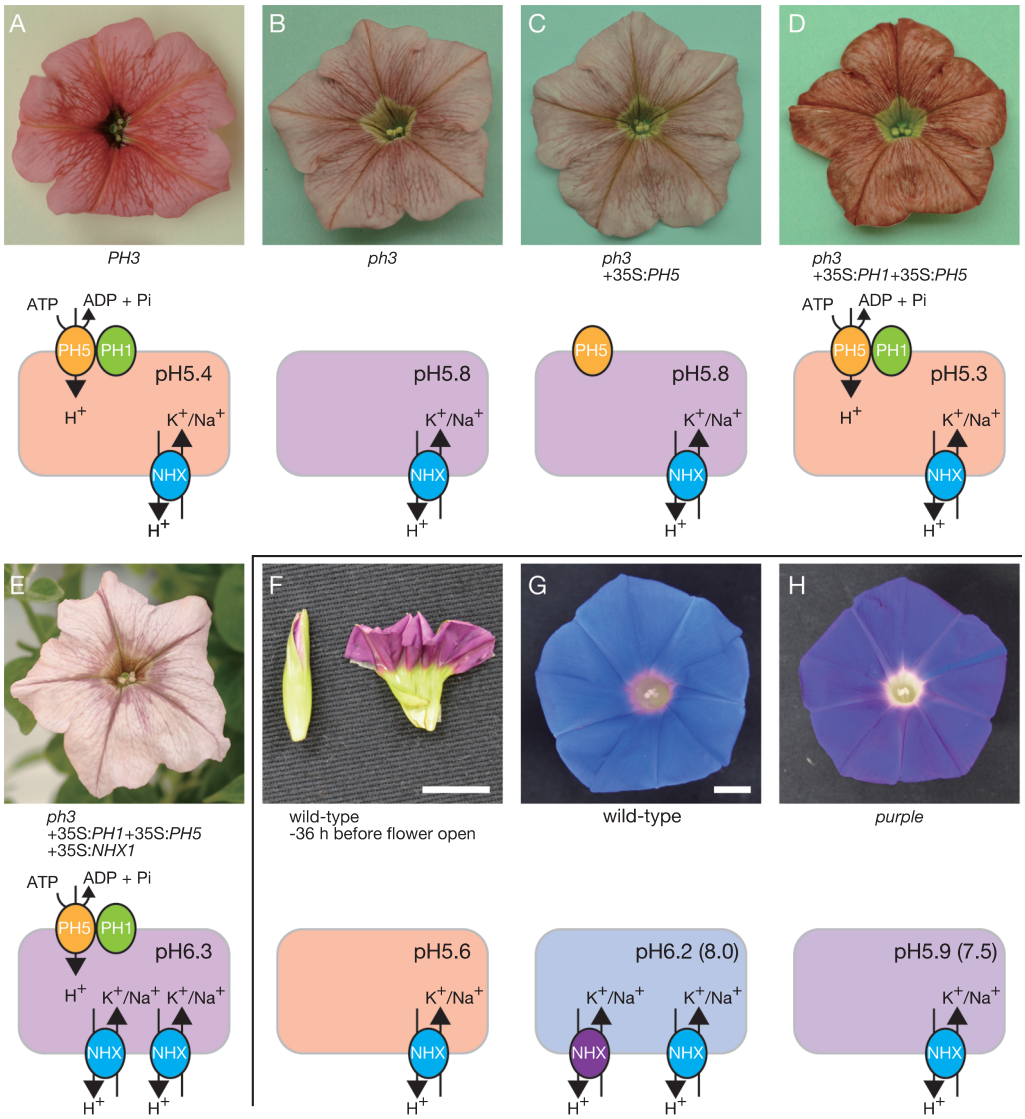


**Fig. 4.** $H^+$ transporters regulate vacuolar pH, affecting the flower color of petunias (A–E) and *I. nil* (F–H). (A) In wild-type petunias, PH3 activates *PH1* and *PH5* expression, and the heteromeric complex of two P-ATPases, PH1 and PH5, mediate hyperacidification. (B) In the *ph3* mutant, *PH1* and *PH5* are not expressed, resulting in an increase of vacuolar pH. This mutant line accumulates cyanidin derivatives that exhibit dull gray flowers. (C) PH1 is necessary for the $H^+$ pump activity of PH5, and PH5 alone cannot rescue the *ph3* phenotype. (D) The *ph3* phenotype is rescued by the co-expression of *PH1* and *PH5*. (E) The rescued phenotype in (D) is canceled by the expression of 35S:*NHX1*. NHX exchanges cations and $H^+$, resulting in an increase in vacuolar pH. (F) Flower buds of the wild-type *I. nil* show lower vacuolar pH and red petals. (G) During flower opening, flower color changes from red to blue. In the same stage, PURPLE/InNHX1 (purple circle) is accumulated and mediates vacuolar alkalization. (H) The *pr* mutant shows partial vacuolar alkalization, and red flower buds change into purple flowers. The pH values of petal homogenates are presented, and those estimated from spectra are shown in parentheses. Scale bars represent 1 cm.

purple flower buds change to purple open flowers (**Fig. 4H**). Therefore, InNHX1 is responsible for part of the vacuolar alkalization that results in flower bluing. It was suggested that other proteins, including InNHX2, take part in vacuolar alkalization and flower bluing (Ohnishi *et al.* 2005).

In *I. tricolor*, an *InNHX1* homologue, *ItNHX1*, is expressed in petal cells, and its temporal expression pattern is similar to that of *InNHX1* (Yoshida *et al.* 2005). Protoplast cells prepared from bud petal cells 7.5 hours before flower opening are able to change their color from red to blue when the cells are treated with 50 mM KCl or NaCl. Petal cells accumulate $K^+$ rather than $Na^+$ along with *ItNHX1* mRNA during flower opening (Yoshida *et al.* 2009a). From these observations, it has been thought that ItNHX1 acts as a $K^+/H^+$ exchanger in the flower petals of *I. tricolor*. The vacuolar pH in opened flower is 7.7, which seems to be higher than the general pH (around 7.2) of plant cytosol. In other words, the concentration of cytosolic $H^+$ is higher than that of vacuolar $H^+$. Therefore, we can speculate that ItNHX1, and probably InNHX1, uses $K^+$ gradient rather than $H^+$ gradient

to exchange $K^+$ and $H^+$ mediating vacuolar alkalization. In fact, tomato LeNHX2 recombinant protein reconstituted in liposomes could show $H^+$ efflux activity *in vitro* using a cation gradient in the absence of a preimposed pH gradient (Venema *et al.* 2003). The further characterization of *Ipomoea* NHX activity is essential for better understanding vacuolar alkalization mechanisms.

## Vacuolar pH control–regulatory systems of the proton transporters

In the petunia, the regulatory system's activating transcription of *PH1* and *PH5* has been well characterized. Transcriptional regulatory complexes composed of proteins that contain MYB, bHLH (basic helix-loop-helix), and WD40 (or WDR) domains (so-called MBW complexes) control epidermal cell diversity, including anthocyanin biosynthesis, trichome and root hair formation, and seed coat pigmentation in angiosperms (Koes *et al.* 2005, Lloyd *et al.* 2017, Ramsay and Glover 2005). The transcriptional regulators of the MBW complexes that involve anthocyanin biosynthesis have been isolated in both petunias and *Ipomoea* (deVetten *et al.* 1997, Morita *et al.* 2006, Park *et al.* 2004, 2007, Quattrocchio *et al.* 1999, Spelt *et al.* 2000). Mutants of the genes for transcriptional regulators show white or pale-colored flowers. Of these, AN1 and AN11 are petunia bHLH and WD40 proteins, respectively. These proteins induce vacuolar acidification through the activation of *PH1* and *PH5* transcription. The MYB protein AN2 is also involved in vacuolar acidification, and *an2* mutants show only a partial hyperacidification defect (Tornielli *et al.* 2009). *AN1* and *PH6* are two names for the same gene, and the *ph6* allele expresses truncated AN1 proteins that can activate anthocyanin pigmentation but not vacuolar acidification (Spelt *et al.* 2002). AN1 and AN11 make an MBW complex with an MYB protein of PH4 (Quattrocchio *et al.* 2006b, Spelt *et al.* 2002). The MBW complex activates the expression of the gene for PH3 of a WRKY protein (Verweij *et al.* 2016). PH3 can bind to AN11 and is necessary for the transcription of *PH5*. It is still unclear whether PH3 is a component of the MBW complex or a component of another complex that includes AN11 but not AN1 and PH4 (Lloyd *et al.* 2017).

To date, neither transcriptional regulators for *Ipomoea* NHXs nor transcriptional regulators for anthocyanin biosynthesis involving vacuolar pH alkalization have been reported. The transcriptional activation of *Ipomoea* NHXs seems to be connected with flower opening when a large influx of water and ion into petal cells and cell expansion are caused. A regulatory system for such an influx and cell expansion process may be involved in the activation of *Ipomoea* NHX. Yoshida *et al.* (2009a) suggested that *Ipomoea* NHXs contribute to the increase in vacuolar osmotic pressure through $K^+$ accumulation, inducing water influx for cell expansion growth and flower opening (**Fig. 4F**, **4G**). However, since the *pr* mutant lacking InNHX1 still shows normal cell expansion and flower opening (**Fig. 4G**), InNHX1 is not essential for such processes (Fukada-Tanaka *et al.* 2000, Pittman 2012, Yamaguchi *et al.* 2001).

## Diversity and universality of the vacuolar pH regulatory systems in flower color

Vacuolar pH regulation by $P_{3A}$-ATPase/$P_{3B}$-ATPase and NHX proteins is thought to be not specific to the flowers of petunias and *Ipomoea*, respectively. Functional *PH5* and *PH1* homologues are widely spread in angiosperms; however, independent losses of these homologues have occurred in many angiosperms (Li *et al.* 2016). We found that these homologues are absent in the *I. nil* genome (data not shown, Hoshino *et al.* 2016). *PH5* homologues were present not only in angiosperms but also in gymnosperms, and they were shown to have evolved from plasma membrane $P_{3A}$-ATPases. The constitutive expression of Arabidopsis, carnation, grape, and rose *PH5* homologues could complement the *ph5* mutation of the petunia (Appelhagen *et al.* 2015, Li *et al.* 2016). However, *PH5* homologues are absent in a number of angiosperms, even in Solanaceae plants (e.g., tomato, potato, pepper). PH1 homologues are found in some groups of bacteria and fungi. Since PH1 homologues are absent in most algae, it was suggested that several cases of gene loss and horizontal transfer events have been involved in their evolution (Li *et al.* 2016). Grape and rose *PH1* homologues could complement the petunia *ph1* mutant. Takahashi *et al.* (2013) showed that soybean mutants that exhibit purple-blue flowers have a nonsense mutation in the apparent *PH4* homologues. These observations suggest that the vacuolar hyperacidification systems, including P-ATPases as well as MBW complexes, are partially conserved among angiosperms and that some plants use the systems to create red flowers.

In contrast to the distribution of *PH5* and *PH1* homologues, vacuolar *NHXs* are ubiquitous in plants (Bassil and Blumwald 2014). Arabidopsis has two vacuolar *NHX* genes, and double mutants of the genes exhibit the acidification of vacuoles in the root cells (Bassil *et al.* 2011). *PhNHX1* of the petunia is expressed in flower petals (Yamaguchi *et al.* 2001), and PhNHX1 can reduce the pH gradient across the tonoplast (Faraco *et al.* 2014). The dull gray flower phenotype of the *ph3* mutant (**Fig. 4B**) was rescued by the co-expression of *PH1* and *PH5* (**Fig. 4D**). The rescue was canceled by the overexpression of *PhNHX1* in the rescued mutant, and the *ph3* mutant co-expressing *PH1*, *PH5*, and *PhNHX1* showed dull gray flowers like those of the *ph3* mutant (**Fig. 4E**). Interestingly, the pH value (around 6.2) of the petal homogenate of the *PH1*-, *PH5*-, and *PhNHX1*-expressed *ph3* mutant is higher than that of the *ph3* mutant (around 5.8).

## Future perspectives

In *I. nil*, flower color modification using the genome editing tool, CRISPR/Cas9, has recently been reported (Watanabe *et al.* 2017). In one of the three *DFR* genes, *DFR-B*, in the *I. nil* cv. Violet CRISPR/Cas9 induced mutations, and the flower color of the cultivar changed from white from red. The genome editing technology will be widely applied to ornamental flower breeding in the near future. Disruption of *EFP* or *3GT* genes in ornamental plants seems to be an ideal method to create pale flower varieties.

As in the case of *I. nil dk-2* mutants (**Fig. 3A**), transposons can epigenetically control gene expression in land plants. Transposons are generally distributed among angiosperms, and sometimes act as endogenous mutagens and generate floral color variations in ornamental plants. As the *dk-2* mutant, transposons integrated within promoter sequences of flower pigmentation genes might be involved in particular flower variegation in ornamental plants other than *I. nil*.

Bicolor floral traits of *P. hybrida* Picotee and Star are regulated by spatiotemporal production of siRNA molecules from tandemly orientated flower pigmentation genes. It is considered that trans-acting regulatory factors control the spatiotemporal production of siRNA molecules and the regulatory locus is introduced by interspecific hybridization breeding. Characterization of the trans-acting regulatory factors is important for understanding of the RNA-silencing mechanism in flower pigmentation patterns and utilization in breeding of ornamental plants.

The creation of blue flowers in a species with no blue variation is one of the biggest goals of plant breeders. The overexpression of vacuolar *NHX*s and the suppression of *PH5* homologues could be a useful strategy for creating blue flowers through molecular breeding. The first trial has been reported by Kasajima and Sasaki (2016). They introduced expression cassettes of *InNHX1*, *InNHX2*, and a chimera repressor of petunia *PH4* into torenia. However, neither blue flowers nor the pH increase of petal exudates was observed in transgenic plants. It is possible that the species specificity of the proteins resulted in inhibited $H^+$ transporter activities. The modification of endogenous vacuolar *NHX* genes and/or endogenous homologues of petunia *PH* genes are possible ways to overcome such species specificity problems.

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