

REVIEW

A Research Strategy to Understand the Mechanisms that Govern Flower Color Pattern Formation

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Abstract

The coloration pattern of flower tissue affects the commercial potential of floricultural plants and is also a subject of fundamental biological interest. Transposon insertion or excision and post-transcriptional gene-silencing are well-studied mechanisms involved when flower color patterns form. In this paper, I present a research strategy to understand the mechanisms that govern the formation of flower color patterns. First, I discuss the significance of flower color pattern-formation research and then go on to describe a research system in the following six sections: Observation of flower patterns, Comparison of pigment components, Gene expression analysis, Regulation of target gene expression, Genomic structure, and Factors that can change color pattern-formation. In these sections, reference is made to my own studies on the marginal picotee pattern of *Petunia* flowers. Post-transcriptional gene-silencing of the *chalcone synthase* gene is responsible for the formation of white tissue in the white marginal picotee pattern in *Petunia* flowers. The unusual genomic structure of *chalcone synthase* is probably related to the operation of position-specific post-transcriptional gene-silencing. In the colored marginal picotee pattern of *Petunia* flowers, the higher expression of *flavonol synthase* is responsible for the central white tissue formation. I also provide a research perspective from which to resolve the remaining questions.

Discipline: Horticulture

Additional key words: anthocyanins, chalcone synthase, flavones, flavonol synthase, post-transcriptional gene-silencing, transposon

Preface

Beautiful flower color patterns are a key commercial characteristic of floricultural plants. Financial chaos triggered by an extraordinary price trade of tulip bulbs of a novel flower color pattern, which were generated by a viral infection, in the Netherlands in the 17th century is a famous accident (Blunt 1950). Even now we can often find such flower color patterns in virus-infected *Tulipa* flowers (Fig. 1). Although this accident is often cited as an example of human folly, I prefer to consider it in terms of the beauty of flower color patterns. Flower color patterns comprising multiple colors can be found in many floricultural species and their cultivars. The aim of researching flower color patterns is primarily to obtain practical information to generate beautiful floricultural materials.

Pigments are color compounds whose concentration and occurrence can be visually detected, which has made



Fig. 1. Flower pattern generated by viral infection in *Tulipa*
Left, virus-infected flower. Right, normal flower.

them indispensable for developing fundamental biological concepts. Mendel's law, cytoplasmic inheritance, transposons as well as post-transcriptional gene-silencing (PTGS)

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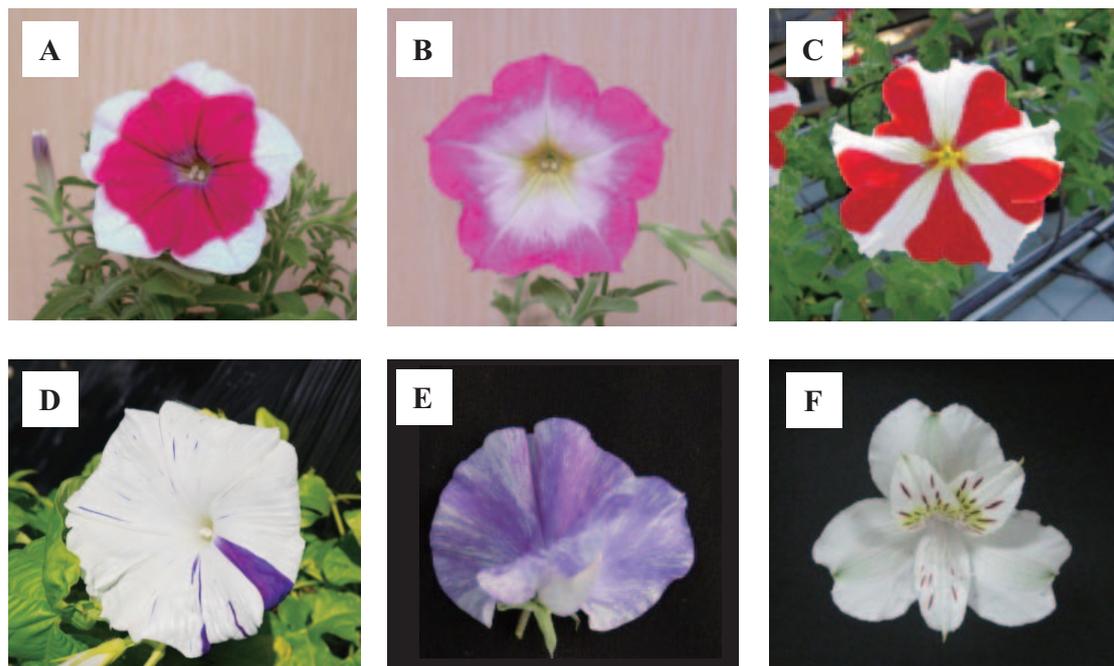


Fig. 2. Flower pattern variety

A, white marginal picotee (*Petunia*). B, colored marginal picotee (*Petunia*). C, star type (*Petunia*). D, funnel sector (*Pharbitis*). E, flaked-variegation (*Lathyrus*). F, blotch (*Alstroemeria*).

(Jorgensen 1990) were all discovered based on plant pigment studies. In many cases, a flower color pattern is understood as a form of differentiation related to variation in pigment biosynthesis ability. As a general rule, each cell of an individual organism has the same genes, so that tissue differentiation is generated by differences in transcription among cells, which is regulated endogenously and spontaneously. There are also exogenous factors such as viral infection and endogenous factors such as transposon and PTGS, which cause flower color pattern formation. The latter are probably the simplest research objects when studying differentiation mechanisms

In this paper, I will present a general procedure to elucidate the mechanisms responsible for flower color pattern formation, referring to my own studies on the marginal picotee pattern of *Petunia* flowers to explain the idea in detail (Morita et al. 2012, Saito et al. 2006, Saito et al. 2007). While pigments popularly distributed in plants are anthocyanins and carotenoids, those comprising flower color pattern are mainly anthocyanins or betalains. The flower color patterns I cover here are thus relate to transposon and PTGS targeting anthocyanin biosynthesis.

Flower pattern types

Anthocyanins are a major category of flower pigments. However, because they lack a physiologically fetal role, we can find various types of anthocyanin patterns in flowers.

Representative color patterns include the picotee type, in which different colors appear between the flower's margin and center (Figs. 2A, B); the star type, in which different colors appear along the veins (Fig. 2C); the funnel sector type, in which different colors appear from the center to the margin in different parts of the flower (Fig. 2D); the flaked-variegation type, in which numerous tiny spots are scattered through the flower (Fig. 2E); and the blotch type, in which discrete and easily countable spots appear (Fig. 2F). The color pattern-formation mechanisms have been studied in restricted species, such as *Petunia* (Hosokawa et al. 2013, Koseki et al. 2005, Morita et al. 2012, Saito et al. 2006, Saito et al. 2007, van Houwelingen et al. 1998), *Dianthus* (Itoh et al. 2002, Momose et al. 2013), *Pharbitis* (Iida et al. 2004, Inagaki et al. 1994), *Camellia* (Tateishi et al. 2010), and *Dahlia* (Ohno et al. 2011). In these species, the chemical structures and biosynthetic genes of anthocyanins have been well studied.

Research Strategy

Here I discuss general strategies to study flower color pattern-formation mechanisms in the following six sections:

1. Observation of flower patterns

The type of flower color pattern reflects its formation mechanism. Transposons are often involved in the formation of the funnel sector pattern (Fig. 3A). Cell divergence

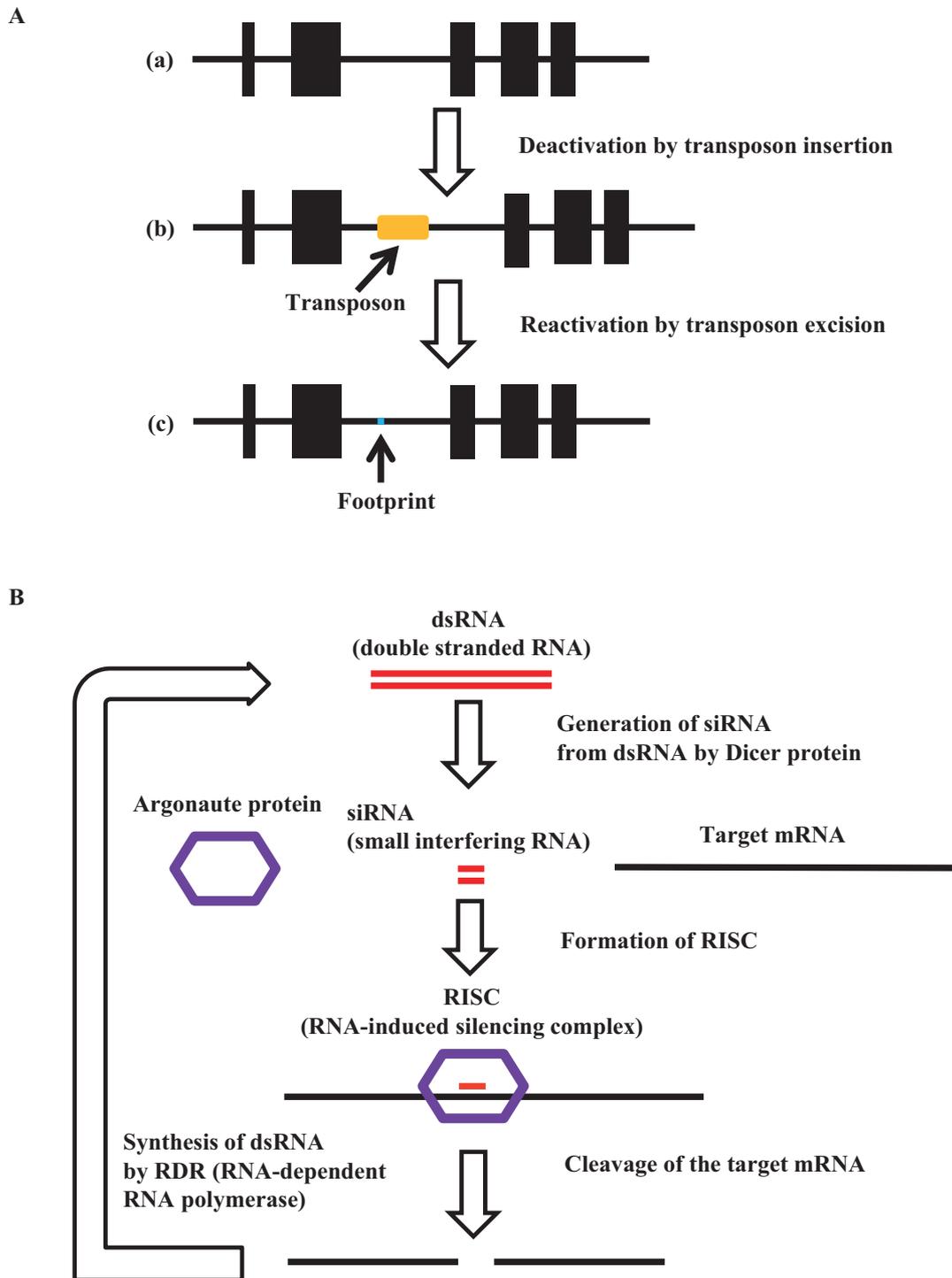


Fig. 3. Simplified schemes of transposon insertion and excision (A) and PTGS (B)

In (A), black squares indicate exon regions. a, gene encoding an active enzyme. b, a transposon-inserted gene encoding an inactive enzyme. c, a transposon-excised gene encoding the active enzyme where the footprint remains.

occurs along radial lines from the margin to the center in petals. Insertion of a transposon into a target pigment biosynthetic gene prevents the production of the active enzyme encoded by that gene, causing white tissue formation. When the transposon is excised from the altered gene, the enzyme

becomes active again and pigmentation appears. Cells that retain or lack the pigment biosynthetic activity divide along radial lines, resulting in the formation of the funnel sector pattern. Because transposon excision at an earlier petal formation stage leads to wider patterns, various sector sizes

appear depending on the timing of transposon insertion or excision (Iida et al. 2004). Patterns that constantly appear are often PTGS-related phenomenon (Fig. 3B). PTGS is a sequence-specific RNA degradation mechanism (Voinnet 2008) and is now understood as an important system involved in various plant phenomena, for example, leaf shape, lateral root formation, fertility, and flowering time (Jones-Rhoades et al. 2006). PTGS induced by introducing anthocyanin biosynthetic genes was also used to regulate anthocyanin biosynthesis (Jorgensen et al. 1996, Napoli et al. 1990, Van der Krol et al. 1990).

Microscopic observation has also provided considerable information on color pattern-formation mechanisms. In transposon-based flower color patterns, the coloration changing area often comprises clearly distinguished white and colored cells, because the pigment biosynthetic activity differs drastically among the cells. For example, Figure 4A shows a microscopic image of the funnel sector color pattern in *Pharbitis* flowers. In PTGS-based flower color patterns meanwhile, unlike those that are transposon-based, the coloration changing area generally comprises cells in which the color intensity gradually changes (Jorgensen 1995). This phenomenon was observed in areas of both types of marginal picotee patterns of *Petunia* flowers subject to changes in coloration (Fig. 4B), suggesting the involvement of PTGS in the pattern formation (Saito et al. 2007). Based on these differences, we can tentatively define the formation mechanism for a color pattern *via* microscopic observation.

2. Comparison of pigment components

Anthocyanin pigments and their biosynthetically-related compounds can be compared between tissues with different colors. In such studies, we often observe the accumulation of compounds upstream of the inhibited step in the pigment biosynthetic pathway, so that the inhibited step is detectable based on the variation in composition (Gerates et al. 1982). Among anthocyanin-related compounds, flavones, flavonol and cinnamic acid derivatives are contained in plant tissues as glycosides at similar levels to anthocyanins and their biosynthetic relationship is shown in Fig. 5. These compounds must be analyzed at various growth stages in flower tissues, from the small bud to the opened flower, to understand the changes of these components over time, since these compounds often accumulate at different stages (Saito et al. 2006, Saito et al. 2007).

These compounds can be analyzed using the same techniques as for anthocyanins. They also have distinctive absorption spectra, so we can tentatively identify the compounds using HPLC equipped with a photodiode-array-detector. These compounds can also be quantified based on their absorbance spectra. Thin-layer chromatography (TLC) is another useful analytical technique, which we can use to detect anthocyanins as color spots. Flavones and flavonols

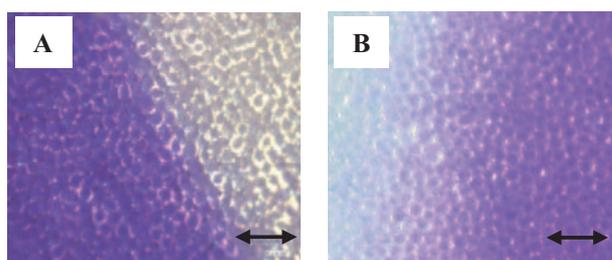


Fig. 4. Cells in the coloration changing area of the flower patterns

A, funnel sector pattern created by transposon in *Pharbitis*. The coloration changing area comprised clearly distinct white and colored cells. B, the white marginal picotee pattern caused by PTGS in *Petunia*. The coloration changing area comprised cells of gradually changing color density. The same phenomenon is observed in the *Petunia* flower with the colored marginal picotee pattern. Bars represent 100 μ m.

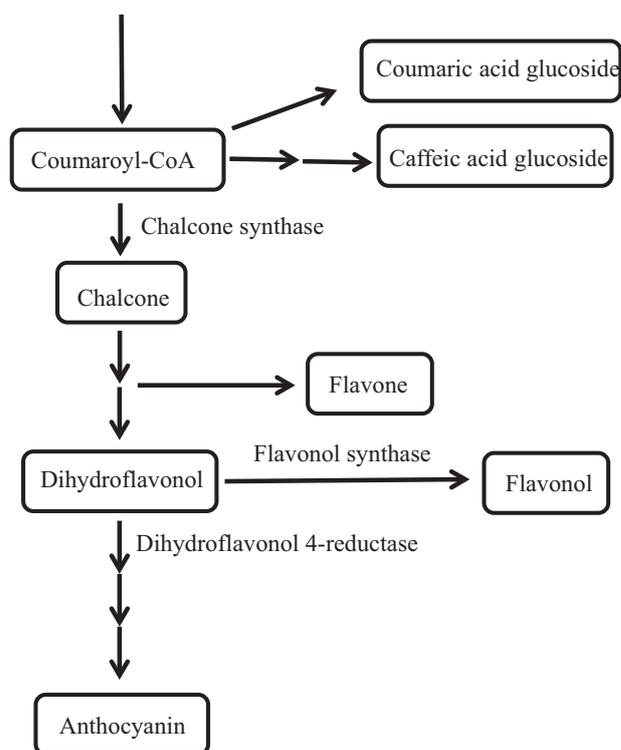


Fig. 5. Anthocyanin biosynthetic pathway and related enzymes

Chalcone synthase and *flavonol synthase* are genes responsible for the formation of white tissues of the white and colored marginal picotee patterns in *Petunia* flowers, respectively.

are generally detected as dark non-fluorescent spots, while cinnamic acid derivatives are detected as blue-purple spots in TLC under ultraviolet light. We recently developed a cross-TLC method to easily detect co-pigments, which are colorless compounds that influence color tone and density

when coexisting with anthocyanins (Shimizu-Yumoto et al. 2012).

In *Petunia* flowers with a white marginal picotee pattern, anthocyanins, flavonols, caffeic acid glucosides, and coumaric acid glucosides were detected in similar concentrations in the colored tissue (Saito et al. 2006, Saito et al. 2007). In the white tissue, concentrations of anthocyanins and flavonols decreased drastically compared with levels in the colored tissue, but caffeic and coumaric acid glucosides accumulated (Fig. 5). The specific accumulation of caffeic and coumaric acid glucosides in the white tissue was observed from early in flower growth, before pigmentation emerged, suggesting that the metabolic activity of coumaroyl-CoA (Fig. 5), which is a precursor of coumaric and caffeic acid glucosides, to chalcone, which is a precursor of flavonols and anthocyanins, decreased in the white tissue.

In *Petunia* flowers with the colored marginal picotee pattern, concentrations of anthocyanins decreased drastically but flavonols accumulated in the white central tissue (Saito et al. 2006, Saito et al. 2007) (Fig. 5). The specific accumulation of flavonols in the white tissue is observed from the middle flower growth stages, when pigmentation begins to appear, suggesting that the metabolic activity of dihydroflavonols, flavonol precursors (Fig. 5), to anthocyanin decreases in the white tissue.

3. Gene expression analysis

Major genes concerned with anthocyanin biosynthesis have been cloned from several plant species. The transcriptional levels of these genes were analyzed *via* hybridization- and PCR-based analyses. A gene whose expression level differs drastically between white and colored tissues is likely a target for pigment biosynthesis regulation. When no target gene can be detected, other regulatory mechanisms should be examined; differences in translation, modification or degradation of enzymes may also affect anthocyanin biosynthesis. Differences in transport of anthocyanin into vacuoles by glutathione-S-transferase are also possible (Larsen 2003, Sasaki et al. 2012). When the expression levels of multiple genes vary, the involvement of transcription factors that regulate the expression of anthocyanin biosynthetic genes should be examined. Otherwise, the expression level of a single gene may influence the expression of others. In any case, we must seek a rational explanation for the compositional changes.

In *Petunia* flowers with the white marginal picotee pattern, the expression of the gene encoding chalcone synthase (*CHS*) was specifically repressed, while genes encoding other anthocyanin biosynthetic enzymes were expressed at similar levels in colored and white tissues (Saito et al. 2006) (Fig. 5). This repression was synchronized with the accumulation of caffeic and coumaric acid glucosides and decreased concentrations of anthocyanins and flavonols.

These findings demonstrate that the repression of *CHS* expression leads to the formation of white tissue.

In *Petunia* flowers with the colored marginal picotee pattern, the expression levels of the genes encoding dihydroflavonol 4-reductase (*DFR*), which catabolize dihydroflavonol to anthocyanin, were similar in the colored and white tissues (Saito et al. 2006) (Fig. 5). Instead, the expression levels of the gene that encodes flavonol synthase (*FLS*), which catalyzes the catabolism of dihydroflavonol to flavonol, increased in the white tissue. The increased expression of *FLS* was synchronized with the accumulation of flavonols and decline in anthocyanin concentrations in the white tissue. These changes demonstrate that the increased expression of *FLS* inhibits anthocyanin biosynthesis and leads to the formation of white tissue. Flavonols in which glucose is bound at the 3'-position are found only in white tissue, meaning over-expression of *FLS* may change the glycosylation of flavonols.

4. Regulation of target gene expression

After identifying the target gene, the next step is to clarify the mechanism responsible for the change in the position-specific expression level. Inserting transposons into the promoter region of the target gene causes a decline in transcription activity while insertion into the coding region truncates the transcript and prevents the enzyme from functioning (Itoh et al. 2002) (Fig. 3A). When the target gene sequence remains unchanged between white and colored tissues, the mRNA of the target gene could be degraded by PTGS operation.

When PTGS operates, similar transcription activity of the target gene could be detected between white and colored tissues. Run-on assays using isolated nuclei (Koseki et al. 2005) or analysis of pre-mRNA levels (Morita et al. 2012) are performed to evaluate the transcription activity. PTGS operation will involve an increased concentration of small interfering RNA (siRNA), which is homologous with the target gene, specifically in the white tissue (Fig. 3B). This phenomenon was detected in the white marginal picotee of *Petunia* flowers (Morita et al. 2012). The siRNA detected in the white tissue mainly comprised 21-nucleotide RNAs, known to guide the cleavage and degradation of mRNA of highly homologous genes, and re-generated from the degraded mRNA. The siRNA detected in the white tissue was derived from both sense and antisense sequences of the exon region, indicating that PTGS operating in this tissue is involved in generating double-stranded RNA of *CHS*. Degradation of *CHS* mRNA prevents synthesis *CHS* and compositional change in anthocyanins and related compounds, resulting in the formation of white tissue in the white marginal picotee *Petunia* flower.

The regulation mechanism to increase the expression levels of *FLS* in the white central tissue of *Petunia* flowers

with the colored marginal picotee pattern has not been studied. This phenomenon can be understood as repression of *FLS* in colored marginal tissue. We speculate that PTGS of *FLS* operates within the colored marginal tissue of this cultivar.

5. Genomic structure

The genomic structure of a target gene should be elucidated to further understand the color pattern-formation mechanisms. The length of the targeted gene increases as a result of transposon insertion (Fig. 3A). Even after excision of the transposon, a footprint comprising a small number of nucleotides often remains in the gene sequence as evidence of the previous presence of a transposon. Both insertion and excision of transposons with a remaining footprint deactivate the target gene.

The formation of double-stranded RNA is a PTGS process (Fig. 3B). The gene introduced in transgenic plants becomes the target of double-stranded RNA formation due to the operation of an RNA-dependent RNA polymerase. In PTGS operating non-transgenic plants, an inverted repeat of the target gene is often found, which probably causes double-stranded RNA of the gene to form (Della Vedova et al. 2005, Kusaba et al. 2003, Senda et al. 2004). There is the *CHS* gene family in *Petunia* plants, among which *CHSA* is responsible for anthocyanin biosynthesis in flower tissue (Koes et al. 1989). All *Petunia* plants with the white marginal picotee flowers had two *CHSA* genes forming a tandem-repeat structure, suggesting that these *CHSAs* are required for the PTGS position-specific operation in the margin of *Petunia* flowers (Morita et al. 2012). Because the tandem-repeat structure cannot form double-stranded structures alone, the PTGS mechanism of *CHSA* is of interest. We note that the genomic structure of *FLS* in *Petunia* flowers with colored marginal picotee pattern has not been studied.

6. Factors that can change color pattern formation

Some plants change their flower color pattern according to growth conditions, e.g. the flower color patterns of some *Petunia* cultivars change in response to light intensity or temperature (Griesbach et al. 2007, Harder 1938). Clear changes in flower color pattern in response to temperature are observed in *Eustoma* (Fukuta & Nakayama 2008). In flowers of the 'Magic Samba' *Petunia* cultivar, the white area in the white marginal picotee pattern increases due to a phosphate deficiency (Hosokawa et al. 2013). It is important to note that the regulation of color density (i.e. the pigment concentration) and the location where the coloration develops (i.e. where anthocyanin is biosynthesized) are essentially different; these changes sometimes do not appear together (Fukuta & Nakayama 2008).

Perspectives

Recent studies of flower color patterns in several plant species indicate that PTGS targeting *CHS* is involved in color pattern formation in many of them (Koseki et al. 2005, Morita et al. 2012, Ohno et al. 2011, Tateishi et al. 2010). However, one profound question, still unresolved, is what determines the position of the flowers in which PTGS operates? As mentioned in the preface, the location where PTGS operates is likely to be endogenously and spontaneously determined. Furthermore, the location determination mechanism appears to be regulated by several environmental factors. I am currently screening chemical compounds that can change the white marginal picotee pattern of *Petunia* flowers to understand the position-specific mechanism of PTGS. Developing a technique for regulating PTGS-based flower color patterns would boost the horticultural industry and also help resolve fundamental questions about tissue differentiation mechanisms.

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