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Review

Transcriptional regulators of flavonoid biosynthesis and their application to flower color modification in Japanese gentians

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Abstract Japanese cultivated gentians (*Gentiana triflora*, *G. scabra* and their hybrids), some of the most important ornamental flowers in Japan, have vivid blue flowers that accumulate polyacylated anthocyanins such as gentiodelphin. To breed attractive flower colors in Japanese gentians, our research group has been studying the molecular mechanisms that control flower pigmentation. Flavonoids, including anthocyanins, are widely distributed in the plant kingdom and are found in almost all plant organs. Along with longstanding genetic and molecular biological analyses of flavonoid biosynthesis, recent studies have revealed that transcription activators and repressors are involved in sophisticated control of temporal and spatial flavonoid accumulation in various plant organs. In this review, we summarize recent research on the transcriptional regulation of flavonoid biosynthesis in flowers, with a special focus on our findings using Japanese gentians. We also introduce and discuss the potential application of these transcription factor genes as novel tools to engineer flower color intensity and patterns in floricultural plants.

Key words: Flavonoid, flower color, Japanese gentian, MYB, transcription factor.

Introduction

The genus *Gentiana*, belonging to the gentian family (Gentianaceae), is widely distributed all over the world except for Africa (Ho and Liu 2001; Kohlein 1991). It includes about 400 species, some of which are important as traditional medicinal plants. *Gentiana* species are also valuable for ornamental purposes because of their attractive flower colors, especially their vivid blue-violet pigmentation. In fact, the term "gentian blue" is derived from gentian flower colors and describes a purplish blue color (Figure 1A). In Japan, "Rindo", Japanese gentians, are some of the most popular blue flowers and are economically important in the market for cut flowers or potted flowering plants from early summer to late autumn. Two gentian species, *G. triflora* Pall var. *japonica* and *G. scabra* Bunge var. *buergeri* have been used as parents for breeding and successive cultivation has been established since the 1960s in Japan (Kodama 2006; Yoshiike 1992). Typically, the flower colors of gentian cultivars are blue to purplish, but occasionally pink or white-flowered cultivars have been bred using natural mutations. Recently, Japanese gentians have been introduced to foreign countries such as Chile and New Zealand, and come to be recognized internationally. However, variation in flower color, morphological and physiological characters is scarce. Thus, we have attempted to develop molecular breeding techniques, including genetic engineering and tissue-culture, to create novel gentians with useful traits such as novel flower colors, plant shape and disease resistance (Nishihara et al. 2008).

Flower coloration is mainly due to the accumulation of three major classes of plant pigments: flavonoids, carotenoids, and betalains. Depending on plant species, these chemical compounds are also responsible for pigmentation of various plant organs, such as fruits, seeds, stems, and roots (Delgado-Vargas et al. 2000; Gandia-Herrero and Garcia-Carmona 2013; Grotewold 2006a; Tanaka et al. 2008). These pigments usually accumulate in certain defined tissues during plant development in a programmed manner, and can sometimes be induced in response to various biotic or abiotic environmental stresses. Among these plant pigments, flavonoids are widely distributed in the plant kingdom and are known to have various biological functions, such as UV protection, antimicrobial activities, antioxidant properties, and chemical regulation of plant development (Falcone Ferreyra et al. 2012; Koes et al. 2005; Taylor and Grotewold 2005;

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Winkel-Shirley 2002). These plant natural products have become targets of genetic engineering because of their potential beneficial effects on human health (Falcone Ferreyra et al. 2012; Wang et al. 2011). Because flavonoid pigments are also frequently associated with a wide range of colors, such as yellow, red, purple, and blue, in various organs including fruits and flowers, they have long been subjects of interest to many plant scientists (Grotewold 2006a, b; Koes et al. 2005). In most cases, the colors are attributed to the anthocyanins, one of the major subgroups of flavonoids, with more than 500 anthocyanins currently characterized (Veitch and Grayer 2011). The accumulation of chalcones and aurones also contributes to yellow flower coloration. Different subclasses of flavonoids, including flavonols and flavones, are also known as copigmentation cofactors (so-called 'copigments'). The presence of these copigments together with anthocyanins affect color hue and intensity in several flowers (Martens and Mithofer 2005; Ono et al. 2010; Saito et al. 2011).

The study of flavonoid chemistry has a long history, with recent studies having revealed the flavonoid biosynthetic pathways in higher plants at the molecular level. The enzymatic genes required for flavonoid biosynthesis (also known as structural genes) have been proved to be transcriptionally regulated. The coordination of these transcription factor genes is essential for the sophisticated regulation of flavonoid biosynthesis in vegetative organs, including leaves, stems, and roots, and reproductive organs such as seeds, fruits, and flowers (Hichri et al. 2011; Petroni and Tonelli 2011). In recent years, we have also attempted to characterize crucial transcription factor genes regulating flower pigmentation in Japanese gentians possessing vivid blue flowers that accumulate anthocyanins and flavones. Through our characterization of the flower pigmentation mechanism, we found that these transcription factor genes are useful for modulating flavonoid biosynthesis in flowers and can be applied in genetic engineering to alter the flower color intensity and patterning of gentians and heterologous plants. Here, we summarize current knowledge on the sophisticated regulation of flavonoid biosynthesis in flower organs, focusing on Japanese gentians. We also provide some examples of flower color modification using gentian transcription factors and discuss the potential usage of transcriptional regulation to generate novel flowers in floricultural plants.

The flavonoid biosynthetic pathway in Japanese gentian flowers

The blue color of Japanese gentian flowers arises from gentiodelphin, the anthocyanin characteristic of *Gentiana* species of the Asia region. Gentiodelphin is composed of a delphinidin chromophore, three glucoses, and two caffeoyl moieties, and is formally known as delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-caffeoyl-*β*-D-glucoside)-3′-*O*-(6-*O*-caffeoyl-*β*-D-glucoside) as shown in Figure 1B (Goto et al. 1982). Of the two molecules of caffeic acid, the caffeic moiety at the 3′-position is more important for blue pigmentation (Yoshida et al. 2000). In pink-flowered gentian, cyanidin derivatives designated as gentiocyanins A, B, and C have also been reported (Hosokawa et al. 1995). The interspecies hybrid cultivar 'Albireo' (*G. triflora*×*G. scabra*) has also been studied and found to contain seven acylated anthocyanins, namely gentiodelphin, albireodelphins A–E, and gentiocyanin A (Hosokawa et al. 1997). Among these anthocyanins, gentiodelphin has the most complex structure and accumulates abundantly in most blue-flowered gentians. Flavonoids are needed as co-pigments and/or metal ions for blue flower coloration in several plant species, such as cornflower (*Centaurea cyanus*), Asiatic dayflower (*Commelina communis*), and Himalayan blue poppy (*Meconopsis grandis*) (Yoshida et al. 2009). The accumulation of flavone derivatives has been reported in Japanese gentian flowers; in addition, *C*-glycosylflavones

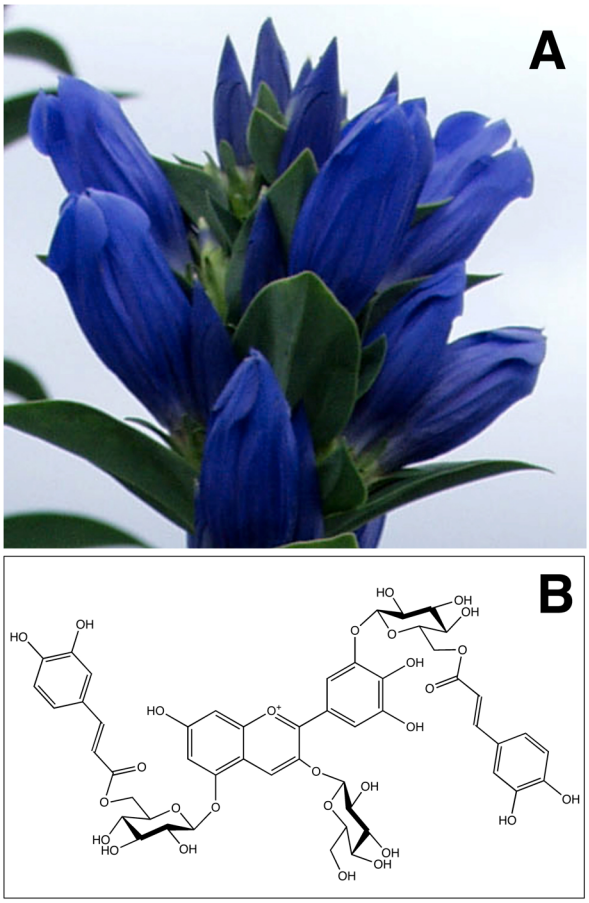

Figure 1. Typical flowers of Japanese cultivated gentian and the chemical structure of gentiodelphin. (A) Flowers of a gentian cultivar (*G. triflora* cv. Iwate). (B) Representation of the major floral anthocyanin, gentiodelphin, in blue-flowered gentian.

have been detected in several gentian species (Jankovic et al. 2009; Lin et al. 1997; Nakatsuka et al. 2012). In gentian, however, the contribution of these flavones to flower color remains to be clarified. Other biological functions of flavones in gentian are also still unclear.

The flavonoid/anthocyanin biosynthetic pathway is one of the most well-studied metabolic pathways (for a review, see Tanaka et al. 2008). Anthocyanin aglycones (anthocyanidins) are commonly synthesized via ten enzymatic reactions from the amino acid phenylalanine. Different types of anthocyanin molecules are formed by various combinations of the resulting aglycones with sugars and organic acids (Yoshida et al. 2009). Gentiodelphin is generated from delphinidin aglycone by three glucosylating and two caffeoyl-transferring reactions. After production of delphinidin aglycone, the 3-position of delphinidin is first glucosylated by UDP-glucose dependent glucosyltransferase (UGT) (Tanaka et al. 1996) followed by glucosylation at the 5-position by the other UGT (Nakatsuka et al. 2008b). Two subsequent routes have been proposed. In one route, 3′-glucosylation occurs first; in the other, glucosylation occurs after acylation of the glucose in the 5-position (Fukuchi-Mizutani et al. 2003). Both routes are possible for gentiodelphin synthesis. The genes that encode the enzymes catalyzing each anthocyanin modification step have been identified and their corresponding enzymes have been characterized (Nakatsuka et al. 2008b; Nishihara et al. 2008). The glucosylations at each 3-, 5-, and 3′-position are mediated by different UGTs (Fukuchi-Mizutani et al. 2003; Nakatsuka et al. 2008b; Tanaka et al. 1996). Acyl-CoA dependent anthocyanin 5-aromatic acyltransferase (A5AT) was first identified as the enzyme responsible for the acyl transfer to the glucosyl group at the 5-position (Fujiwara et al. 1997; Fujiwara et al. 1998). This enzyme was later revealed to also catalyze the 3′-acylation of anthocyanin (Mizutani et al. 2006). This result implies that both acylations are carried out during gentiodelphin biosynthesis by the same enzyme, which was accordingly renamed as anthocyanin 5,3′-aromatic acyltransferase (A5/3′AT). Recently, vacuolar-type glucosyltransferases (GTs) and ATs, which utilize acyl-glucose as the glucosyl and acyl donors, respectively, have been identified to be involved in anthocyanin modification steps in several species, including Arabidopsis, carnation, agapanthus, and delphinium (Matsuba et al. 2010; Miyahara et al. 2013; Miyahara et al. 2012; Nishizaki et al. 2013). These GTs and ATs belong to a completely distinct protein family of cytosolic UGTs and acyl-CoA dependent ATs. However, no enzymatic reaction steps in gentiodelphin biosynthesis, as described above, seem to be mediated by such enzymes. With respect to flavone biosynthesis, two genes responsible for biosynthesis of flavone aglycones apigenin and luteolin, namely flavone synthase (*FNSII*) and flavonoid 3′-hydroxylase (*F3′H*), have been characterized in Japanese gentian (Nakatsuka et al. 2005). Subsequent steps involved in flavone modification remain unknown, however, and the steps involved in flavonoid transport and accumulation have also not been elucidated in gentian.

Transcription factors responsible for anthocyanin biosynthesis in flowers

Flavonoid enzymatic genes can be divided into two groups: early and late biosynthetic genes (Pelletier et al. 1997; Quattrocchio et al. 1993). Early biosynthetic genes constitute enzymatic genes involved in flavonol, flavone, and phlobaphene biosynthetic pathways, whereas late biosynthetic genes comprise those involved in anthocyanin and proanthocyanidin biosynthesis. The individual gene sets categorized into the two groups differ among plant species (Pelletier et al. 1997).

Most flavonoid biosynthetic genes are regulated by transcription factor genes belonging to R2R3MYB, basic helix-loop-helix (bHLH), and WD-repeat (WDR) protein families (Baudry et al. 2004; Broun 2005). Previous research has revealed that late flavonoid biosynthetic genes, specific for biosynthesis of anthocyanins, proanthocyanidins, and condensed tannins, are regulated by a ternary complex (MYB-bHLH-WDR) composed of these protein types (Baudry et al. 2004; Broun 2005; Ramsay and Glover 2005; Xu et al. 2013). In Arabidopsis, for example, the TRANSPARENT TESTA2 (TT2, R2R3MYB)-TT8 (bHLH1)-TRANSPARENT TESTA GLABRA1 (TTG1, WDR) complex activates the expression of *DFR*, *ANS*, *BANYULS*, and *TT19* genes involved in proanthocyanidin biosynthesis (Xu et al. 2014), whereas the PRODUCTION OF ANTHOCYANIN PIGMENTATION1 (PAP1)-TT8/GLABRA3 (GL3)-TTG1 complex activates genes involved in anthocyanin biosynthesis (Gonzalez et al. 2008; Tohge et al. 2005). Three other R2R3MYB proteins, PAP2, MYB113, and MYB114, can also form a transcription complex with TT8/GL3-TTG1 involved in anthocyanin biosynthesis (Gonzalez et al. 2008). In addition to MYB-bHLH-WDR complexes, the B_{sister} MADS-box protein TT16, which is closely related to *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), has a dual regulatory function in both proanthocyanidin accumulation and endothelial development in Arabidopsis and *Brassica napus* (Chen et al. 2013; Nesi et al. 2002). During light- and sugar-induced anthocyanin accumulation, the bZIP protein HY5 binds directly to the promoter region of anthocyanin structural genes (Shin et al. 2007) and activates anthocyanin biosynthesis via PAP1 (Shin et al. 2013).

In the case of floral organs, transcription factors

regulating flower pigmentation have been exclusively identified in petunia and snapdragon (Koes et al. 2005; Schwinn et al. 2006). Regulatory genes have been also characterized in several ornamental plant species, including Japanese morning glory (Morita et al. 2006), gerbera (Elomaa et al. 1998; Elomaa et al. 2003), torenia (Nishijima et al. 2013), *Mimulus aurantiacus* (Streisfeld et al. 2013), and peach (Uematsu et al. 2014). Several reports have appeared on the regulation of floral anthocyanin pigmentation in non-ornamental plants, such as soybean (Takahashi et al. 2013), kiwifruit (Fraser et al. 2013), and tobacco (Pattanaik et al. 2010). Little is known regarding the regulation of the early flavonoid biosynthetic pathway in flowers. We introduce the case of gentian flower pigmentation in the following sections.

Transcriptional regulators of anthocyanin biosynthesis in gentian flowers: late flavonoid biosynthesis

As described above, flavonoid biosynthetic structural genes are classified as early or late biosynthetic genes, which correspond in gentian flowers to flavone and gentiodelphin biosynthetic genes, respectively (Nakatsuka et al. 2005).

In gentian, temporal expressions of *GtMYB3* and *GtbHLH1* are well correlated with those of late biosynthetic genes (Nakatsuka et al. 2008a). On the other hand, *G. triflora* WDR ortholog 1 (*GtWDR1*) is expressed throughout the entire plant (our unpublished data). A yeast two-hybrid analysis has actually confirmed a protein–protein interaction between GtMYB3 and GtbHLH1, while a transient expression assay has demonstrated that cotransformation of GtMYB3 and GtbHLH1 can enhance the promoter activities of the late anthocyanin biosynthetic genes *GtA5/3′AT* and *GtF3′5′H*

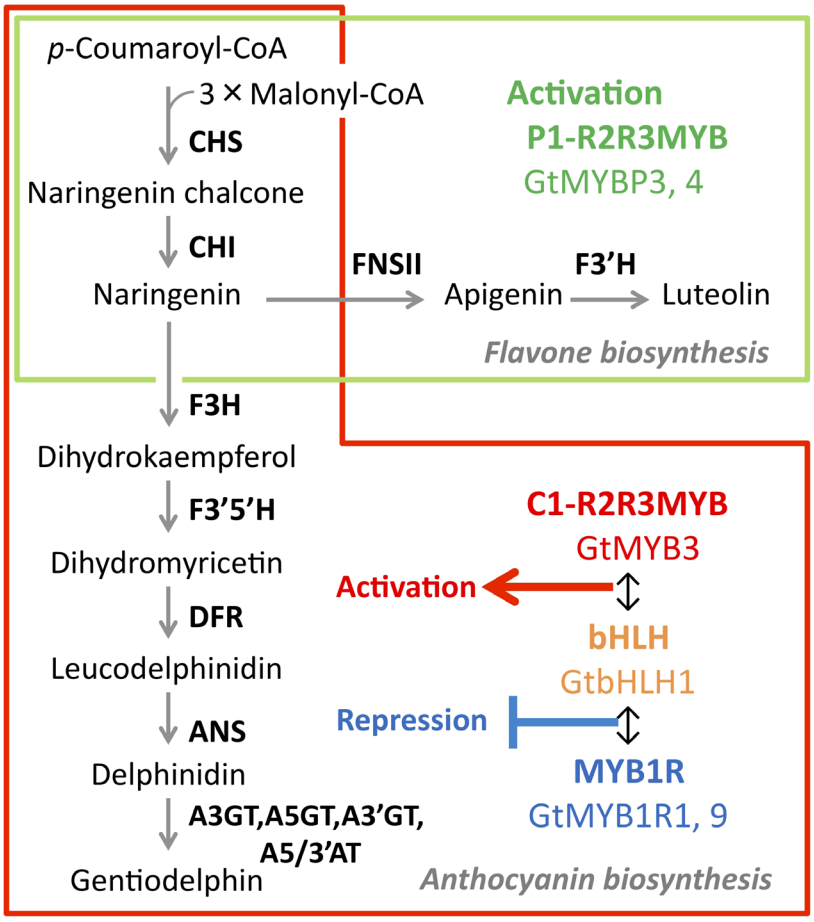

Figure 2. Diagrammatic representation of transcriptional regulation of flavonoid biosynthesis in gentian flowers. The biosynthetic pathway can be divided into flavone biosynthesis (early flavonoid biosynthesis) and anthocyanin biosynthesis (late flavonoid biosynthesis) in gentian. Abbreviations are as follows: CHS, chalcone synthase; CHI, chalcone isomerase; FNSII, flavone synthase II; F3′H, flavonoid 3′-hydroxylase; F3H, flavanone 3-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; A3GT, UDP-glucose:anthocyanin 3-*O*-glucosyltransferase; A5GT, UDP-glucose:anthocyanin 5-*O*-glucosyltransferase; A3′GT, UDP-glucose:antocyanidin 3-*O*-glucosyltransferase; A5/3′AT, anthocyanin 5,3′ -aromatic acyltransferase.

(Nakatsuka et al. 2008a). In addition, the white-flowered cultivar 'Polarno-White' has been characterized as a *GtMYB3*-deficient mutant derived from the insertion of a transposable element into the coding region of the *GtMYB3* gene. Furthermore, this gene undoubtedly contributes to anthocyanin pigmentation in gentian flowers, as evidenced by the fact that other white-flowered gentian cultivars/lines also contain mutations, such as In/Dels, in the *GtMYB3* gene (Nakatsuka et al. 2011a) and that suppression of the *GtMYB3* gene by a chimeric repressor can reduce flower pigmentation (see below). Yeast two-hybrid analysis showed that GtMYB3 interacted strongly with both GtbHLH1 and GtWDR1 (Nakatsuka et al. 2008, unpublished data). We therefore speculate that the GtMYB3/GtbHLH1/GtWDR1 complex controls gentiodelphin biosynthesis in gentian petals as well as anthocyanin biosynthesis in flower petals of other ornamental plants (Figure 2). Nevertheless, no mutants of either GtbHLH1 or GtWDR1 have been identified in gentian thus far; the detailed functions of these transcription factors in combination with GtMYB3 remain to be studied. Anthocyanin pigmentation induced by stress conditions such as cold or senescence is sometimes observed in gentian flowers, suggesting that there might be other transcription factors regulating such stress-induced pigmentations. The elucidation of other transcription factors is necessary to fully understand the molecular mechanism underlying anthocyanin pigmentation in gentian flowers.

Floral pigmentation intensity and patterning in snapdragon are also regulated by MYB transcription factor genes (Schwinn et al. 2006; Shang et al. 2011). A few novel R2R3MYB genes have recently been characterized in petunia: DEEP PURPLE (DPL) regulating vein-associated anthocyanin pigmentation in flower tubes and PURPLE HAZE (PHZ) determining light-induced anthocyanin accumulation on exposed petal surfaces (bud-blush) (Albert et al. 2011). In Asiatic hybrid lilies, LhMYB6 and LhMYB12 together with LhbHLH2 regulate anthocyanin pigmentation in specific organs (Yamagishi et al. 2010). LhMYB6 was first reported to correlate with anthocyanin spots in tepals (Yamagishi et al. 2010), but LhMYB12-Lat, an another allele of *LhMYB12*, has also been shown to be involved in splatter-type spot formation in flower tepals (Yamagishi et al. 2014). Japanese gentians might also have other transcription factor genes regulating color intensity and patterning, a point that should be considered as a future research theme.

Transcriptional regulators of flavone biosynthesis in gentian flowers: Early flavonoid biosynthesis

Flavonoid compounds synthesized in early biosynthetic steps differ depending on plant species, and may include flavonols (Arabidopsis, petunia, maize, and lisianthus), flavones (snapdragon, torenia, and maize), isoflavones (legumes), and phlobaphenes (maize and sorghum). Flavone biosynthesis categorized into the early biosynthetic pathway is thus thought to be regulated by different transcription factor(s) than those controlling gentiodelphin biosynthesis. Transcription factors involved in the early flavonoid biosynthetic pathway have been reported in several plant species, including Arabidopsis AtMYB11/12/111 (Mehrtens et al. 2005; Stracke et al. 2007), maize ZmP1 (Grotewold et al. 1994), grape VvMYBF1 (Czemmel et al. 2009), and tomato SlMYB12 (Ballester et al. 2010). *GmMYB12B2*, which is expressed in tissues that synthesize isoflavonoids, has also recently been isolated and characterized in soybean (Li et al. 2013). All these transcription factors belong to R2R3MYB subgroup 7 (Dubos et al. 2010; Stracke et al. 2001). Unlike R2R3MYB subgroups 5 and 6 involved in anthocyanin and proanthocyanidin biosyntheses, the activation of these R2R3MYB proteins does not depend on an interaction with bHLH proteins (Grotewold et al. 1994). AtMYB11/12/111 transcription factors can activate the expression of flavonol biosynthetic genes, including *CHS*, *CHI*, *F3H*, and *FLS1* (Mehrtens et al. 2005; Stracke et al. 2007). On the other hand, ZmP1 regulates phlobaphene biosynthesis to activate the transcription of the flavanone reductase (*FNR*, *A1*) gene in parallel with *C*-glycosyl flavone accumulation (Cocciolone et al. 2005; Grotewold et al. 1994). In one of our studies, we isolated two R2R3MYB subgroup 7 genes, termed *GtMYBP3* and *GtMYBP4*, from gentian petals (Nakatsuka et al. 2012). *GtMYBP3* and *GtMYBP4* transcripts were detected at the early flower development stages prior to pigmentation, and correlated well with the expression profiles of flavone biosynthetic genes (*FNSII* and *F3′H*). A transient expression assay showed that *GtMYBP3* and *GtMYBP4* enhanced the promoter activities of early biosynthetic genes of gentian flavonoid biosynthesis, but not those of late biosynthetic genes. Overexpression of *GtMYBP3* or *GtMYBP4* resulted in increased flavonol but not anthocyanin accumulation in Arabidopsis seedlings. Moreover, when these two genes were transformed into tobacco, the transgenic plants showed reduced anthocyanin and increased flavonol accumulation, resulting in reduced flower coloration (Nakatsuka et al. 2012). These findings suggest that *GtMYBP3* and *GtMYBP4*, the first characterized R2R3MYB subgroup 7 orthologs in plant petal organs, are the transcription factors involved in early flavonoid biosynthesis in gentian petals (Figure 2). Although our results strongly suggest that *GtMYBP3* and *GtMYBP4* are involved in the regulation of early flavonoid biosynthesis of gentian flowers, these genes have not definitively been proven to be the actual regulators of flavone biosynthesis.

Further elucidation is necessary to gain insights into the functions of these genes in gentian petals.

Negative regulators of anthocyanin biosynthesis in gentian flowers

In addition to the above-described positive transcription factors of flavonoid biosynthesis in gentian flowers, our recent research has revealed the existence of negative transcription factors in gentiodelphin biosynthesis (Nakatsuka et al. 2013). Arabidopsis AtMYBL2, petunia PhMYB27, and strawberry FaMYB1 have been reported as negative regulators of anthocyanin biosynthesis, and contain EAR and L2R repressor motifs in their C-terminal regions (Aharoni et al. 2001; Albert et al. 2014; Matsui et al. 2008). AtMYBL2 protein interacts with TT8 protein to form the AtMYBL2-TT8-TTG1 complex (Matsui et al. 2008). Similarly, jasmonate ZIM-domain (JAZ) protein interacts with the PAP1-TT8/GL3-TTG1 complex and represses anthocyanin accumulation (Qi et al. 2011). PhMYBx (R3-MYB) is also proposed to act as an inhibitor of anthocyanin biosynthesis in vegetative tissues of petunia (Albert et al. 2014; Albert et al. 2011). We have also isolated two single-repeat R3MYB transcription factors, GtMYB1R1 and GtMYB1R9, from a gentian petal cDNA library. In contrast to AtMYBL2, PhMYB27, and FaMYB1, GtMYB1R1 and GtMYB1R9 do not contain EAR and L2R repressor motifs within their amino acid sequences (Nakatsuka et al. 2013). Both *GtMYB1R1* and *GtMYB1R9* are preferentially expressed in petal tissues. In our study, GtMYB1R1 expression was also slightly detected in stems, pistils, and especially leaves, whereas the *GtMYB1R9* transcript was barely detected in any tissues except for petals. Interestingly, the overexpression of either *GtMYB1R1* or *GtMYB1R9* in transgenic tobacco plants led to reduced anthocyanin accumulation, resulting in white flower colors. These transgenic tobacco plants had significantly decreased *ANS* and *DFR* transcripts among anthocyanin biosynthetic genes. The NtAN2 transcription factor regulates anthocyanin biosynthesis in tobacco plants (Pattanaik et al. 2010). The expression level of *NtAN2* did not significantly change between control and transgenic plants, indicating that this native MYB transcription factor was not responsible for the anthocyanin reduction in transgenic tobacco flowers. Our yeast two-hybrid analysis showed that GtMYB1R1 and GtMYB1R9 form a transcription factor complex interacting with GtbHLH1 (Nakatsuka et al. 2013). The transient expression assay also showed that activation of the gentian *DFR* promoter by coexpression of *GtMYB3* and *GtbHLH1* was significantly suppressed by the addition of *GtMYB1R1* or *GtMYB1R9*. We therefore propose that these proteins regulate the flavonoid biosynthetic pathway by acting as antagonistic transcription factors (Figure 2). Heterologous expression of *AtCPC*, a single MYB transcription factor regulating trichome and hairy root development, has been found to induce suppression of anthocyanin accumulation in tobacco flowers and to alter trichome and hairy root density (Zhang et al. 2009). In addition to transcription factors with repressor motifs, such as AtMYBL2 and FaMYB1 (Aharoni et al. 2001; Matsui et al. 2008), GtBYB1R1 and GtMYB1R9 lacking such motifs are likely to work as antagonistic transcription factors in anthocyanin pigmentation in petals. It is unknown, however, whether GtMYB1R1 and GtMYB1R9 actually have the same function to modulate anthocyanin pigmentation in gentian flowers. We are currently producing transgenic gentians to investigate the effects of up- and down-regulation of these genes. More recently, transcription repressors regulating anthocyanin pigmentation have been comprehensively studied in petunia (Albert et al. 2014). Under non-inductive anthocyanin pigmentation conditions, the PhMYB27 repressor is associated with a potential double-lockdown mechanism for reducing anthocyanin pigmentation that prevents the formation of an MBW complex and converts the MBW activation complex into a repressive form. Under inductive conditions, the PhMYBx repressor seems to move between cells via diffusion trough plasmodesmata, and then depletes bHLH protein by a passive repressive mechanism (so-called squelching) (Albert et al. 2014). The AtCPC protein also moves from hairless cells to hairy cells and induces root hair formation (Wada et al. 2002). A sophisticated braking system to avoid extra pigmentation or to allow partial pigmentation might also exist in gentian flowers to attract pollinators. To fully elucidate the flavonoid biosynthetic pathway in gentian flowers, further studies are necessary to address how and when individual regulatory genes are expressed.

Application of transcription factors to genetic engineering of flower pigmentation

In the course of our analyses of gentian transcription factor genes, we produced several lines of transgenic plants, including those of tobacco and gentian. Transgenic tobacco and gentian flowers displaying typical changes in pigmentation levels and patterning are shown in Figure 3. Production of novel flower colors by genetic engineering has been successfully applied to many ornamental flowers (Nishihara and Nakatsuka 2011; Tanaka et al. 2010), but has been exclusively performed by introduction of flavonoid biosynthesis structural genes with the aim of accumulating non-native pigments in target flowers. Although manipulation of transcription factor genes cannot be similarly used to accumulate novel pigments from different species, the approach still

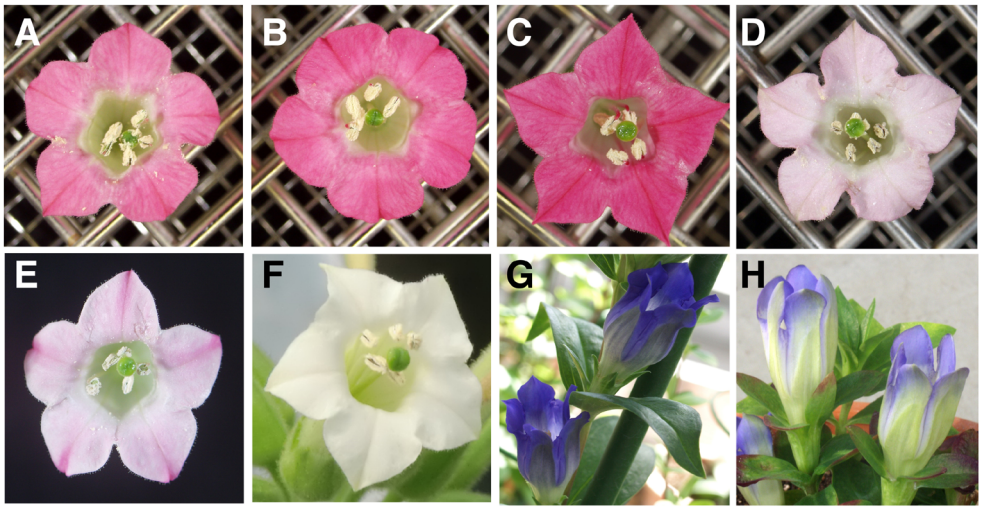

Figure 3. Modification of flower color by gentian transcription factor genes in tobacco (A–F) and gentian (G and H). (A) Wild-type tobacco cv. SR1. (B) A tobacco flower overexpressing *GtMYB3*. (C) A tobacco flower overexpressing both *GtMYB3* and *GtbHLH1*. (D) A tobacco flower overexpressing a chimeric repressor of *GtMYB3*. (E) A tobacco flower overexpressing *GtMYBP3*. (F) A tobacco flower overexpressing *GtMYB1R9*. (G) Wild-type gentian cv. Albireo. (H) A gentian flower overexpressing a chimeric repressor of *GtMYB3*.

has great potential for controlling the contents of flower pigments and changing flower colors and patterns. In some cases, qualitative as well as quantitative changes can be achieved. To enhance pigment accumulation, the control of transcriptional levels of a series of genes is thus more likely to be effective than manipulation of a single structural enzyme gene. The utility of this approach has been previously demonstrated in flowers of several plant species, including tobacco, tomato, Arabidopsis, and petunia, by introducing transcription factors derived from maize or snapdragon (Bradley et al. 1998; Lloyd et al. 1992; Mooney et al. 1995). The coexpression of *GtMYB3* and *GtbHLH1* genes from gentian can also be successfully applied to enhance flower pigmentation in tobacco (Figure 3C).

Arabidopsis AtMYB75, termed PAP1, is one of the most characterized transcription factors functioning as an activator of anthocyanin biosynthetic genes (Borevitz et al. 2000). In the study, overexpression of *PAP1* brought about an amazing color change in transgenic Arabidopsis and tobacco leaves. These transgenic plants also produced flowers with enhanced pigmentation. Transformation with *PAP1* genes has been subsequently applied to many plant species to enhance anthocyanin accumulation. Interestingly, *PAP1* overexpression can enhance not only anthocyanin accumulation but also emission of floral volatiles in petunia and rose (Ben Zvi et al. 2008; Ben Zvi et al. 2012); coengineering of flower color and scent is thus also possible through ectopic expression of transcription factor genes. Furthermore, *PAP1* overexpression leads to the enhancement of oxidative and drought tolerance in Arabidopsis (Nakabayashi et al. 2014). *PAP1*-overexpressing tobacco plants are also defensive against herbivores (our unpublished results). The engineering of elite floricultural plants harboring novel flower color, scent, and tolerance against abiotic and biotic stress may be possible in the future.

Some transcription factors can be used for suppression of flower pigmentation in heterologous species. For example, *AtMYB12*-overexpressing tobacco plants show pale color pigmentation (Luo et al. 2008), and gentian GtMYBP3, GtMYBP4, GtMYB1R1, and GtMYB1R9 can reduce anthocyanin pigmentation in tobacco flowers (Nakatsuka et al. 2012, 2013). In the case of an RNAi or antisense strategy, isolation of the homolog genes in each plant species is prerequisite and sometimes requires time and effort. Because transcription factors generally regulate a series of flavonoid biosynthetic genes in all plant species, engineering of flower color is possible even in heterologous plants. Furthermore, Chimeric REpressor gene Silencing Technology (CRES-T), an efficient gene silencing system using chimeric repressors, can be used not only for functional analysis of plant transcription factors, but also for modification of plant traits (Mitsuda et al. 2011a). This system has been successfully applied to the production of bicolor flowers in gentian (Nakatsuka et al. 2011b; Figure 3H). CRES-T can also be used for many ornamental flowers, including torenia, carnation, rose, chrysanthemum, cyclamen, morning glory, and lisianthus (Mitsuda et al. 2011b). The use of transcription factors that are not directly responsible for flower pigment biosynthesis is thus a promising approach. In addition, flower pigmentation patterns such as spots and sectors might be controlled if regulation under suitable promoters can be achieved.

Finally, it is noteworthy that coengineering of transcription factors and structural genes can modify

flavonoid biosynthesis. (Xie et al. 2006) have reported that coexpression of Arabidopsis *PAP1* and the *Medicago* anthocyanidin reductase gene can promote proanthocyanidin formation in tobacco flowers. The transgenic tobacco flowers show decreased anthocyanin levels compared with flowers overexpressing *PAP1* alone. In other instances, coexpression of Arabidopsis *AtMYB12* and the soybean isoflavone synthase gene can lead to enhanced biosynthesis of isoflavones and flavonols both in leaves and petals (Pandey et al. 2014). For the future, the combination of transcription factors and structural genes is a more attractive approach for the engineering of specific metabolic flow and the creation of novel flower colors and patterns. Taken together, genetic engineering of flower pigmentation and patterning is now partially achievable, but more detailed studies are necessary to create the beautiful flower colors found in nature. We hope that such research will proceed so that deliberate engineering of desired flower colors, like artists painting on canvas, becomes possible.

Perspectives

Recently, our research revealed that the proper gene expression of the flavonoid biosynthetic pathway was regulated by transcription factors in Japanese cultivated gentians, which are important ornamental flowers in Japan. Precise transcriptional regulation is essential for flower pigmentation as demonstrated in other flowers such as petunia and snapdragon. Our results provide important insights into the function of each gene involved in flower pigmentation regulation in gentians. In particular, we identified several different transcription factor genes involved in the regulation of the early and late biosynthetic pathways in gentian flowers. Moreover, the activation and repression of flavonoid biosynthetic genes seems to occur concurrently in the same flower. The reason(s) is not clearly understood yet, but it gives a clue to help fully understand the complex flavonoid biosynthesis in gentian flowers. It is also noteworthy that multiple genes with similar functions are present in gentian flowers, e.g. *GtMYBP3* and *GtMYBP4*, *GtMYBR1R1* and *GtMYBR1R9*. Detailed functional analyses of these genes would give us a definitive answer about redundancy and flower pigmentation in highly heterozygous plant species such as gentians. We have also demonstrated the possibility of controlling flower color intensity and patterning through transcriptional regulation of flavonoid biosynthetic genes. This approach to creating desirable flower colors will also have a significant impact on plant biotechnology and may be used in breeding programs for most flowers. Recent next-generation sequencing technologies have been applied to several flower species including petunia (Zenoni et al. 2011), rose (Kim et al. 2012), carnation (Tanase et al. 2012), and orchid (Chou et al. 2013), yielding data that have provided a foundation for novel research. It is noteworthy that the whole genome sequence of carnation, one of the major cut-flowers sold worldwide, has recently been reported (Yagi et al. 2013); the data will be useful for the discovery of novel genes and the elucidation of the complex metabolic networks of flavonoid biosynthesis in flowers. We also aim to deepen our understanding of flavonoid biosynthesis in Japanese gentian and to promote a Japanese gentian breeding program in the near future.

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