Biotechnol Lett (2011) 33:433–441
DOI 10.1007/s10529-010-0461-z

REVIEW

Genetic engineering of flavonoid pigments to modify flower color in floricultural plants

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Received: 1 August 2010 / Accepted: 13 October 2010 / Published online: 4 November 2010

Abstract Recent advances in genetic transformation techniques enable the production of desirable and novel flower colors in some important floricultural plants. Genetic engineering of novel flower colors is now a practical technology as typified by commercialization of a transgenic blue rose and blue carnation. Many researchers exploit knowledge of flavonoid biosynthesis effectively to obtain unique flower colors. So far, the main pigments targeted for flower color modification are anthocyanins that contribute to a variety of colors such as red, pink and blue, but recent studies have also utilized colorless or faint-colored compounds. For example, chalcones and aurones have been successfully engineered to produce yellow flowers, and flavones and flavonols used to change flower color hues. In this review, we summarize examples of successful flower color modification in floricultural plants focusing on recent advances in techniques.

Keywords Flavonoid · Flower color · Genetic engineering · Transcription factor · Transgenic plants

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Introduction

There are many beautiful flowers in nature and they show a variety of shapes and colors. Such diversity is acquired through evolutionary processes to ensure successful reproduction by attracting pollinators or by promotion of wind pollination (Henry 2005). Flower color in most angiosperms is especially crucial to attract pollinators such as insects and birds. From a horticultural point of view, flower color is one of the most important targets for plant breeders and many different-colored cultivars have been bred using natural mutants or genetically related species. However, some plants are incapable of yielding particular flower colors by traditional breeding, for example, a blue color in rose. This is because only conspecifics and closely related plant species can be crossed and thus genetically distant genetic resources are unavailable for introduction of flower colors of interest by traditional breeding methods. Plant cell and tissue culture techniques, such as embryo rescue and protoplast fusion, can overcome the reproductive barrier in some cases, but such techniques are not applicable to all plant species.

Accumulation of secondary metabolites such as flavonoids, carotenoids and betalains is most responsible for flower color development. Spontaneous changes in accumulated metabolite contents cause variation in flower colors. Most commercially available ornamental flowers have been bred exploiting such natural mutation. However, we have no control

over the attempts to create new flower colors by mutation induction, because the mutations occur at random. Usually, selection of one particular mutated flower color from among many mutated populations is required. For successful breeding of novel flower colors, one challenge is genetic engineering by transgenic approaches of which many such studies are in progress (reviewed in Davies 2009; Tanaka et al. 2009; Nishihara and Nakatsuka 2010).

Flavonoids are the most well-studied plant secondary metabolites and their biosynthetic pathway has been elucidated in higher plants such as *Arabidopsis*, maize and petunia (Koes et al. 2005; Grotewold 2006a, b). Knowledge of the molecular basis of the biosynthetic pathways makes a transformation approach feasible since Meyer et al. (1987) successfully modified the flower color in petunia by overexpressing a heterologous maize dihydroflavonol 4-reductase (*DFR*, *A1*) gene. Subsequently, there have been many attempts to produce plants bearing novel flower colors, including such important floricultural crops as rose and carnation. Although carotenoids and betalains are important for flower pigmentation (Grotewold 2006b; Tanaka et al. 2008b), there are few examples of successful flower color modification by regulating the carotenoid biosynthetic pathway and no reports of successful manipulation of betalain biosynthesis. Carotenoids are a widespread family of plant pigments that, like flavonoids, play vital roles in plant photosynthesis in chloroplasts. Carotenoids are also precursors for the synthesis of the plant hormones abscisic acid, strigolactone and gibberellin (Grotewold 2006b; Umehara et al. 2008); therefore, their genetic manipulation is rather difficult. In contrast, flavonoids are not critical for plant survival and it is comparatively easy to modify the metabolic pathway. Although betalains are also not essential, the betalain biosynthetic pathway is less well understood and is difficult to utilize for flower color modification at present. In addition, betalains are present only in a limited number of plant species of the Caryophyllales. Along with recent advances in genetic engineering technology, rapid progress in molecular engineering of flower color has also been achieved and many useful methods to modify flower color have been developed. We here summarize recent studies on modification of flower color by genetic engineering of flavonoid pigments in floricultural species. In addition, this review will refer to fundamental studies in model plants, such as tobacco and *Arabidopsis*, to discuss the potential use of flower color modification in the future.

Flavonoid biosynthetic pathway in higher plants

Flavonoids are derived from the phenylpropanoid biosynthetic pathway from which many plant-specific secondary metabolites are derived, such as lignin, stilbenoids, coumarins and phytoalexins. The typical flavonoid biosynthetic pathway in higher plants is shown in Fig. 1. Briefly, the first step is a condensation reaction of one *p*-coumaryl-CoA and three malonyl-CoA molecules catalyzed by chalcone synthase (CHS), resulting in a pale yellow pigment, 4,2′,4′,6′-tetrahydroxychalcone. Anthocyanidin aglycons (e.g. pelargonidin, cyanidin and delphinidin), which elicit red to blue colors, are synthesized from the chalcones by several enzymes through several additional reactions, including hydroxylation, reduction and oxidation. It is well known that blueness is enhanced with increasing numbers of hydroxyl groups in the B-ring of anthocyanidins, and the hydroxylation reactions are catalyzed by two enzymes, flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H), in flavanones or dihydroflavonols. Generally, chalcones and anthocyanidins are subjected to further modifications, such as glycosylations, malonylations and acylations, and then transported into vacuoles. Chalcone 4′-*O*-glucoside is converted to the bright yellow pigment aureusidin, 6-*O*-glucoside, by aureusidin synthase (AS) in the vacuoles of yellow snapdragon flowers (Nakayama et al. 2000; Ono et al. 2006). In addition, 3-deoxyanthocyanins are synthesized from flavanones in several steps in orange to red flowers in a few plant species such as *Sinningia cardinalis* (Winefield et al. 2005; Nakatsuka and Nishihara 2010).

In addition to the pigmented flavonoids discussed above, flavones and flavonols also contribute to flower color hue. Both groups of compounds comprise unpigmented or pale yellow flavonoids, and play important roles in the stability and development of blue color by anthocyanin pigments by so-called ‘copigmentation’. Flavones and flavonols are synthesized from flavanones and dihydroflavonols by flavone synthase (FNS) and flavonol synthase (FLS), respectively (Fig. 1).

In studies of model plants, expression of the flavonoid biosynthesis genes is revealed to be regulated

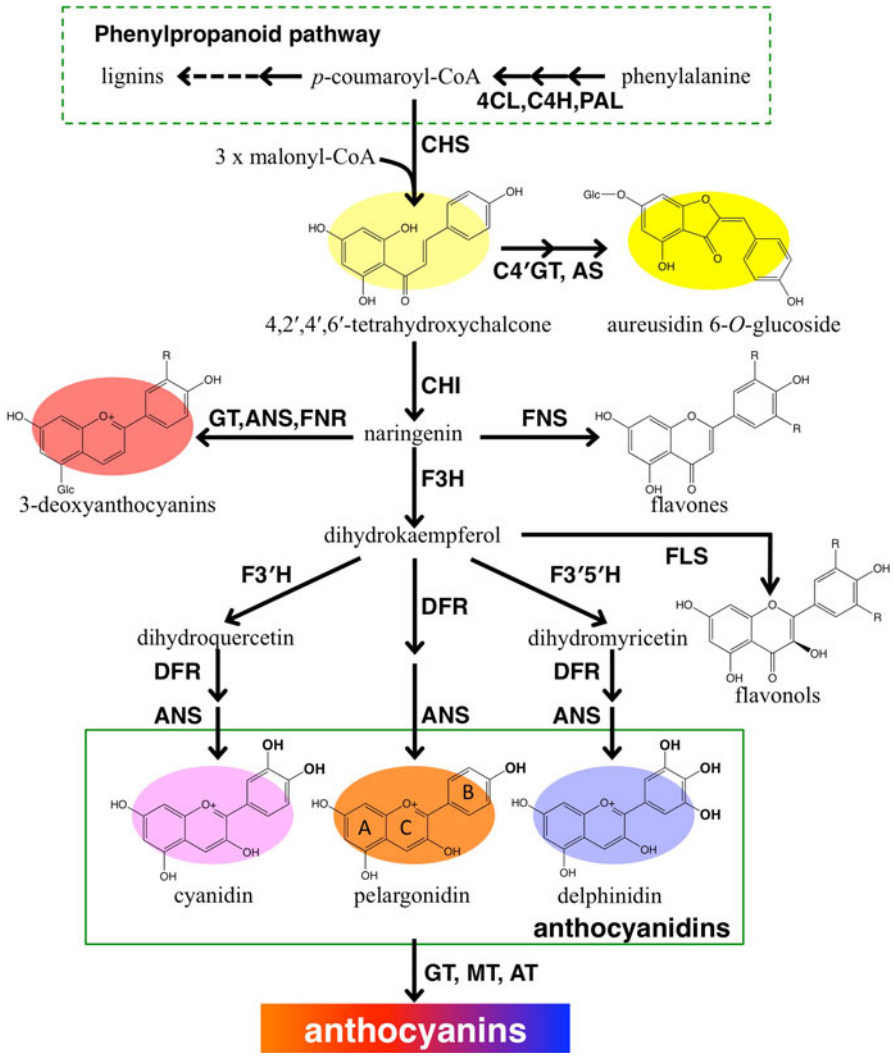

Fig. 1 Flavonoid biosynthetic pathway and flavonoid compounds accumulated in flowers. A simplified pathway derived from several plant species is depicted for ease of explanation. The painted colors shows image of each compound. *ANS* anthocyanidin synthase, *AS* aureusidin synthase, *AT* acyltransferase, *C4′GT* chalcone 4′-*O*-glucosyltransferase, *C4H* cinnamate-4-hydroxylase, *CHI* chalcone isomerase, *CHS* chalcone synthase, *4CL* 4-coumarate:CoA ligase, *DFR* dihydroflavonol 4-reductase, *F3H* flavanone 3-hydroxylase, *F3′H* flavonoid 3′-hydroxylase, *F3′5′H* flavonoid 3′,5′-hydroxylase, *FLS* flavonol synthase, *FNR* flavanone 4-reductase, *FNS* flavone synthase, *GT* glycosyltransferase, *MT* methyltransferase, *PAL* phenylalanine ammonia-lyase

by the complex of R2R3-MYB, basic helix-loop-helix (bHLH) and WD40 repeats (WDR) transcription factors (Grotewold 2005; Quattrocchio et al. 2006; Davies 2009). Such triplet-complex transcription factors are indicated to regulate the petal pigmentation of floricultural plants including snapdragon, petunia, morning glory, gerbera and gentian (e.g. Morita et al. 2006; Nakatsuka et al. 2008a; Davies 2009).

Strategies for modification of flower color by genetic engineering of the flavonoid biosynthetic pathway

There are two main strategies to modify flower color using a transgenic approach. One is the control of endogenous flavonoid pigments in flowers both quantitatively and qualitatively, the other is an approach to accumulate non-native pigments in flowers. Both strategies are effective, and in the following section we introduce recent examples of successful flower color modification by each strategy, mainly focusing on floricultural plants (summarized in Table 1).

Control of endogenous flavonoid pigments to modify flower color

The simplest strategy to modify flower color is to reduce the amounts of endogenous flower pigments by suppressing the essential enzymes required for their biosynthesis. Sometimes in nature, individuals

Table 1 Examples of flower color modifications by regulating flavonoid biosynthesis in Agricultural plants published over the past 5 years

Plant species	Original colors	Gene sources	Methods	Produced flower colors	References
Cyclamen persicum	Purple	Endogenous *F3′5′H*	Antisense	Red to pink	Boase et al. (2010)
Gentiana sp.	Blue	Endogenous *CHS*	Antisense	White	Nishihara et al. (2006)
	Blue	Endogenous *CHS*	RNAi	Pale blue to white	Nakatsuka et al. (2008b)
	Blue	Endogenous *ANS*	RNAi	Pale blue	Nakatsuka et al. (2008b)
	Blue	Endogenous *F3′5′H*	RNAi	Magenta	Nakatsuka et al. (2008b)
	Blue	Endogenous *F3′5′H*	RNAi	Lilac to pale blue	Nakatsuka et al. (2010)
		Endogenous *A5/3′AT*	RNAi		
Nierembergia sp.	Violet	Endogenous *F3′5′H*	Antisense	Pale blue	Ueyama et al. (2006)
Ostespermum hybrida	Magenta	Endogenous *F3′5′H*	RNAi	Reddish	Seitz et al. (2007)
		Gerbera hybrida DFR	Over expression		
Petunia hybrida	Blue	*Mazus pumilum CHS*	Dominat negative	Pale blue	Hanumappa et al. (2007)
	Red	*Lotus japonicus PKR*	Over expression	Variegated red	Shimada et al. (2006)
Rosa hybrida	Red to pale cyanic	*Viola* sp. *F3′5′H*	Over expression	Bluish	Katsumoto et al. (2007)
		Iris×*hollandica DFR*	Over expression		
		Endogenous *DFR*	RNAi		
Torenia hybrida	Blue	Endogenous *ANS*	RNAi	White to pale blue	Nakamura et al. (2006)
	Blue	Endogenous *ANS*	Antisense	Pale blue	Nakamura et al. (2006)
	Blue	*Antirrhinum majus AS*	Over expression	Yellow	Ono et al. (2006)
		Antirrhinum majus C4′GT	Over expression		
		Endogenous *F3H* or *DFR*	RNAi		

with white or faint-colored flowers arise by natural mutations in gene(s) involved in flavonoid biosynthesis. To induce this phenomenon artificially, *CHS* was suppressed using an antisense method. The first, well-known example of application of this technique was the successful production of white flowers in tobacco and petunia (van der Krol et al. 1988). Subsequently, antisense and sense cosuppression methods were proven to be useful for modifying the flower color to white or faint colors in many plants. For example, recently the antisense strategy targeting *CHS* or anthocyanidin synthase (*ANS*) was applied to produce a white-flowered gentian and pale blue torenia (Table 1; Nakamura et al. 2006; Nishihara et al. 2006).

The antisense and cosuppression methods are little used nowadays owing to development of the more efficient RNA interference (RNAi) method. With regard to flower color, Fukusaki et al. (2004) first demonstrated that flower color modification in torenia was possible by downregulation of *CHS* by RNAi. We also showed that chalcone isomerase (*CHI*) suppression resulted in flower color change in transgenic tobacco plants (Nishihara et al. 2005). Thus, RNAi silencing is proven to be effective to modify flower color as well as other traits and has become one of the most popular methods to down-regulate gene expression in higher plants (Tanaka et al. 2008a; Frizzi and Huang 2010).

Depending on the gene or species, the suppression frequency and obtained flower color can differ between antisense and RNAi methods, as shown in gentian and torenia (Nakamura et al. 2006; Nakatsuka et al. 2008b). Thus, to produce stable and completely white flowers, researchers should test which silencing technology is suitable for the particular plant material under study. The regulation of endogenous modification genes can also be the target of genetic engineering. For example, suppression of *F3′5′H* in cyclamen, *Nierembergia* and gentian by antisense or RNAi methods resulted in modification of flower color hue (Table 1; Ueyama et al. 2006; Nakatsuka

et al. 2008b; Boase et al. 2010; see also next section). More recently, we also demonstrated that suppression of both *F3′5′H* and *A5/3′AT* (anthocyanin 5,3′-aromaticacyltransferase) genes by RNAi in gentian induced novel flower colors (Nakatsuka et al. 2010). Hanumappa et al. (2007) reported a unique strategy to reduce flower pigmentation. These authors produced transgenic petunia plants harboring a mutated *CHS*, which induced a dominant-negative effect, resulting in reduced flower color intensity. It is also possible to enhance pigment biosynthesis and to produce deeper-colored flowers by overexpression of rate-limiting biosynthetic enzymes, but successful examples are limited. The manipulation of *FLS* and *DFR*, which are competing steps for flux towards flavonols and anthocyanins, resulted in a decrease or increase, respectively, in anthocyanin content in the flower (reviewed in Davies and Schwinn 2010). We also reported that gentian *F3′H* overexpression in tobacco caused an increase in floral anthocyanin content (Nakatsuka et al. 2006).

The choice of promoters for gene regulation is another important factor. In most cases, the Cauliflower mosaic virus 35S (CaMV35S) promoter, which can induce constitutive expression of transgenes, has been used in production of transgenic plants, and modification of flower color is also conducted using this promoter. However, CaMV35S is not functional in some floricultural plants such as gentian (Mishiba et al. 2010) and chrysanthemum; therefore other promoters must be developed. Candidates are the promoters of the flavonoid biosynthesis-related genes or *rol*C from *Agrobacterium rhizogenes*, and these promoters have been successfully used in gentian and tobacco (Nakatsuka et al. 2007b, 2008b). In these cases, depending on the different localization of promoter activity, the patterns of flower pigmentation can be designed and adoption of several promoters will be useful to produce a variety of flower color patterns in the future.

Production of non-native flavonoid pigments in flowers

Flavonoid components in flowers vary greatly among species and cultivars. Some plants can produce certain anthocyanins, whereas others do not. Thus, a promising approach is to produce non-native flavonoids (especially pigmented anthocyanins) by introducing foreign genes into the plant of interest. This approach was first demonstrated in petunia by introducing a heterologous maize *DFR* gene (Meyer et al. 1987). The foreign maize DFR can reduce dihydrokaempferol (DHK) effectively in petunia to produce pelargonidin-type anthocyanins, resulting in production of brick-red-colored flowers (Fig. 1). Hydroxylation patterns of anthocyanin aglycons greatly influence their color development. The number of B-ring hydroxyl groups is controlled by *F3′H* and *F3′5′H*, which belong to the cytochrome P450 family, and these genes are now available from many plant species (Grotewold 2006a, b). The idea to control the hydroxylation pattern of anthocyanin skelton for production of new flower colors is reasonable because the naturally accumulated anthocyanins depend on the plant species and cultivar. By controlling the hydroxylation pattern, transgenic carnations and roses have been produced to accumulate delphinidin-type anthocyanins that do not accumulate naturally (Tanaka et al. 2005; Katsumoto et al. 2007). These flower-color-modified carnations and roses are now commercially available in accordance with the Cartagena Protocol on Biosafety. The strategy to introduce foreign *DFR* and *F3′5′H* gene is prerequisite for production of delphinidin. However, it is not so easy to achieve exclusive and dominant accumulation of delphinidin and, in the case of carnation, DFR-deficient white lines are used as the transformation host, whereas in the case of rose suppression of the endogenous *DFR* gene was achieved by RNAi (Table 1). For reference, commercially available GM roses in Japan do not contain RNAi construct of rose *DFR* gene. They are derived from overexpression of both pansy *F3′5′H* and torenia anthocyanin 5-acyltransferase genes. More recently, delphinidin-containing violet chrysanthemums have been produced by some research groups, although the details have not been published yet.

In most cases, multiple regulation of biosynthetic genes is necessary in floricultural plants where desirable mutations are rare. The approach of manipulating multiple genes has now been utilized in many species. In *Osteospermum*, suppression of *F3′H* by RNAi and introduction of gerbera *DFR* resulted in pelargonidin accumulation (Table 1; Seitz et al. 2007). We also reported manipulation of three genes, namely gerbera *DFR* overexpression and *F3′H* and *FLS* suppression, in tobacco to produce red

flowers (Nakatsuka et al. 2007a). In the case of torenia and petunia, multiple genes are manipulated to produce the flower color of interest.

Others flavonoids useful for modifying flower color

Here examples of flower color modification by manipulation of specific plant flavonoid pigments are summarized. One such pigment is the yellow aurone (Fig. 1). Ono et al. (2006) revealed that regulation of aurone biosynthesis led to production of yellow flowers in torenia. Specifically, suppression of the endogenous *F3H* or *DFR* genes and overexpression of snapdragon *C4′GT* (chalcone 4′-*O*-glucosyltransferase) and *AS* genes caused abundant accumulation of aureusidin 6-*O*-glucoside in the petals. In this case, preventing accumulation of endogenous anthocyanin pigments by suppression of *F3H* or *DFR* is necessary to achieve a visible yellow flower color by accumulation of the aureusidin 6-*O*-glucoside.

In another example, 6′-deoxychalcones are yellow pigments accumulated in a limited number of plant species belonging to the Asteraceae or Leguminosae. We cloned a novel gene (*PKR1*) from *Lotus japonicus*, which is a leguminous model plant, and introduced the gene into a red-flowered cultivar of petunia (Shimada et al. 2006). The flower of the transgenic plant showed a variegated red color and accumulation of isoliquiritigenin derivatives was observed. Such rare flavonoids can be useful to create cream- to yellow-flowered plants as shown by Davies et al. (1998). Successful production of yellow flowers by genetic manipulation is still at a developing stage compared with development of red- or blue-colored flowers. To produce yellow flowers, it is important to control the metabolic flow strictly to accumulate the yellow pigments abundantly in the petals.

It is rare for unpigmented compounds, such as flavone and flavonol, to be controlled but these compounds contribute to copigmentation and are useful for flower color modification. In particular, copigmentation is responsible for development of blue flowers (Yoshida et al. 2009). In torenia, Aida et al. (2000) first demonstrated that introduction of the antisense *DFR* gene induced accumulation of flavones and bluer flower colors owing to copigmentation effects. Ueyama et al. (2002) reported that suppression of *FNSII* led to reduction in flavone contents in torenia flowers. Interestingly, reduction of anthocyanin contents was observed in the *FNSII*-suppressed torenia, though the reason is not clearly understood yet. Overexpression of gentian *FNSII* in tobacco led to accumulation of flavones and reduction of anthocyanin content (Nakatsuka et al. 2006). As mentioned in the above section, manipulation of *FLS* enables control of the metabolic flux between flavonols and anthocyanins. In the future, control of flavones, flavonols and other copigments might be increasingly important for changing flower color hues, especially to blue, by copigmentation effects in many floricultural plants.

Various modification genes, including glycosyltransferases, BAHD or serine carboxypeptidase-like (SCPL) acyltransferases, that are capable of glycosylating or acylating a wide variety of substrates are now becoming available (Milkowski and Strack 2004; Gachon et al. 2005; D'Auria 2006). Anthocyanin modifications, such as glycosylation, acylation and malonylation, are also potential targets for genetic engineering of flower colors. However, manipulating anthocyanin modifications is not reported to change flower color dramatically. In most cases, flavonoid component analysis clearly detected the accumulation of novel pigments, but a visible effect on flower hue was scarcely observed (reviewed in Davies 2009). We also showed introducing the gentian anthocyanin 5-*O*-glucosyltransferase (*A5GT*) gene into tobacco modified the flower color but the change was subtle (Nakatsuka et al. 2008c). However, as in the case of production of a yellow-flowered torenia by introducing snapdragon *C4′GT* mentioned above, introduction of a modification enzyme might be a prerequisite to accumulate certain flavonoids in the vacuole. We recently characterized 3-deoxyanthocyanidin-specific glucosyltransferase (ScUGT5) in *Sinningia cardinalis* flowers (Nakatsuka and Nishihara 2010) and this enzyme might be useful to produce orange to red flowers by accumulation of 3-deoxyanthocyanins in the petals.

Recent strategies to modify flower colors

Transcription factors regulating genes involved in the flavonoid biosynthetic pathway have been identified not only in model plants but also in floricultural or food crops. For example, R2R3 MYB transcription

factors regulating anthocyanin biosynthesis have been successfully cloned from petunia (Quattrocchio et al. 2006), members of the Rosaceae, e.g. apple, strawberry, plum and rose (Lin-Wang et al. 2010), lily (Yamagishi et al. 2010), snapdragon (Jackson et al. 1991; Schwinn et al. 2006), morning glory (Morita et al. 2006), gentian (Nakatsuka et al. 2008a) and tobacco (Pattanaik et al. 2010). These genes usually regulate sets of genes in the flavonoid biosynthetic pathway; therefore it is possible to control multiple genes effectively by manipulating these transcription factors. R2R3-MYB and bHLH genes are utilized to control flavonoid biosynthesis in some plant species such as *Arabidopsis*, tomato, petunia and tobacco, but many flavonoids are synthesized in fruits or leaves and few are of importance in flowers (Davies 2009; Nishihara and Nakatsuka 2010). For example, *AtMYB12*-expressing transgenic tobacco displayed reduced floral pigmentation (Luo et al. 2008). Recently, a purple-colored creeping bentgrass was successfully produced using the maize transcription factors *Pl* and *Lc* (Han et al. 2009). Combinations of these two classes of transcription factors are effective to induce abundant accumulation of anthocyanins, and conversely suppression of these genes will be useful for reduction of flower color.

Although RNAi and antisense methods are useful for gene silencing as mentioned above, chimeric repressor gene-silencing technology (CRES-T) targeting transcription factors is also a promising approach for flower color modification. A chimeric repressor dominantly represses the transcription of its target genes; therefore phenotypes can be easily observed even when RNAi and antisense methods are ineffective (Mitsuda et al. 2006). In fact, genetic engineering of a variety of traits including plant height, flower shape, color, male sterility, and hormone susceptibility, is possible using CRES-T in many horticultural plants such as torenia, morning glory, chrysanthemum, lisianthus, gentian and cyclamen (Mitsuda et al. 2008). Not only *R2R3-MYB* and *bHLH* genes but also other kinds of transcription factors can induce a change in flower color, probably due to pleiotropic effects. We have developed novel types of a variety of floricultural plants and the results will be published soon (*Plant Biotechnology* Special Issue 2011). In addition, recent molecular-biological studies reveal that other factors also influence flower color in floricultural plants, including a vacuolar ion transporter in tulip (Momonoi et al. 2009), vacuolar acidification in morning glory and petunia (Verweij et al. 2008; Yoshida et al. 2009), and cell shape in snapdragon, petunia and *Thalictrum* (Baumann et al. 2007; Di Stilio et al. 2009). Many studies in which these factors are targets for flower color modification are currently in progress.

Conclusion

In the past 20 years, transgenic technology has been vastly improved and transgenic floricultural plants with modified flower colors have become commercially available, as have many other edible crops. Beyond the earliest studies in which a single gene was manipulated to modify flower color, the technology now allows modification of complex biosynthetic pathways to create a variety of flower colors by regulating multiple genes simultaneously. Generally, genetically modified (GM) flowers are more acceptable to consumers than GM food crops; therefore many floricultural plants are at advanced stages of development. However, biosafety assessment remains a crucial step to be cleared in many cases. The law obliges biosafety experiments and the cost for the last phase of development is very expensive in many countries (Tanaka et al. 2009). This is because floricultural crops often comprise many plant species and each evaluation must be performed individually using a trial-and-error process. Knowledge from model plants is less helpful for this process, and greater patience is required for their biosafety assessment. Thus, in addition to fundamental research on genetic engineering of novel flower colors, the generation and refinement of practical assessment systems for GM flowers is urgently needed.

Acknowledgments The authors acknowledge funding from KAKENHI (Grant-in-Aid for Scientific Research; 21658013) and the Programme for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry.

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