Seeing is believing: engineering anthocyanin and carotenoid biosynthetic pathways
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The biosynthetic pathways of flavonoids and carotenoids have been well established, and the biosynthetic genes have been mostly isolated. Metabolic engineering of their biosynthetic pathways has provided not only novel colored or health-beneficial plants but also excellent models to study the efficacy of such engineering. In order to achieve a specific color by accumulating a corresponding compound, it is necessary to upregulate the pathway leading to the compound and downregulate the competing pathway. The regulation of gene expression has to be optimized in a target crop as well.

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Introduction
Flavonoids and carotenoids are ubiquitously distributed plant pigments \([1,2]\). Flavonoids together with a class of flavonoids, anthocyanins, confer a wide spectrum of color that includes pale yellow, scarlet, red, magenta, violet, and blue to flowers and fruits. Carotenoids furnish flowers and fruits with distinct colors ranging from yellow to red and are essential components for photosynthesis \([3]\). Flower color has been an important trait in the floriculture industry. Flavonoids and carotenoids also protect plants by absorbing UV and scavenging free radicals. Flavonoids have attracted interest because they offer health benefits to humans as well as act as plant developmental regulators via modulating auxin transport \([4]\). Carotenoids also play an important role in human nutrition and health, participating in pro-vitamin A and anti-cancer activities \([5]\).

The conceptual and technological bases of plant metabolic engineering and the difficulty and challenges of engineering plant natural products have been discussed \([6]\). In practice, successful engineering consists of three technical elements: (1) the isolation of useful genes, (2) the development of an efficient transformation system of a target crop, and (3) the sophisticated regulation of gene expression in the target crop. Twenty years have passed since the color of a plant was modified for the first time through genetic engineering: the brick red petunia accumulating pelargonidin that expressed the maize DFR gene \([7]\). Over the years, flavonoid and carotenoid biosynthetic pathways have been extensively studied, and the relevant genes in the pathways are mostly available. Transformation systems have been developed for economically important crops, including many floricultural species \([8]\), although their efficiency varies depending on the plant species and cultivars. Great progress has been made in regulating heterologous or endogenous genes in transgenic plants. For example, candidates of many tissue-specific promoters are easily available \(\textit{in situ}\) \([6]\), although their activity should be experimentally examined. RNAi (RNA interference by double-stranded RNA) has enabled the efficient downregulation of a target gene (usually called knocked down) \([9]\). Still, the optimization of gene regulation is necessary for a target species. For example, a widely used cauliflower mosaic virus 35S (CaMV35S) promoter is silenced in gentian, requiring that other promoters be developed \([10]\). The selection of a source for the structural genes is often crucial, as described here. In this review, we summarize the achievements in this field by focusing on a limited number of reports and discuss the tactics and practical problems of metabolic engineering. Recent, more comprehensive reviews focusing on flower color are available \([11,12]\).

Engineering a flavonoid biosynthetic pathway
The flavonoid biosynthetic pathway has been well characterized (Figure 1), and the main pathway is shared among higher plants, enabling the engineering of the pathway for the purposeful accumulation of compounds. A list of the successful implementation is shown in Supplementary Table 1.

Downregulation of anthocyanin biosynthesis
The higher efficiency of the downregulation by RNAi than of antisense or sense suppression (co-suppression) was shown when the three methods were compared by suppressing the anthocyanidin synthase gene in \textit{Torenia hybrida} \([13]\) (Figure 2a). Chalcone isomerase (CHI) catalyzes the isomerization of tetrahydroxy chalcone to naringenin very efficiently. Suppression of the CHI gene by RNAi resulted in depletion of anthocyanins, which indicates that the technology is useful for complete gene
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suppression [14]. Since knockout of a target gene in a plant genome is still difficult to achieve and plants are often polyploid, RNAi remains the best way to silence target genes, although the stability of suppression by RNAi has not been well examined in field conditions. Allele-specific knockdown experiments have been carried out by utilizing the 3'-untranslated region of the chalcone synthase mRNA [15].

The downregulation of the flavanone 3-hydroxylase gene in carnation resulted in white or paler flower color than that of the host. Unexpectedly, the carnations had an altered floral scent and emitted more methyl benzoate than the control plants [16]. The downregulation of a lignin biosynthetic gene increased the metabolic flux into flavonoids in Arabidopsis [17]. These results indicate that engineering a pathway may have unintended effects on another pathway and that there is cross-talk between pathways.

The downregulation and overexpression of flavonol synthase genes in petunia predictably led to the increase and decrease of anthocyanins, respectively [18]. Downregulation of flavone synthase in torenia resulted in accumulation of flavanones as expected but somehow a decrease in anthocyanins [19]. The latter result implies that there might be a feedback inhibition type of interaction in the flavonoid pathway, or downregulation of a biosynthetic enzyme might destabilize a suggested metabolic biosynthetic complex [20].

Upregulating anthocyanin/flavonoid biosynthesis

Elevation of the amount of flavonoids can be achieved by overexpression of one of the biosynthetic genes. Overexpression of the petunia CHI gene in tomato resulted in an increase of up to 78-fold of flavonols [21]. The overexpression of transcriptional factors that regulate the transcription of flavonoid biosynthetic genes is more commonly utilized to elevate the amount of anthocyanins. R2R3 Myb, the basic helix loop helix (bHLH), and WD40-type transcriptional factors regulate the expression of structural genes in the flavonoid biosynthesis [22]. The constitutive expression of the maize Lc gene (a bHLH) resulted in ectopic accumulation of anthocyanins, redder flower color in tobacco [23], and purple leaves in petunia [24]. An increase in flavonols and flavanones in tomato fruits was achieved by fruit-specific transcription of the maize Lc and C1 (a R2R3 Myb) genes [25]. The constitutive expression of the tomato ANT1 (a R2R3 Myb) gene in tomato and tobacco resulted in purple color owing to the increase of anthocyanins [26].

Modification of B-ring hydroxylation

The B-ring hydroxylation pattern greatly influences the color of anthocyanins and, thus, flower/fruit color. Pelargonidin, cyanidin, and delphinidin (Figure 1) tend to give brick red/scarlet, red/magenta, and violet/blue, respectively. Flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H) determine the hydroxylation patterns (Figure 1).

Engineering the pathway toward pelargonidin

In some plants, the substrate specificity of dihydroflavonol 4-reductase (DFR) contributes to determine the hydroxylation pattern; petunia DFR efficiently utilizes dihydromyricetin but does not utilize dihydrokaempferol (DHK) and thus petunia does not accumulate pelargonidin. This is the reason that petunia and probably some other plants, such as gentian and iris, lack the orange or bright-red color varieties. Maize and gerbera DFR can utilize DHK as a substrate. Transgenic petunias accumulating pelargonidin and bearing brick red and orange color have been generated by expressing the maize and gerbera DFR genes, respectively, in a petunia that accumulates DHK owing to the deficiency of the F3'H and F3'5'H genes [7]. It is noteworthy that such a deficiency of competing enzymes (F3'5'H and F3'H) against DFR is necessary for pelargonidin accumulation. The gerbera DFR gene gave more intense and stable color changes than maize DFR gene in petunia. Such phenotypic difference was derived from their different transcriptional level. The maize DFR gene in transgenic petunia plants was prone to be silenced by methylation [27]. Pelargonidin accumulating orange petunia was obtained from cyanidin accumulating red plant by the downregulation of the endogenous F3'H gene and overexpression of the rose DFR gene [18]. Overexpression of the gerbera DFR gene and knockdown of chimeric F3' and F3'H genes resulted in pelargonidin accumulating tobacco [28]. Downregulation of the F3'5'H gene and overexpression of the gerbera DFR gene in Osteospermum hybridum resulted in pelargonidin accumulation, while overexpression of the gerbera or strawberry DFR gene alone did not result in phenotypic changes [29]. Downregulation of F3'5'H and F3'H genes in a violet torenia accumulating delphinidin and cyanidin resulted in pale-pink color accumulating pelargonidin, and additional expression of the pelargonium DFR gene led to more pelargonidin and a darker pink color (unpublished results, Figure 2b). A similar strategy should work to generate red gentian and iris accumulating pelargonidin. These results indicate that the overexpression of a gene leading to a target compound is not enough and that it is necessary to downregulate competing pathways to accumulate a compound in purpose.

Engineering toward delphinidin

Roses, carnations, chrysanthemums, lilies, and gerbera, all of which are top-selling cut flowers, lack delphinidin-based anthocyanins and violet to blue flower colors. This is attributed to their lack of the F3'5'H gene. Expression of the Campanula medium F3'5'H gene resulted in more efficient accumulation of delphinidin (up to 99%) than that of the petunia or lisanthus F3'5'H gene in tobacco
Major anthocyanidins and flavonoid biosynthetic pathway relevant to color. Anthocyanidins are further modified by glycosylation, acylation, and methylation to anthocyanins that are subsequently transported to vacuoles [1,2]. The color depends mainly on the structure of flavonoids. Typical...
which indicates that the choice of the gene source is to be considered for successful engineering. Such different performance of the genes or derived enzymes may be due to the efficiency of transcription and translation, enzymatic properties, and so on. A hardship is that it is difficult to predict the performance of an exogenous gene or enzyme in a target plant although prior biochemical characterization of the enzymes may be useful.

Transgenic violet carnations accumulating delphinidin (Figure 2c) have been successfully commercialized by expressing a petunia or a pansy $F3'5'H$ gene and a petunia $DFR$ gene in white carnations that were deficient in the $DFR$ gene [31].

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Carotenoids
Carotenoids are lipophylic isoprenoid compounds with polyene chains that may contain up to 15 conjugated double bonds (Figure 3). More than 700 naturally occurring carotenoids have been identified [34].

Biosynthesis of carotenoids
Figure 3 is a summary of the carotenoid biosynthesis pathway. Genes encoding enzymes of the carotenoid biosynthetic pathway were first identified in bacteria followed by various kinds of organisms [5,35]. There is increasing evidence that carotenogenesis in plant tissues is predominantly regulated at the transcriptional level [5,35], but further study is needed as to the molecular components that control the expression of carotenogenic genes. The carotenoid levels are also post-transcriptionally regulated. Lu et al. demonstrated that the sink capacity of the chromoplast is important for the control of the carotenoid content in the tissue [36*].

Carotenoid biosynthesis pathway. Enzymes are abbreviated as follows: CrtB, bacterial phytoene synthase; CrtI, bacterial phytoene desaturase; DXPS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; IPI, isopentenyl pyrophosphate isomerase; GGDP, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, *z*-carotene desaturase; LCYB, lycopene *b*-cyclase; LCYE, lycopene *e*-cyclase; CHYB, *b*-ring hydroxylase; CHYE, *e*-ring hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; CRTISO, carotenoid isomerase; NSY, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid dioxygenase.
Metabolic engineering of carotenoids biosynthesis

Carotenoids play essential roles in plant life, providing photoprotective functions during photosynthesis [3] and surviving as substrates for the biosynthesis of the plant growth regulator abscisic acid [37]. In addition, the carotenoid biosynthetic pathway shares geranyl geranyl pyrophosphate (GGPP) with other essential metabolic pathways that lead to the synthesis of gibberellic acid, chlorophylls, and vitamin E. Altering the carotenoid content and/or composition in vegetative tissues will have detrimental effects on plant growth. More sophisticated regulation of transgenes is necessary for modification of carotenoid biosynthetic pathway than flavonoid one. Transgenic tomato constitutively overexpressing the phytotene synthase (PSY) gene increased the carotenoid level in vegetative tissues. The transformants, however, showed a dwarf phenotype owing to the depletion of the endogenous GGPP pool, resulting in a shortage of gibberellins [38]. The bacterial phytotene synthase gene (crtB) was introduced under the control of a ripening-specific promoter [39]. The spatial and temporal expression of PSY successfully increased the carotenoid content in ripening fruit without dwarfism. These results suggest that the manipulation of carotenoid biosynthesis should be performed in a tissue-specific manner.

Early attempts have focused on the manipulation of the carotenoid content in food crops in order to improve their nutritional value for the human diet [35]. ‘Golden rice’, genetically modified rice that produces β-carotene, is the best example. The first attempt was to introduce the daffodil PSY gene under the control of the endosperm-specific glutelin promoter and bacterial crtB with a plastid targeting sequence under the control of the CaMV35S promoter [40]. The carotenoid level in the transgenic endosperm was estimated at 1.6 μg/g. Although the enzymatic activity of crtI is to convert phytotene to lycopene (Figure 3), the synthesized carotenoids were mostly β-carotene. Schaub et al. later showed that the endogenous genes encoding phytotene desaturase, α-carotene desaturase, lycopene β-cyclase, β-ring hydroxylase, and carotenoid isomerase are expressed in the wild-type rice endosperm, whereas PSY transcripts are virtually absent [41]. A higher carotenoid level (<37 μg/g) was achieved by substituting daffodil PSY by maize PSY [42]. Seed-specific overexpression of the crtB gene with the napin promoter produces transgenic canola containing up to a 50-fold increase in total carotenoids in their seeds [43].

Carotenoid levels were also upregulated by suppression of one of the genes involved in the biosynthesis. In potato, tuber-specific silencing of lycopene ε-cyclase increased the total carotenoid level in tubers up to 2.5-fold and β-carotene level up to 14-fold [44].

Most plants do not contain ketocarotenoids such as astaxanthin and canthaxanthin. Genetic engineering aimed at ketocarotenoid production in plants is of great commercial interest for its antioxidative property and vivid orange-red color. Production of ketocarotenoid was successfully achieved in plants by introduction of the β-ketolase gene from microorganism. The most recent examples are carrot [45] and Lotus japonicus [46]. In later case, flower color changed from yellow to orange.

Successful engineering of carotenoids by manipulating light signal transduction pathway components was reported [47,48]. A fruit-specific silencing of a light signal transduction pathway component (DET) results in simultaneous elevation of both flavonoid and carotenoid contents in tomato fruits [48].

Engineering carotenoid degradation

The carotenoid content can be also altered by manipulating the degradation. Ohmiya et al. have demonstrated that in the white petals of chrysanthemum carotenoids were synthesized but subsequently degraded by carotenoid cleavage dioxygenase (CcMCCD4a), resulting in the white color. Suppression of CcMCCD4a gene expression converted the petal color from white to yellow (Figure 2f) [49]. By contrast, overexpression of CcMCCD4a altered petal color from yellow to white (unpublished data).

Some carotenoid cleavage dioxygenases (CCD) contribute to the formation of aroma and pigment compounds. The style branches of crocus, which yields a spice called saffron, accumulate red-colored apocarotenoids, crocin glycosides, picrorocin, and safranal [50]. These apocarotenoids are produced by 7,8(7,8) cleavage of zeaxanthin catalyzed by the zeaxanthin cleavage enzyme. The seed of Bixia orellana accumulated red apocarotenoids called bixin, a color additive used in foods and cosmetics. Bixin was produced by the cleavage of lycopenae at 5,6(5’,6’) catalyzed by lycopenae dioxygenase [51]. Among the members of the CCD family, PhCCD1 from petunia [52] and LeCCD1 from tomato [53] were shown to cleave carotenoids at 9,10(9’,10’) and contribute to the formation of β-ionone and geranylacetone, important constituents of flavor. The genes encoding these enzymes will serve as new genetic tools for the alteration of color and/or aroma of flowers.

Conclusions

Metabolic engineering of flavonoids and carotenoids has generated the plants with novel flower color or health benefits by purposely accumulating specific desirable compounds. In general, the results of the engineering are predictable at least to some extent. However, it is not easy to predict the amount of the compounds accumulated. The engineering of flavonoid and carotenoid biosynthetic pathways may affect other metabolic pathways in plants and result in detrimental effects. This can be solved by achieving a comprehensive understanding of the interaction of the metabolic pathways and sophisti-
cated transgene regulation. Knowledge obtained from the engineering of the two pathways will pave the way to engineer other pathways.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.copbio.2008.02.015.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- *of outstanding interest*


This paper shows that the expression of gerbera or strawberry DFR cDNA in Osteospermum that was dominant in F3′/S′ activity did not produce pelargonidin. When the F3′/S′ activity of the transgenic Osteospermum plants expressing these DFR genes was chemically inhibited, pelargonidin was produced. The knockdown of F3′/S′ activity by RNAi in the plant expressing gerbera DFR successfully redirected the delphinidin pathway to pelargonidin.


Blue roses are the ‘Holy Grail’ in plant breeding. There are many rose cultivars that are claimed to be blue, but they are generally pink, gray, or mauve. Blue flowers tend to have polycataylated delphinidin, strong copigments, and higher vacuolar pH or metal ions, all of which roses lack. The authors of this paper achieved almost exclusive accumulation of delphinidin in rose petals and a novel violet color by selecting rose cultivars that authors of this paper achieved almost exclusive accumulation of delphi-


