The Genetics and Biochemistry of Floral Pigments

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Abstract

Three major groups of pigments, the betalains, the carotenoids, and the anthocyanins, are responsible for the attractive natural display of flower colors. Because of the broad distribution of anthocyanins (synthesized as part of the flavonoid pathway) among the flowering plants, their biosynthesis and regulation are best understood. However, over the past few years, significant progress has been made in understanding the synthesis and participation of carotenoids (derived from isoprenoids) and betalains (derived from tyrosine) in flower pigmentation. These three families of pigments play important ecological functions, for example in the attraction of pollinating animals. Anthocyanins in particular have also been the target of numerous biotechnological efforts with the objective of creating new, or altering the properties of existing, coloring compounds. The focus of this review is to examine the biosynthesis, regulation, and contribution to flower coloration of these three groups of pigments.

Contents

INTRODUCTION

Fruit and flower colors are of paramount importance in the ecology of plants and in their ability to attract pollinators and seed-dispersing organisms (43). In addition, plants also play an important aesthetic function by providing flowers with a broad spectrum of colors. Not surprisingly, ornamentals were among the first plants to be hybridized to alter specific color traits, and fruit and flower color have contributed to elucidating fundamental genetic principles. Today, the market for ornamental plants and cut flowers is rapidly expanding and totals over $70 billion in annual sales (5). Although increasing postharvest life, altering scent, and modifying flower shape are areas where progress is being actively pursued, much of the novelty in the cut flower industry continues to be targeted toward the generation of new colors (87). The high stakes associated with the development of new floral traits are best exemplified by the passions awaked in the seventeenth century by the unique pigmentation patterns of the "broken tulips," which led to the "tulipomania" for which records are preserved in many classical paintings (47). Florigene's Moonseries of genetically engineered carnations (**http://www.florigene.com/**), marketed in the United States, Australia, Canada, Japan, and some European countries, provide the first genetically engineered commercial flowers.

Three types of chemically distinct pigments, betalains, carotenoids, and anthocyanins are responsible for the colors of flowers (**Figure 1**). Of the three, the anthocyanins have been studied the most in the context of flower pigmentation, reflecting their broader distribution among the angiosperms. There are several excellent reviews documenting the complex biochemistry and distribution of flavonoid pigments (e.g., 16, 30, 32, 82) and their biosynthesis and regulation (e.g., 56, 70a, 101, 102). Much of the molecular information available on the regulation and biosynthesis of flower pigments derives from studies performed in model systems that include maize, *Arabidopsis*, petunia, and snapdragon. However, nonclassical plant models continue to provide unique insights into the ecophysiological regulation and functions of flower pigmentation. This review describes recent advances in our understanding of the biosynthesis, storage, and regulation of the different flower pigments.

DOPA: dihydroxyphenylalanine

BETALAINS

Biosynthesis

Betalains are water-soluble, nitrogen-containing compounds synthesized from tyrosine by the condensation of betalamic acid (**Figure 2**), a central intermediate in the formation of all betalains, with a derivative of dihydroxyphenylalanine (DOPA). This reaction results in the formation of the red to violet betacyanins, such as those found in red beets or in the flowers of portulaca (**Figure 1*a***). The condensation of betalamic acid with an amino acid (e.g., Ser, Val, Leu, Iso, and Phe) or amino acid derivative

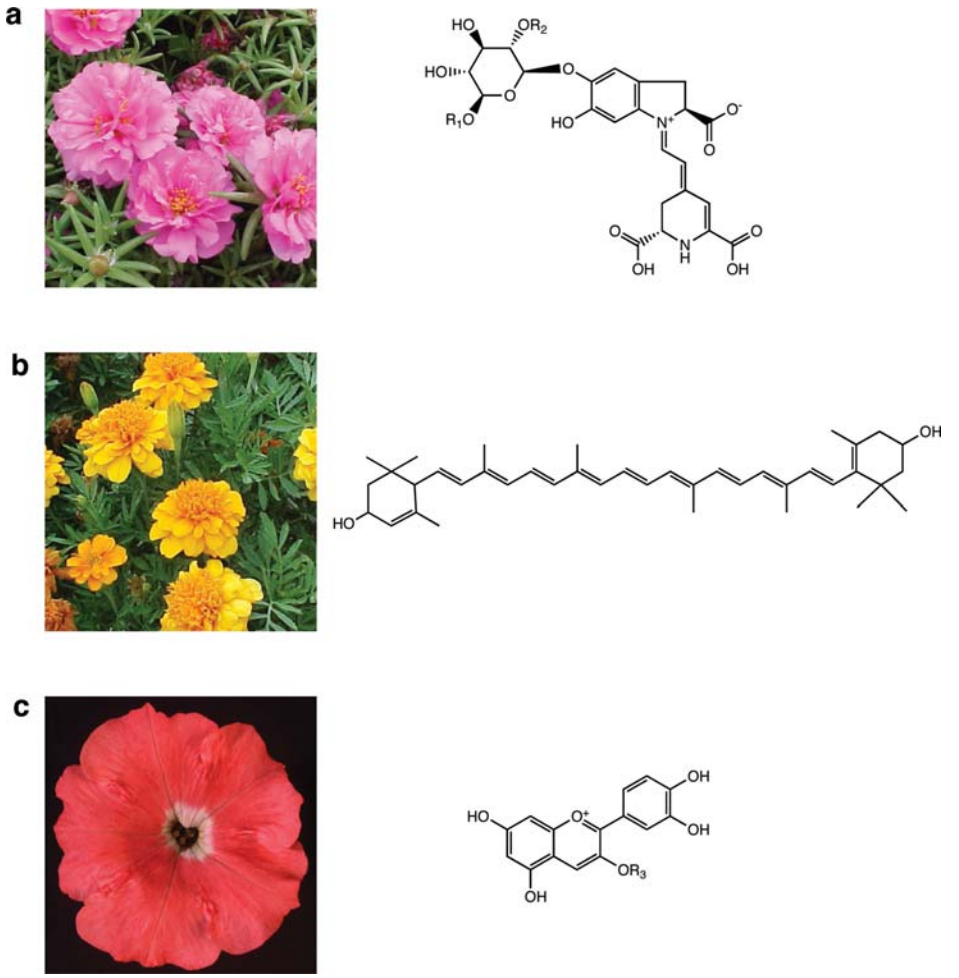

Figure 1

Flower displaying the three major types of pigments and the corresponding structures. (*a*) Portulaca (*Portulaca grandiflora*) flowers accumulating primarily the betalain pigment, betanin ($R_1 = R_2 = H$). (*b*) Marigold (*Tagetes patula*) flowers accumulating the carotenoid pigment, lutein. (*c*) Petunia (*Petunia hybrida*) flower accumulating an anthocyanidin, cyanidin. Pictures were kindly provided by the Missouri Botanical PlantFinder and F. Quattrocchio.

(e.g., 3-methoxytyramine) (**Figure 2**) results in the formation of the yellow to orange betaxanthins. Betacyanins and betaxanthins can be further classified into several subclasses, based on the chemical characteristics of the betalamic acid conjugate (86). Recent advances in the separation and analysis of betalains, which are unstable under the acidic conditions normally used for Nuclear Magnetic Resonance (NMR) spectra analyses, are likely to shed additional light on the existence of novel conjugates (85). As is common for many other phytochemicals, light and hormones have a dramatic effect on the accumulation of betalains (70).

The conversion of tyrosine to DOPA (**Figure 2**) is carried out by a tyrosinase-type phenoloxidase (84), a group of copper-containing bifunctional enzymes involved in the hydroxylation of phenols to *o*-diphenols. In addition to participating in the formation of the betalamic acid core, the tyrosinase enzyme also oxidizes DOPA to dopaquinone, contributing to the biosynthesis of *cyclo*-DOPA, which conjugates with betalamic acid to form the chromophore of all betacyanins, betanidin (86). The formation of betalamic acid from DOPA requires the extradiol cleavage of the 4,5 bond carried out by a DOPA dioxygenase, first identified in the basidiomycete fly agaric (*Amanita muscaria*) (35). The plant

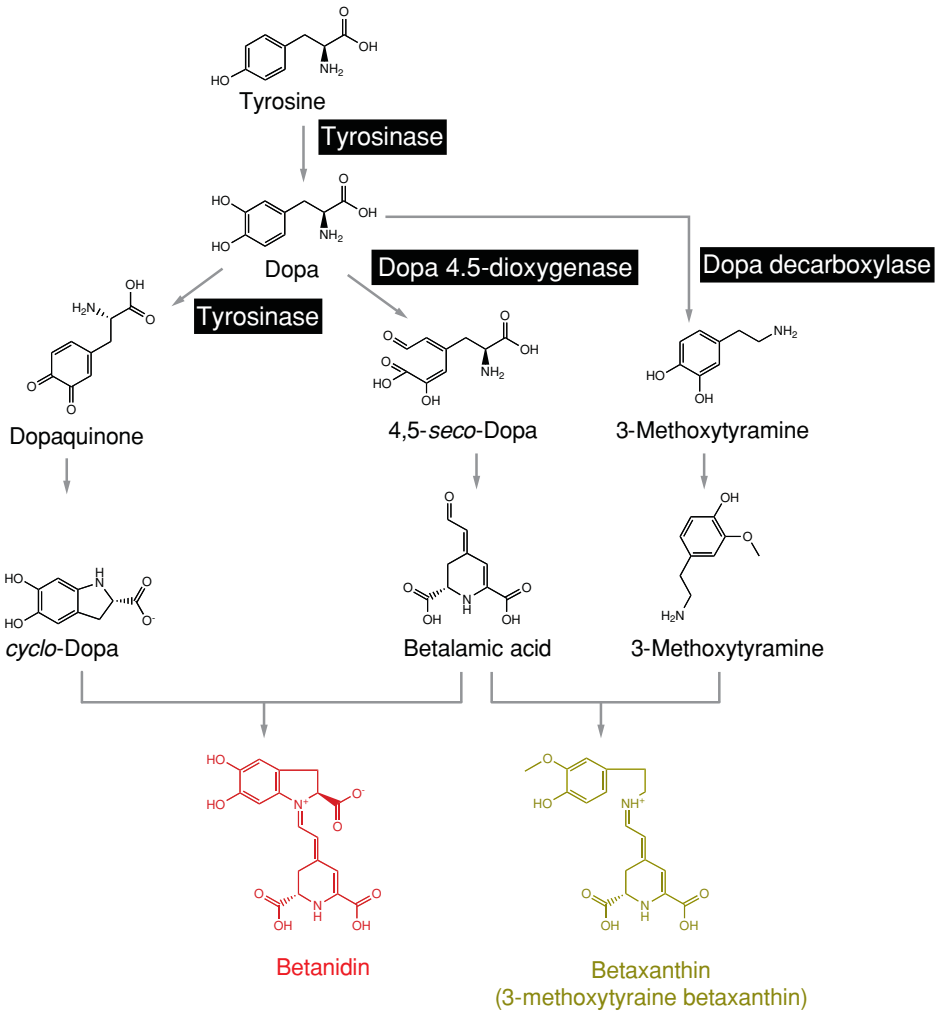

Figure 2

Schematic representation of the biosynthetic pathway of some betalain pigments. The known enzymes are indicated in black boxes and the compound names are shown.

enzyme was subsequently cloned by a subtractive cDNA approach using *Portulaca grandiflora* isogenic lines with different color phenotypes (7). The plant enzyme exhibits no obvious sequence or structural similarity with the fungal enzymes. Moreover, the plant enzyme displays regiospecific extradiol 4,5-dioxygenase (7), in contrast to the 2,3- and 4,5 dioxygenase activity of the *Amanita muscaria* enzyme (35). The 4,5-*seco*-DOPA is subsequently recyclized, a step likely to occur spontaneously (86). This different activity of the plant and fungal enzymes permits *Amanita muscaria* to accumulate muscaflavin, in addition to betalain, in the cuticle of the cap. The introduction of the DOPA dioxygenase from *Amanita muscaria* into *Portulaca grandiflora* petals by particle bombardment resulted in the accumulation of various betalains, and also of muscaflavin, a pigment normally not found in plants (59), which is synthesized by the extradiol ring cleavage of the 2,3 bond followed by recyclization into the 6-atom, N-containing ring muscaflavin.

The next step in the biosynthesis of betalains involves the formation of an aldimine link between betalamic acid and *cyclo*-DOPA (to make betanidin) or an amino acid derivative (to make betaxanthin) (**Figure 2**). No enzyme capable of carrying out the aldimine

reaction has yet been identified, opening the possibility that this step occurs spontaneously in vivo (86). It remains unclear how the spontaneous condensation of betalamic acid with various different DOPA or amino acid derivatives results in the specific patterns of betalains consistently obtained in the same plant.

As is the case with many other plant natural products (28), betalains are stored in the vacuole as glycosides. Glycosylation of betacyanins occurs both at the level of the *cyclo*-DOPA (74, 104) and by the glucosylation of betanidin (91, 92). The cloned *Dorotheanthus bellidiformis* 5- and 6-*O*-glucosyltranserases transfer glucose with similar efficiency from UDP-glucose to betanidin, to form betanin, and to several flavonoids (91, 92), raising the provocative possibility that there are evolutionary links between these two pathways (see below). Although yet to be tested for its ability to glycosylate flavonoids, the *cyclo*-DOPA 5-*O*-glucosyltransferase belongs to a group of enzymes very distinct from those involved in the phenylpropanoid pathway (75). Tyrosine feeding experiments suggest a strict compartmentalization for the betacyanin biosynthetic pathway, with the possibility of forming multienzyme complexes (83). However, it remains unknown whether there is a single or multiple pools of betalamic acid responsible for the formation of both betacyanins and betaxanthins, or how these compounds are transported to the vacuole, their ultimate site of accumulation.

Occurrence and the Mutual Exclusion with the Anthocyanins

Anthocyanin pigments are broadly distributed among the flowering plants (see below), but betalains are restricted to the order of the Caryophyllales. Within this order, betalains are absent in a couple of families including the Caryophyllaceae, which comprises genera such as *Lychnis* and *Dianthus* (e.g., carnations, *Dianthus caryophyllus*), widely used as ornamentals and cut flowers for their colorful anthocyanin pigmentation. Remarkably, anthocyanins are not present in any of the families accumulating betalains, an observation that has puzzled scientists for several decades, and which resulted in a model in which anthocyanins and betalains are mutually excluded (44, 48, 83). While this exclusion probably makes sense from a functional perspective, since both types of pigments have overlapping absorption spectra, and hence colors, the molecular basis of this exclusion is not clear. Plant-accumulating betalains express at least some of the flavonoid biosynthetic enzymes (e.g., 79) and can accumulate significant quantities of flavonols, other flavonoids, and in some cases even proanthocyanidins, suggesting that it might be the last step in the flavonoid pathway, the anthocyanidin synthase (ANS), the only enzyme "missing" in betalain-accumulating plants (83). The origin of the betalain biosynthetic pathway in just one order of the angiosperms is even more puzzling given that these pigments are also found in some basidiomycetes. One possibility is that the anthocyanin and betalain pigments have coexisted in ancestral plant species and that one of the two pigments has been selectively lost because of similar redundant pigmentation functions. Alternatively, the betalain biosynthetic pathway could have been acquired independently and more recently in the fungi and plants. The evolution of this pathway would have made unnecessary the presence of the anthocyanins, resulting in the observed exclusion of both pigments in the Caryophyllales. The paraphyletic relationship of the betanidin 5- and 6-*O*-glucosyltransferases from *Dorotheanthus bellidiformis* with other glucosyltransferases, together with their ability to utilize both betanidin and flavonoids as substrates (91, 92), was interpreted to indicate that these enzymes, originally involved in the glycosylation of flavonoids, were later recruited to glycosylate betacyanins. If so, these findings would suggest that betalains originated later in the evolution of plants than the anthocyanins. This model raises the question of how betalains appeared independently in the fungi,

with a fungal betacyanin biosynthetic enzyme being able to function in the plants (59). An alternative model is that anthocyanins and betalains coexisted in an ancestral plant, and that during the evolution of the angiosperms, selective loss of ANS or of an enzyme necessary for betalain formation resulted in the current distribution. It remains to be established what selective advantage, if any, betalains provide over anthocyanins.

CAROTENOIDS

Functions

Carotenoids are plastid-synthesized and localized lipid-soluble C_{40} tetraterpenoids universally distributed in the plant kingdom. In contrast to the dispensable anthocyanins and betalains, carotenoids are essential for plant life, providing important photoprotective functions during photosynthesis and serving as precursors for the biosynthesis of the phytohormone abscisic acid (ABA) (11, 36). Carotenoids are also very significant nutraceutical components of the animal diet, serving, for example, as precursors for vitamin A biosynthesis and as antioxidants (17). Animals are unable to synthesize carotenoids de novo, yet recent studies indicate that plants and animals share multiple carotenoid-modifying enzymes (55). Birds, fish, and marine invertebrates frequently utilize carotenoids present in their diets for pigmentation purposes (78). For example, carotenoids color the red plumage of house finches and flamingos, and the ketocarotenoid astaxanthin is responsible for the orange color of salmon meat. Astaxanthin also provides the bluish color to lobster shells; the bathochromic shift from red to blue is the result of the binding of this carotenoid to the crustacyanin macromolecular complex (9). Boiling restores the red color by denaturing the β-crustacyanin protein and relaxing the proximity of the astaxanthin chromophore (90). The hue alteration resulting from the interaction of proteins and pigments remains to be exploited for the manipulation of flower color.

Beyond their essential biological activities, carotenoids have long been recognized as flower pigments (26). Carotenoids are responsible for most of the yellow to orange flower colors in ornamentals that include marigold (*Tagetes*) (**Figure 1*b***), daffodil (*Narcissus*), *Freesia*, *Gerbera*, *Rosa*, *Lilium*, and *Calendula*. More important and less recognized is the ability of carotenoids to coexist with red or purple anthocyanins, resulting in brown and bronze hues that neither pigment would be able to provide by itself (16). From the more than 600 carotenoid structures known, the carotenes (hydrocarbons) and their oxygenated derivatives, the xanthophylls, are most commonly associated with flower pigmentation. Because of the many important functions that carotenoids have for plants and animals, most of the enzymes in the carotenoid biosynthetic pathway have been identified (11, 17, 103).

Biosynthesis

As is the case for other isoprenoids, isopentenyl diphosphate (IPP) provides the five-carbon building block for carotenoids. In the plastids, where carotenoid biosynthesis takes place, IPP is synthesized through the plastid-specific DOXP (1-deoxyxylulose 5-phosphate) pathway (47a). The first committed step in the carotenoid pathway is catalyzed by phytoene synthase (PSY), resulting in the condensation of two C_{20} geranylgeranyl diphosphate (GGPP) molecules to form phytoene (**Figure 3**). Four desaturation reactions, two each catalyzed by the membrane-associated phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), result in the formation of the pink lycopene from the colorless phytoene (**Figure 3**). In addition to the desaturases, the formation of lycopene (*trans* configuration) requires the action of the carotenoid isomerase (CRTISO) enzyme, cloned from the *tangerine* tomato mutant (41), which is responsible for converting

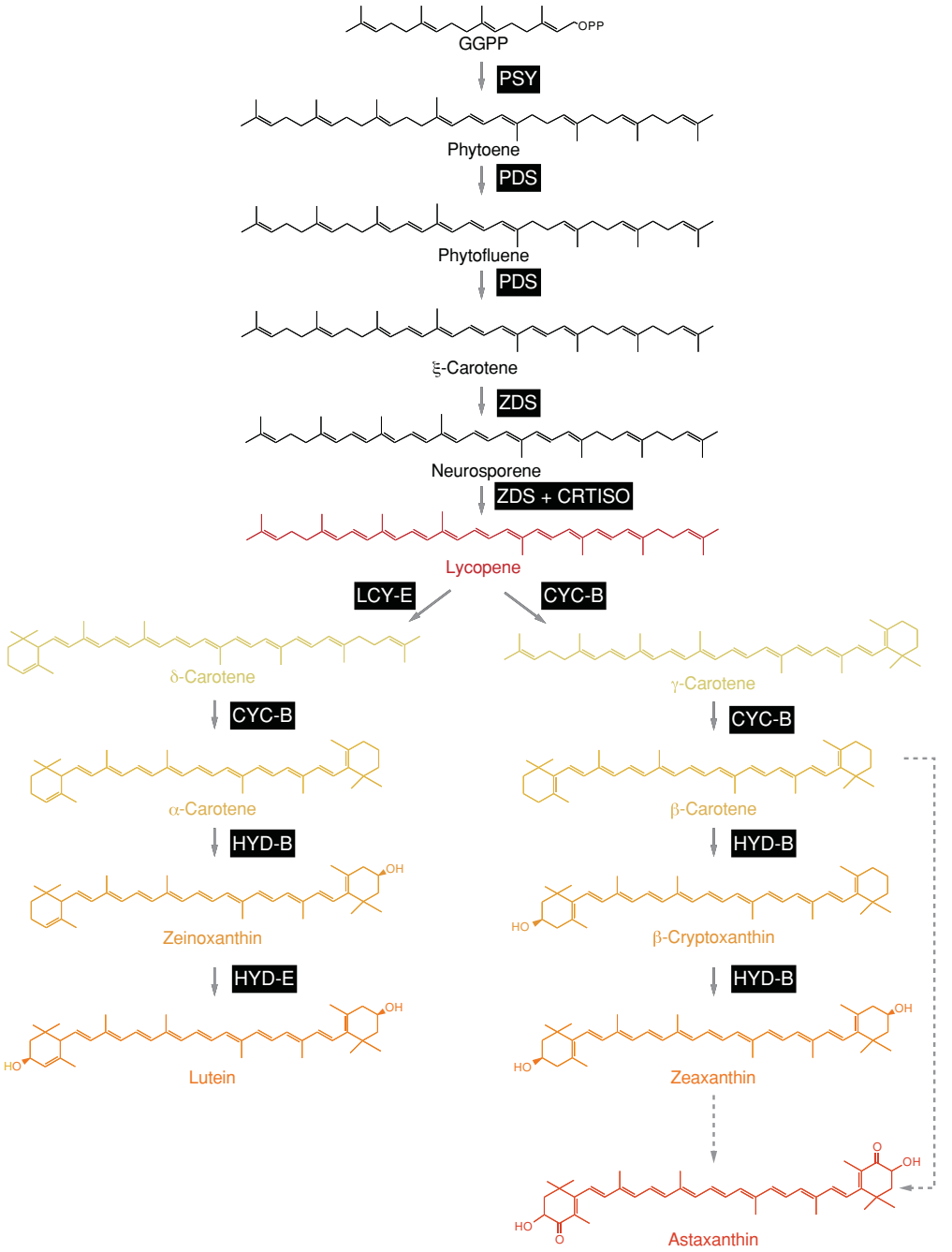

Figure 3

Schematic representation of the biosynthetic pathway of some major carotenoid pigments. The names of the compounds are indicated. GGPP corresponds to geranylgeranyl diphosphate. The enzyme names, in black boxes, are PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; CYC-B, chromoplastic form of lycopene β-cyclase; LCY-E, lycopene ε-cyclase; HYD-B, carotenoid β-ring hydroxylases; HYD-E, carotenoid ε-ring hydroxylase.

poly-*cis*-lycopene (prolycopene) to lycopene. A single enzyme, phytoene desaturase (CRTI), carries out all four desaturation and isomerization reactions in bacteria and fungi. Although plant desaturases have no homology to CRTI, CRTISO does (41, 68). The cyclization of lycopene represents a branch point in the pathway, and two products can be formed depending on the position of the double bond on the cyclohexane ring. On one hand, lycopene β-cyclase, for which there are two forms in tomato, one specific to green tissues (LCY-B) and the other to chromoplasts (CYC-B), first produces γ-carotene containing one β-ring (**Figure 3**), which is subsequently converted to β-carotene by the same enzyme. On the other hand, lycopene ε-cyclase (LCY-E) produces δ-carotene. The formation of α-carotene, the precursor for lutein, involves formation of a β-ring on δ-carotene by lycopene β-cyclase (36).

The α- and β-carotenes are the precursors for the xanthophylls, which are oxygenated carotenoids generated by β- and ε-ring-specific hydroxylases. β-carotene is converted to zeaxanthin by the carotenoid β-ring hydroxylases (HYD-B), encoding a nonheme diiron enzyme (38) for which there are two genes in *Arabidopsis* (89). The hydroxylation of the ε-ring is carried out by the carotenoid ε-ring hydroxylase (HYD-E), a cytochrome P450 enzyme, CYP97C1, encoded by the *Arabidopsis LUT1* locus. In addition to displaying activity toward the ε-ring, LUT1 can also hydroxylate the β-ring (89). Hydroxylation of the β-ring of α-carotene is also mediated by a P450 enzyme (E. Wurtzel, personal communication). Lutein is the main carotenoid present in the petals of marigold, and the broad range of colors that characterize marigold flowers is due to the very different levels of this xanthophyll. Indeed, marigold varieties with very light flower color (e.g., French Vanilla) have a reduced expression of all the carotenoid biosynthetic genes, suggesting a regulatory mutation, rather than a defect in a single biosynthetic enzyme (54). Interestingly, however, the varieties with reduced xanthophyll accumulation in the petals display normal levels of carotenoids in the leaves, strengthening the notion that the "primary" role of carotenoids is independently regulated from their function as secondary metabolites.

The formation of ketocarotenoids, such as, for example, astaxanthin, requires the addition of keto groups in each β-ring of zeaxanthin (**Figure 3**). The initial engineering of astaxanthin in tobacco flowers was accomplished by the expression of the *CrtO* gene, encoding a β-carotene ketolase, from the algae *Haematococcus pluvialis* (49). Subsequently, the AdKeto enzyme was identified from *Adonis aestivalis* (summer pheasant's eye, Ranunculaceae), which is capable of desaturating the 3,4 positions of the β-ring followed by the 4-hydroxylation and the final keto-enol tautomerization, resulting in the formation of the blood-red pigment astaxanthin, abundantly present in the petals of this plant (12). The identification of AdKeto creates novel opportunities for the metabolic engineering of the commercially important ketocarotenoids from the abundant pools of β-carotenes present in many plants, offering alternatives to current approaches to manipulating the pathway involving the introduction of the 4,4′-oxygenase and 3,3′-hydroxylase from marine bacteria into plants (71).

ANTHOCYANINS

Biosynthesis

Anthocyanins are water-soluble pigments that occur in almost all vascular plants and excellent publications have extensively described their chemistry, distribution, and biosynthesis (16, 30, 82). The anthocyanin pigments are responsible for the majority of the orange, red, purple, and blue colors of flowers (**Figure 1*c***). Anthocyanins are derived from a branch of the flavonoid pathway (**Figure 4**), for which chalcone synthase (CHS) provides the first committed step by condensing one molecule

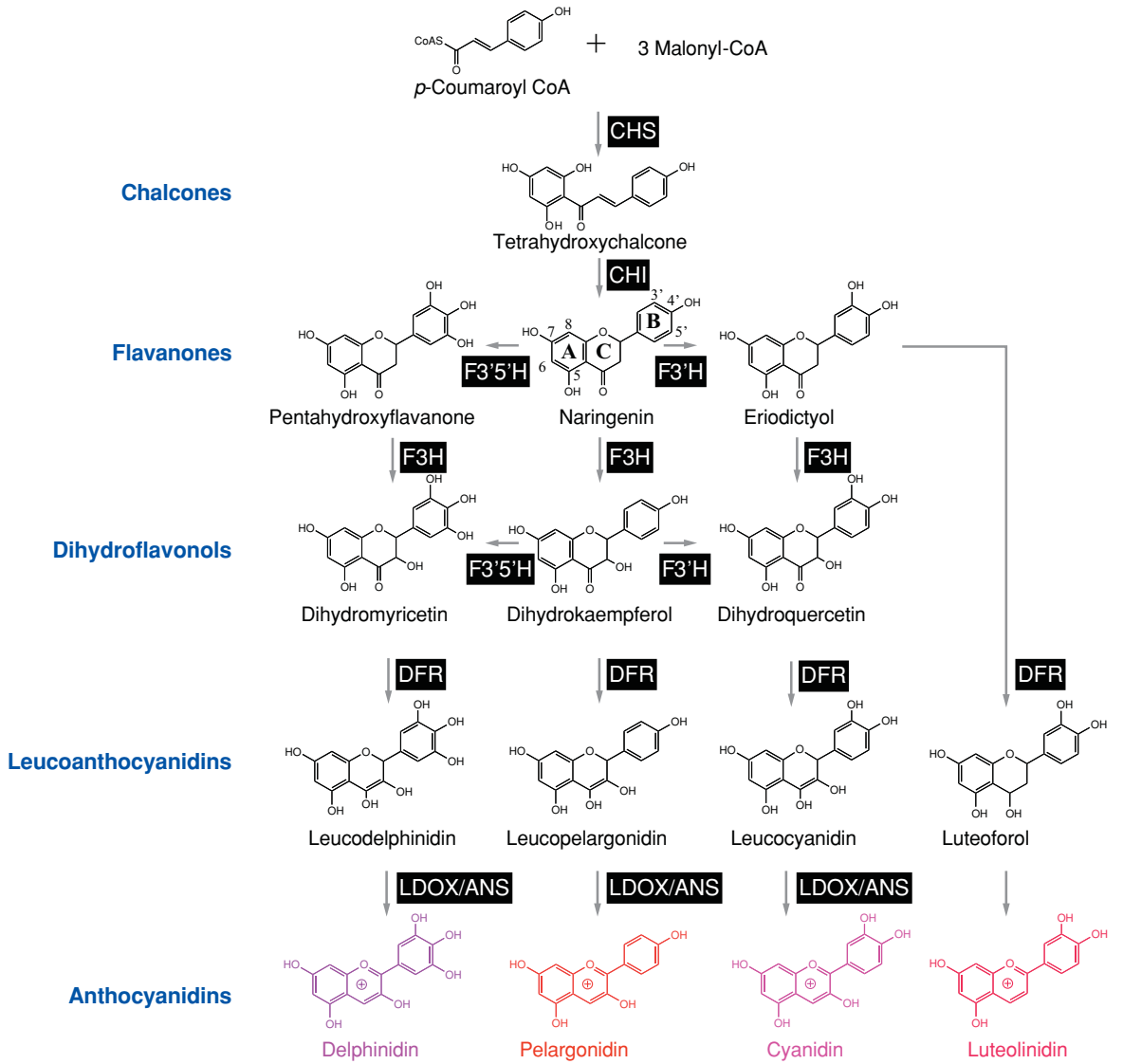

Figure 4

Schematic representation of the biosynthetic pathway of the most abundant anthocyanin pigments. The names of the compounds are indicated. The enzyme names, in black boxes, are CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavanone 3′-hydroxylase; F3′5′H, flavanone 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; LDOX/ANS, leucoanthocyanidin dioxygenase/anthocyanidin synthase. The A-, B-, and C-rings with the carbon numbers are indicated in the structure corresponding to the flavanone naringenin.

of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to produce tetrahydroxychalcone (a chalcone, **Figure 4**). CHS belongs to the family of polyketide synthases and the structure of this enzyme has been solved (2, 15). Chalcone provides the precursor for all classes of flavonoids, which include the flavones, flavonols, flavan-diols, flavan 4-ols, proanthocyanidins (condensed tannins), isoflavonoids, and anthocyanins. The closure

of the C-ring, resulting in the formation of flavanones, is carried out by chalcone isomerase (CHI), an enzyme originally believed to have a structure unique to the plant kingdom (42), but which was also recently found in fungi and prokaryotes (20). In some bacteria, CHI-like enzymes contribute to the degradation of flavonoids by taking advantage of the reversible nature of the isomerization, which permits CHI to also convert flavanones to the corresponding chalcones (33). Flavanones (e.g., naringenin) provide a central branch point in the flavonoid pathway and can serve as substrates for enzymes that introduce –OH groups at the 3′ and 5′ positions of the B-ring (e.g., F3′H and F3′5′H), or for the hydroxylation of the C-ring by flavanone 3-hydroxylase (F3H), a soluble di-oxygenase. Dihydroflavonol 4-reductase (DFR) provides one entry step to the biosynthesis of anthocyanins, and depending on the plant species, it can utilize as a substrate any one or all three of the possible dihydroflavonols, dihydromyricetin, dihydrokaempferol, or dyhydroquercetin, resulting in the formation of the corresponding leucoanthocyanidins, providing structure to the anthocyanin biosynthetic grid (**Figure 4**). In some plant species, an activity that has sometimes been referred to as flavanone 4-reductase (FNR) reduces naringenin to the corresponding flavan 4-ol (e.g., apiferol). However, recent studies in maize suggest that DFR and FNR correspond to the same enzyme (29). The resulting 3-deoxy flavonoids, whose distribution is limited to some bryophytes, a few grasses (e.g., maize and sorghum), and the flowers of the Gesneriaceae (e.g., sinningia), can form 3-deoxyanthocyanin pigments (82), in contrast to the broadly distributed 3-hydroxyanthocyanins. The leucoanthocyanidins are converted into the corresponding anthocyanidins by the action of a leucoanthocyanidin dioxygenase/anthocyanidin synthase (LDOX/ANS). More than 17 different anthocyanidins have been described (31), and the major three are shown in **Figure 4**. Anthocyanidins also serve as substrates for anthocyanidin reductases (e.g., BANYLUS from *Arabidopsis*), key enzymes in the formation of proanthocyanidins (105).

The next step in the anthocyanin pathway is catalyzed by ANS. The structure of the *Arabidopsis* ANS enzyme has been solved (98). ANS, similar to F3H, flavone synthase I (FNSI), and flavonol synthase (FLS), is a member of the nonheme ferrous and 2-oxoglutarate (2OG)-dependent family of oxygenases, sufficient for the conversion of the leucoanthocyanidin (e.g., leucocyanidin) to the corresponding anthocyanidin (e.g., cyanidin) (60). Anthocyanidins, most often represented as the flavylium cation (red), can adopt multiple forms in solution in an equilibrium that primarily depends on the pH and the solvent. In aqueous solutions at pH of 3–6, conditions similar to those present in plant cells, the flavylium cation can be covalently hydrated at position 2, resulting in the corresponding colorless carbinol pseudobases (31). The colored flavylium ion is stabilized in the cell by inter- or intramolecular copigmentation (31). Intermolecular copigmentation involves the interaction of anthocyanins with other noncolored flavonoids (e.g., flavonols), phenylopropanoids, carotenoids, or metals (e.g., Mg^{2+} or Al^{3+}) (16, 31). Noncolored flavonoids provide "depth" to many white or cream flowers. In intramolecular copigmentation, the anthocyanin chromophores are covalently modified by organic acids, other flavonoids, or aromatic acyl groups. These modifications, together with the stacking of planar anthocyanins, add protection from nucleophilic water addition and result in increased anthocyanin pigmentation and hue changes.

Most of the currently known flavonoids are modified at one or several positions by methylation, acylation, or glycosylation. These modifications are often taxa specific and are believed to provide flavonoids with unique properties. For example, flavonoids found in the surface of leaves or flowers (surface flavonoids) are often methylated (67). Although these modifications occur after completion of the skeleton biosynthesis, the most

common is the glycosylation at position 3 with one or multiple sugar residues, followed by acylation. Adding a sugar decoration to anthocyanidins results in a modest hypsochromic shift (to the blue) in the corresponding spectral maxima (31). The most studied glycosylation involves the addition of a glucose group by the UDP-3-*O*-glucosyltransferases (UFGT/3GT), and UFGT/3GT enzymes have been identified and cloned from numerous plant species. In addition to glucose, anthocyanins containing rhamnose and other sugars at the 5, 3′, and 7 positions are found in different plants. The 7GT have high similarity to the betanidin 5GT (92), and to the 3′GT from Gentian (*Gentiana triflora*), which glucosylates the 3′-OH group of delphinidin-type anthocyanins containing glucose groups at the 3′ and 5 positions (19). An interesting variation to the glycosylation of anthocyanidins by different glycosyltransferases in most plants is provided by recent findings in rose (*Rosa hybrida*), in which a single glucosyltransferase, RhGT1, sequentially catalyzes the addition of glucose at the 3-OH and 5-OH positions (65). In addition to glycosylations, anthocyanins can be acylated by a variety of organic acids by a group of enzymes collectively known as anthocyanin acyltransferases (60a). Acylation contributes to intermolecular and/or intermolecular stacking to increase anthocyanin stability and water solubility (81a).

Many of the enzymes in the *Arabidopsis* flavonoid biosynthetic pathway participate in the formation of multienzyme complexes, or metabolons, that may help direct flux into any of the multiple branches of the pathway that can coexist in a cell (3, 100). Although it has not been formally demonstrated for anthocyanin biosynthesis, channeling is involved in the biosynthesis of phenylpropanoids (1, 99). The flavonoid enzymes are associated with the cytoplasmic face of the endoplasmic reticulum (ER), anchored to the membrane through the cytochrome P450 proteins that participate in the pathway (e.g., F3′H) (37, 76, 82). The recent demonstration that several *Arabidopsis* flavonoid biosynthetic enzymes are also located in the nucleus of some cell types (77) may provide clues on nuclear biosynthetic or regulatory activities not previously recognized.

AVI: anthocyanic vacuolar inclusions

Transport and Storage

Because of the visible phenotypes that result from defects in the proper sequestration of anthocyanins, some molecular components involved in the vacuolar trafficking of anthocyanins are starting to emerge (28). The *bz2* locus from maize encodes a glutathione *S*-transferase (GST), which was initially proposed to mediate the transfer of glutathione to cyanidine 3-glucoside (C3G) (51). However, rather than conjugating glutathione to C3G, BZ2 and the equivalent protein in petunia, AN9, appear to serve as carrier proteins, transporting C3G from the cytoplasm to the tonoplast (58), and delivering C3G to MRP3, a maize multidrug resistance-like protein that localizes to the vacuolar membrane (25). The *Arabidopsis TT19* locus also encodes a GST (45), and *tt19* mutants can be complemented by AN9 in their anthocyanin deficiency, but not in their inability to accumulate proanthocyanidins (condensed tannins) in the seed coat (discussed in Reference 46a). In addition to the participation of transporters, vesicles have been implicated in the transport of anthocyanins to the vacuole (28).

Several plant species store anthocyanins within vacuolar inclusions that have been loosely termed anthocyanoplasts, which initiate as vesicles in the cytoplasm and appear to be membrane bound (64, 69). More recently, the intravacuolar structures observed in the flower petals of various plants, including carnation and lisianthus, were termed anthocyanic vacuolar inclusions (AVIs) (50). These inclusions are likely membraneless proteinaceous matrixes that served as anthocyanin traps, preferentially for anthocyanidin 3,5-diglycosides (50) or acylated anthocyanins (10). The expression of the VP24 protein, first identified as encoded by a light-induced gene in sweet potato (*Ipomoea batata*)

AVI-containing cells, correlated with the accumulation of anthocyanins (63). Thus, the VP24 protein, a metalloprotease with aminopeptidase activity (62), likely participates in the transport or accumulation of anthocyanins to the vacuole (106).

Regulation

The regulation of anthocyanin biosynthesis continues to provide a paradigm for the combinatorial control of plant gene expression, providing one of the best-studied plant regulatory systems (40, 46, 56). Basic-helix-loop-helix (bHLH) transcription factors, exemplified by members of the maize R/B family and the *Petunia* AN1 and JAF13 proteins, physically interact with R2R3 MYB proteins (e.g., maize C1 and Petunia AN2) (23) to activate all (in maize) or a subset (in *Petunia* and most other dicots) of the anthocyanin biosynthetic genes. Studies in maize have established that the bHLH-R2R3 MYB interaction serves two purposes: (*a*) it is essential for the activity of the R2R3 MYB partner, either by stabilizing the protein or permitting it to activate transcription, and (*b*) it provides enhanced activity on promoters containing a *cis*-regulatory element conserved in several anthocyanin biosynthetic genes (34). The *PAP1* gene, identified by the pigmentation provided by the enhanced expression in the *PAP1-D* activation-tagged line (2a), encodes the *Arabidopsis* functional ortholog of the maize C1 protein. A combination of RNA and metabolic profiling experiments in *PAP1-D* plants resulted in the identification of two new glycosyltransferases involved in anthocyanin modification in *Arabidopsis* (89a).

In addition to the R2R3 MYB and bHLH regulators, WD40 proteins, exemplified by the *Petunia* An11 (13), the *Arabidopsis* TTG1 (94), the maize PAC1 (4), and the *Perilla* PfWD (80) proteins, play a central role in the activity of the regulatory complex. This cooperation between R2R3 MYB, bHLH, and WD40 proteins is not limited to anthocyanin regulation, and is also involved in the control of multiple developmental processes (72). Little continues to be known on what regulates the regulators. Light and hormones play a central role in the expression of the anthocyanin biosynthetic genes, likely through the activation of the known transcription factors (57, 95).

CELLULAR ARCHITECTURE, pH, AND PIGMENTATION

Although the expression of the pigment biosynthetic genes and the proper subcellular localization of the corresponding pigments are essential, they may not be sufficient for providing the proper hue to flowers. Vacuolar pH plays an important function in coloring anthocyanin pigments. The vacuolar lumen in every cell type is more acidic than the surrounding cytoplasm. In petunia flowers, the acidification of the vacuole results in a red color of the flower and mutations affecting vacuolar pH regulation can be recognized because of the shift of the flower color toward blue. The opposite phenomenon is seen in flowers of *Ipomea*, where development of their normal blue color during petal maturation requires alkalinization of the vacuole by the PURPLE (PR) protein. PR is a putative Na^+/H^+ pump (18) believed to transport sodium ions into and protons out of the vacuole, resulting in the increased vacuolar pH and blue color. Screens have identified many loci in *Petunia* that, when mutated, are deficient in the acidification of the vacuole, and therefore result in hypsochromic shifts. Among them was *ph6* (8), which was an allele of the AN1 bHLH transcription factor (81). Thus, the regulators of the pathway also participate in establishing a vacuolar environment conducive to adequate pigmentation.

In addition to pH, cell shape has a dramatic impact on flower color. The snapdragon (*Antirrhinum majus*) MIXTA R2R3 MYB transcription factor is necessary for the formation of conical cells (61), a characteristic of the petals of many plants. Mutants in the *mixta* locus appear deficient in petal pigmentation,

a consequence of the difference in the way the light is reflected by conical or flat cells (52). Beyond cell shape, the correct packing of anthocyanins in the vacuole is likely to also have a dramatic influence on hue. For example, flowers of the "Rhapsody in Blue" rose cultivar show a change in color induced by age, from red-purple to bluish-purple, and this variation is associated with a progressive accumulation of anthocyanins into AVI-like structures (24). *Lisianthus* flowers also show a correlation between the packaging of anthocyanins into AVIs, the presence of "blackish-purple" pigmentation at the base of the petal, and the reduction or absence of AVIs in the outer zones, associated with a lighter purple color of this region (50). Light affects the way in which anthocyanins are distributed among vacuolar and subvacuolar compartment in maize cells, providing interesting links between environmental signals and anthocyanin pigmentation (39).

FLOWER PIGMENTATION AS A VISUAL CUE

As Darwin noted, "Flowers rank among the most beautiful productions of nature; but they have been rendered conspicuous in contrast with the green leaves, and in consequence at the same time beautiful, so that they may be easily observed by insects. I have come to this conclusion from finding it an invariable rule that when a flower is fertilised by wind it never has a gaily-coloured corolla." Today, it is widely believed that the main function of flower pigments is to attract pollinators and to provide salient signals allowing them to learn the presence of food associated to these signals (53). The relationship between floral traits, among them pigmentation, and the behavior of pollinating animals has been an important factor in the coevolution of plants and the corresponding pollinators. Pollinators seek profitable rewards (e.g., quality and quantity of nectar or pollen) in their foraging visits, and their flower choice is based on a complex decision-making process that involves multiple factors (93). The different color visions of various pollinators make it tempting to speculate that there is a perfect correlation between the pigmentation of flowers and the spectra of colors that a particular pollinator can detect. It has been reported, for instance, that flower-naïve bees prefer hues that seem to be related to highly nectar-rewarding colors in nature in their first foraging flights (21). However, this correlation is far from being demonstrated for different ecosystems and floral varieties. There is a growing debate on whether the association between pollinator vision and flower pigmentation is as strong as believed (6). Indeed, multiple different pollinators can visit flowers with the same color, and it is not rare to find significant variations in flower pigmentation among populations. One possible aspect that needs to be considered is that flavonoids (88) and anthocyanins (27) play a number of important functions, unrelated to pollinator attraction, in vegetative tissues. Thus, the specific hue of a flower could be influenced by the accumulation of flavonoids elsewhere in the plant. On the other hand, our understanding of the specific cues perceived by pollinators in a total floral landscape is very rudimentary, and other factors, in addition to flower pigmentation, likely play a significant role. For example, achromatic cues such as the contrast provided by a corolla to the long-wave receptor type seem to be important for farthest floral detection (22). Also, floral symmetry is more important than color in the visitation preference of naïve bumblebees (73). In contrast, color has a priority over smell in the visitation preference of *Vanessa indica* butterflies (66).

The complexity of the interaction between pollinators and flower pigmentation is nicely illustrated by the phenomenon known as "floral color change," which is widespread in the plant kingdom and which occurs after pollinator visits to a given floral species (96). Floral color change occurs in fully opened, turgid flowers and independently of flower senescence. In some cases, such as in *Viola cornuta*, pollination triggers the accumulation

of anthocyanins, changing the flower color from white to purple. The molecular signals involved in the induction of anthocyanin biosynthesis upon pollination are not known, but light plays a central role (14). In other cases, pigments disappear after pollination (97). Floral color change can involve any of the three types of flower pigments described here, although changes in anthocyanin pigmentation are the most-often recorded (97). It has been argued that color change results in a lost of chromaticity from the pollinator's perspective. This process would thus have an adaptive value, as insects would not need to pay attention to flowers that have been already exploited.

SUMMARY POINTS

1. Three groups of compounds, betalains, carotenoids and anthocyanins, constitute the majority of the flower pigments known.
2. While carotene pigments can coexist with anthocyanins and betalains, there is a mutual exclusion of the latter two.
3. The core biosynthetic pathways for these pigments are well established; only the regulation of anthocyanins is well understood today.
4. Significant biotechnological opportunities are available for additional modifications of the three types of pigments and for targeting them to particular subcellular compartments.
5. Although it is clear that flower pigmentation plays a central role in attracting pollinators, a unique pigment-pollinator relationship likely does not exist.

UNRESOLVED ISSUES AND FUTURE DIRECTIONS

1. There is a need to understand how the accumulation of carotenoids and betalains is regulated.
2. A better understanding of how the transport of these compounds occurs, and how their sequestration in plastid or vacuoles influences pigmentation, would provide unique opportunities for manipulating flower pigmentation.
3. A molecular explanation for the observed exclusion of anthocyanins and betalains is necessary.

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This study opens fundamental questions regarding the possibility that pigment biosynthetic enzymes have other functions in the nucleus.

This is the first evidence that the regulators of a metabolic pathway may also play a role in providing the proper conditions for storing the resulting pigments.

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This study provides evidence for an unexpected link between the biosynthesis of anthocyanins and condensed tannins (proanthocyanidins).

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