Review

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Biosynthesis of betalains: yellow and violet plant pigments

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Betalains are the yellow and violet pigments that substitute anthocyanins in plants belonging to the order Caryophyllales. These pigments have attracted much attention because of their bioactivities, which range from an antioxidant capacity to the chemoprevention of cancer. However, the biosynthetic pathway of betalains remains under discussion; the main steps have been characterized in recent years, but multiple side reactions are possible. The key enzymes involved have only recently been described, providing clues about the regulation of betalain biosynthesis. In this review, we provide a comprehensive view of the biosynthetic scheme of betalains and discuss the different reactions that have been demonstrated experimentally or proposed in the literature.

Pigments of the Caryophyllales

Betalains are hydrophilic pigments responsible not only for the bright coloration of fruits and flowers, but also of roots and leaves of plants belonging to the order Caryophyllales [1–4]. In this order, the only exceptions are the Caryophyllaceae and Molluginaceae, where coloration is due to anthocyanins. Betalains and anthocyanins are two different families of pigments that are never found together in the same plant. The evolutionary reasons for the apparent mutual exclusion have not been properly explained; however, at the biochemical level, it has been demonstrated that the relevant enzymes for the production of anthocyanins are not expressed in betalain-producing plants [5,6].

There are two types of betalains: betaxanthins, which are immonium derivatives of betalamic acid with different amines and amino acids, and betacyanins, where betalamic acid appears condensed with *cyclo*-dihydroxyphenylalanine (*cyclo*-DOPA). Betaxanthins are yellow and their absorbance spectrum has a maximal wavelength (λ_m) at approximately 480 nm, independent of the amino acid nature. By contrast, betacyanins are violet, with an absorbance spectrum centered at λ_m = 536 nm. The presence of betalains in flowers is particularly interesting because of the importance of color in attracting animals for pollination [7,8]. Flowers are bright violet or yellow in coloration depending on the presence of betacyanins or betaxanthins, respectively. The joint presence of the pigments generates orange to red shades and variegated patterns are also possible [9,10]. Figure 1 shows a selection of betalain-containing plants with colors ranging from yellow to violet. The description of visible fluorescence in betaxanthins and its maintenance in plants suggested the possibility of visible-light emission acting as an additional signal to attract pollinators [9,11,12]. Betalain-related pigments have also been described in the fungi *Amanita* and *Hygrocybe* [13,14]. The presence of analogous pigments in plants and fungi implies an evolutionary convergence based on a characteristic aromatic ring-cleaving dioxygenase enzyme of the biosynthetic route.

Betalains are also bioactive molecules with strong antioxidant activity [15,16]. Studies with different cell lines have revealed that the pigments are active in the dose-dependent inhibition of cancer cell growth and proliferation [17,18]. Dietary betalains have also demonstrated strong health-promoting potential by inhibiting the formation of tumors *in vivo* in mice [19,20]. Unlike other families of plant pigments, the biosynthetic pathway of betalains remains to be fully clarified. Earlier reviews have focused on the description of structures [21,22] and the suitability of pigments for industrial applications [23,24]. In this review, we aim to provide a comprehensive view of each biochemical step involved in the biosynthesis of betalains in plants. Recent findings on the enzyme-catalyzed reactions have given a broader view of the pathway involved in the biosynthesis and transformation of betalains and have started to explain its regulation.

Biosynthesis

Betalains are secondary metabolites derived from the amino acid L-tyrosine. Pioneering experiments with radioactively labeled molecules demonstrated its incorporation into the structural units [25,26] and contributed to an early draft of the biosynthetic pathway. Figure 2 shows a full scheme for the biosynthesis of betalains with all reported reactions in the reference list. L-Tyrosine in plants is assumed to derive from arogenic acid, which is not considered in papers dealing with betalains [27].

Formation of betalamic acid

The pathway involved in the biosynthesis of betalains begins with the hydroxylation of L-tyrosine to L-DOPA through the monophenolase activity of the enzyme tyrosinase (or polyphenoloxidase) (Figure 2, reaction 1). Tyrosinase has been characterized from the betalain-forming plants *Portulaca grandiflora*, *Beta vulgaris*, and *Suaeda salsa* [28–31]. The enzyme has also been purified from the fungus *Amanita muscaria*, which produces betalain-related pigments [32]. In all cases, L-tyrosine hydroxylation activity was detected.

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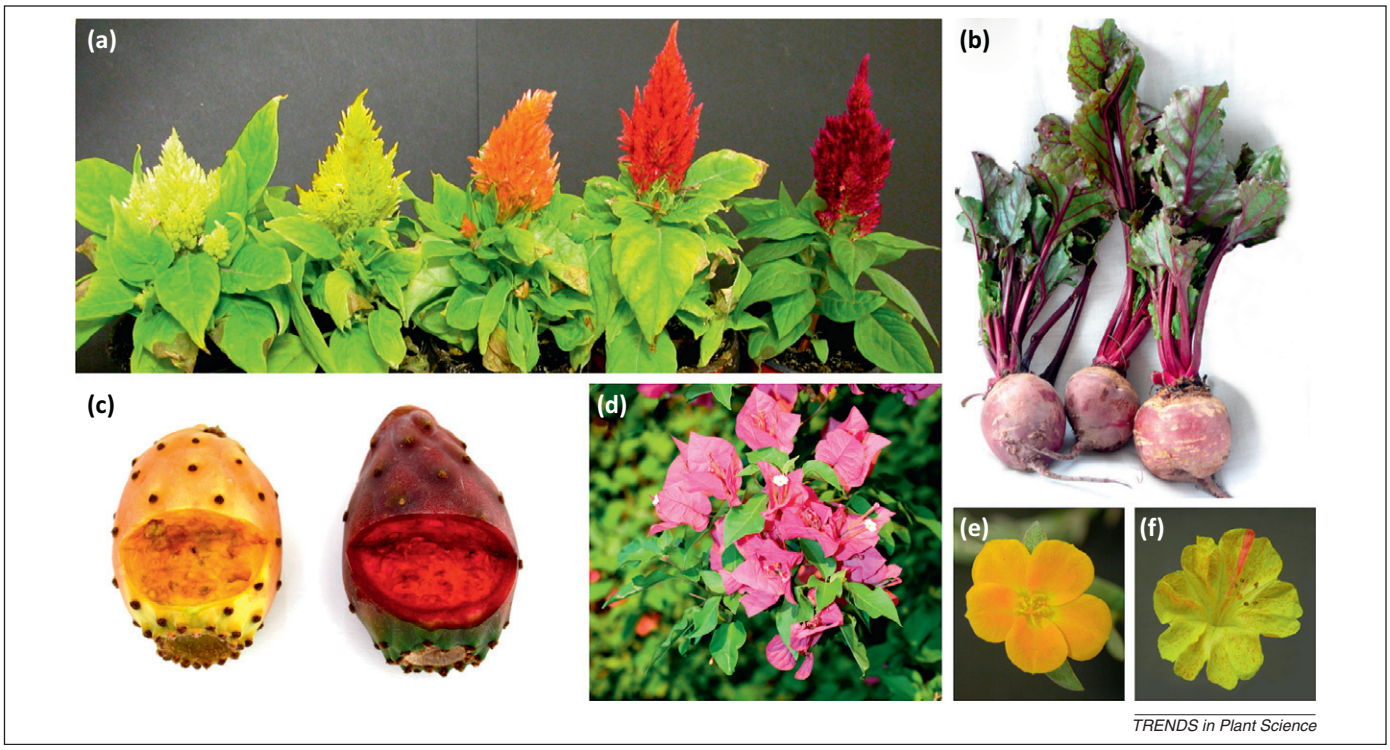

Figure 1. Betalain-containing plants.: **(a)** *Celosia argentea* inflorescences; **(b)** *Beta vulgaris* whole plants; **(c)** *Opuntia ficus-indica* fruits; **(d)** *Bougainvillea glabra* bracts; and **(e)** *Portulaca oleracea* and **(f)** *Mirabilis jalapa* flowers.

The accumulation of L-DOPA, which is necessary for the formation of betalamic acid (Figure 2, reaction 2), requires stopping the oxidation of L-DOPA to *o*-DOPA-quinone (Figure 2, reaction 5) performed by the diphenolase activity of the same enzyme. This reaction may be necessary for the formation of part of the structure of betacyanins, as discussed below, but will consume all of the formed L-DOPA as the substrate [33]. Thus, the presence of ascorbic acid or an analogous reducing agent is necessary to transform *o*-DOPA-quinone back to L-DOPA (Figure 2, reaction 6). This makes L-DOPA available for further steps. Ascorbic acid has been found in significant amounts in vegetable material containing betalains [34–36]. L-DOPA is then the substrate for the second enzyme of the route, 4,5-DOPA-extradiol-dioxygenase, which catalyzes the extradiol cleavage of L-DOPA to form the intermediate 4,5-seco-DOPA (Figure 2, reaction 2). The protein was first characterized from *A. muscaria* extracts [37]. The fungal enzyme produces betalamic acid accompanied by the related pigment muscaflavin, which is found exclusively in the fungus. This is due to an additional 2,3-DOPA-extradiol dioxygenase activity that leads to a 2,3-seco-DOPA intermediate in addition to the plant 4,5-seco-DOPA product [38]. In plants, the enzymes from *P. grandiflora*, *Mirabilis jalapa*, and *B. vulgaris* [39–41] have been functionally characterized, showing 4,5-DOPA-extradiol-dioxygenase activity exclusively.

Betalamic acid is derived from 4,5-seco-DOPA by spontaneous intramolecular condensation between the amine group present in L-DOPA and the enzymatically produced aldehyde group [26] (Figure 2, reaction 3). The acid presents a structural feature not well understood in terms of the chiral carbon configuration. *In planta*, pigments derived from betalamic acid are frequently reported to show 95% of the chiral (S) isomer and 5% of the (R) form. Furthermore, the same proportions have been reproduced *in vitro* for betalamic acid generated from stereopure L-DOPA in dioxygenase enzymatic assays [40,41]. This finding points to reactions 2 and 3 (Figure 2) as being responsible for the isomer proportions found in nature, although a plausible mechanistic explanation has not been given.

Formation of betalains

The variety of betalains described incorporate one unit of betalamic acid in the structure that supports the chromogenic electron resonance system. Depending on the nature of the condensed molecule, different spectroscopic properties are obtained [42]. *In planta*, the yellow betaxanthins are obtained by condensation of betalamic acid with amines and amino acids (Figure 2, reaction 4). It is assumed that the reaction occurs spontaneously between the amine group of the amine and the aldehyde group of betalamic acid to form the corresponding imine [43,44]. This reaction can be reproduced *in vitro* to form semisynthetic betalains [45]. The diversity of amines available in plants makes it difficult to estimate the real number of plausible betaxanthins in nature. Previously unconsidered compounds have been described in recent years owing to the application of increasingly sensitive techniques. Table 1 shows the 31 betaxanthins that have been structurally identified to date in plants. Among the natural betaxanthins, the L-tyrosine- and L-DOPA-derived compounds will receive further attention because of their role as substrates for tyrosinase.

We will first discuss the formation of betacyanins through the condensation with free L-DOPA derivatives.

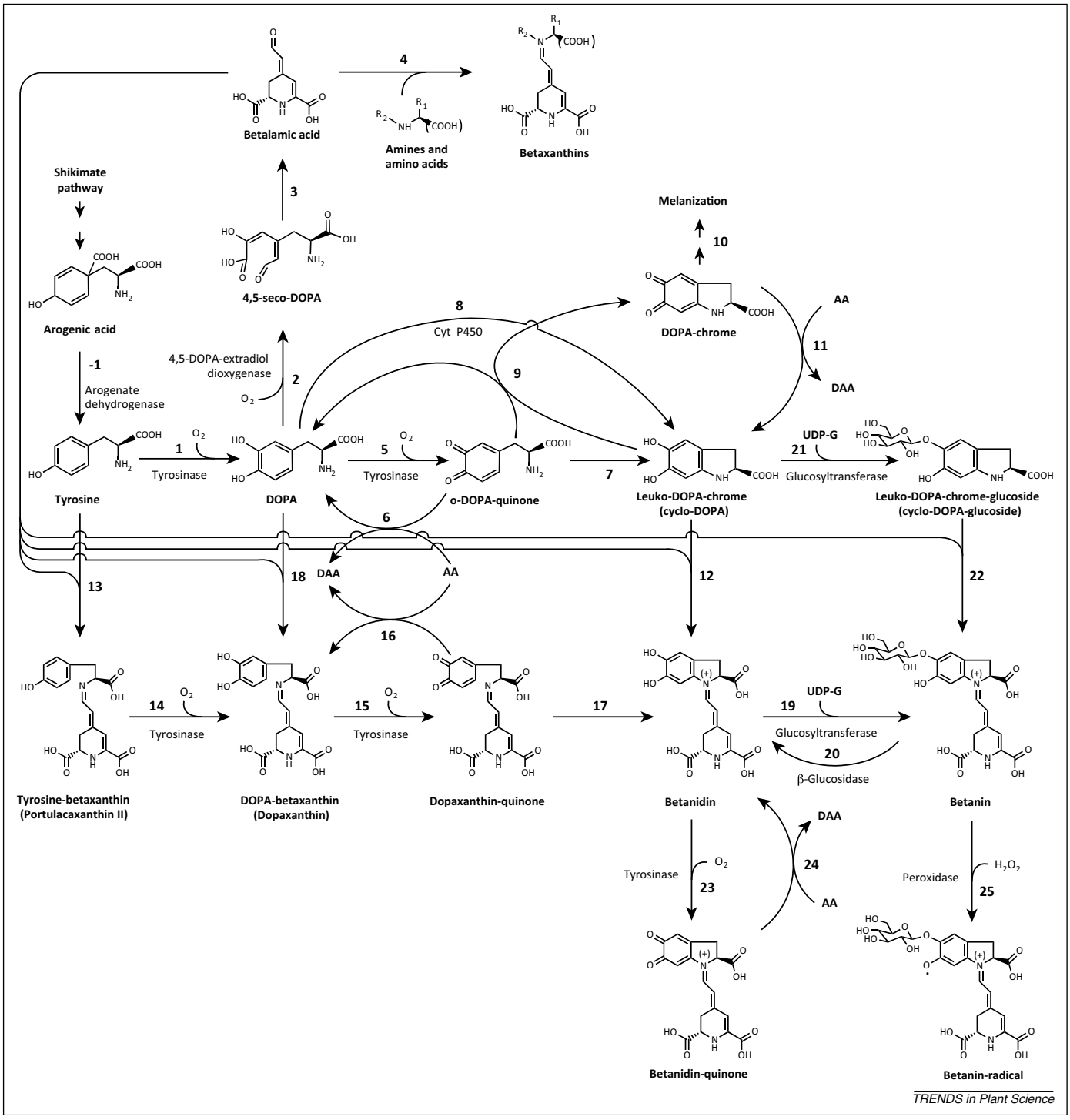

Figure 2. Full scheme for the biosynthetic pathway of betalains. All reactions reported in the bibliography to date are represented. Abbreviations: AA, ascorbic acid; cyt P450, cytochrome P450; DAA, dehydroascorbic acid.

L-DOPA in the absence of a reducing agent is transformed by tyrosinase to *o*-DOPA-quinone using molecular oxygen (Figure 2, reaction 5). Then, the amine group of the *o*-quinone performs an intramolecular nucleophilic attack on the ring, leading to spontaneous cyclization that forms the molecule leuko-DOPA-chrome, also known as *cyclo*-DOPA [46–48] (Figure 2, reaction 7). Alternatively, the conversion of L-DOPA into *cyclo*-DOPA can be performed by a cytochrome P450, as shown in *B. vulgaris* [49]. Although the mechanism involved in the formation of *cyclo*-DOPA has not been identified, only two possibilities seem reasonable: a classical oxidation or a stepwise electron transfer (one-electron oxidation) [50]. In both, DOPA-quinone would be an intermediate oxidation product before the intramolecular nucleophilic attack occurs, in agreement with the reactions described for tyrosinase (Figure 2, reactions 5 and 7). However, because of the lack of information on the DOPA oxidation mechanism for the recently discovered cytochrome P450 subfamily [49], this reaction is shown separately in Figure 2 (reaction 8). In any case, *cyclo*-DOPA has

Table 1. Naturally occurring betaxanthins structurally identified in plants

Trivial name	Amine group	Biological source	Refs
Indicaxanthin	Proline	*Opuntia ficus-indica*	[87]
Vulgaxanthin I	Glutamine	*Beta vulgaris*	[88]
Vulgaxanthin II	Glutamic acid	*B. vulgaris*	[88]
Miraxanthin I	Methionine sulfoxide	*Mirabilis jalapa*	[89]
Miraxanthin II	Aspartic acid	*M. jalapa*	[89]
Miraxanthin III	Tyramine	*M. jalapa*	[89]
Miraxanthin V	Dopamine	*M. jalapa*	[89]
Portulacaxanthin I[a]	Hydroxyproline	*Portulaca grandiflora*	[90]
Dopaxanthin	Dihydroxyphenylalanine	*Glottiphylum longum*	[91]
Humilixanthin	5-Hydroxynorvaline	*Rivina humilis*	[92]
Portulacaxanthin II	Tyrosine	*P. grandiflora*	[93]
Portulacaxanthin III	Glycine	*P. grandiflora*	[93]
Vulgaxanthin III	Asparagine	*B. vulgaris*	[94]
Vulgaxanthin IV	Leucine	*B. vulgaris*	[94]
Muscaaurin VII[b]	Histidine	*B. vulgaris*	[94]
–	Tryptophan	*Celosia argentea*	[67]
–	3-Methoxytyramine	*C. argentea*	[67]
–	γ-Aminobutyric acid	*B. vulgaris, O. ficus-indica*	[95]
–	Serine	*B. vulgaris, O. ficus-indica*	[95]
–	Valine	*B. vulgaris, O. ficus-indica*	[95]
–	Isoleucine	*B. vulgaris, O. ficus-indica*	[95]
–	Phenylalanine	*B. vulgaris, O. ficus-indica*	[95]
–	Alanine	*B. vulgaris*	[96]
–	Histamine	*B. vulgaris*	[96]
–	Methionine	*O. ficus-indica*	[97]
–	Arginine	*Gomphrena globosa*	[98]
–	Putrescine	*Bougainvillea* sp.	[98]
–	Lysine	*Bougainvillea* sp.	[98]
–	Ethanolamine	*B. vulgaris*	[99]
–	Threonine	*B. vulgaris*	[99]
–	Phenethylamine	*O. ficus-indica*	[1]

[a]Originally named 'portulacaxantina'.

[b]This pigment was previously identified in the fungus *Amanita muscaria* [100].

been proposed to react with betalamic acid in the same manner as any other molecule with an amine group [51]. The pigment formed is betanidin (Figure 2, reaction 12), which is the key intermediate in the formation of betacyanins. These proposed reactions must tackle the instability of leuko-DOPA-chrome, which experiences spontaneous oxidation to DOPA-chrome with the concomitant reduction of a molecule of DOPA-quinone back to L-DOPA (Figure 2, reaction 9) [52,53]. This means that, in the absence of a reducing agent, DOPA-chrome and not *cyclo*-DOPA is present in the medium. In addition, DOPA-chrome will evolve further to form the brown polymers characteristic of enzymatic browning [54] (Figure 2, reaction 10). The only way to obtain leuko-DOPA-chrome from L-tyrosine or L-DOPA is by reaching the DOPA-chrome state in the absence of a reducing agent and, before brown polymers are formed, reacting with a reducing agent that is able to transform it to leuko-DOPA-chrome (Figure 2, reaction 11). Although these steps have been conducted *in vitro* and the *cyclo*-DOPA formed yielded betanidin after the addition of betalamic acid [51] (Figure 2, reaction 12), this mechanism seems unlikely to occur at a biological level. In addition, L-DOPA is not accumulated before pigment development occurs in betalain-producing tissues and its concentration is constant. The relevant metabolite accumulated before pigment formation, and which decreases during the process, is L-tyrosine [55].

Formation of betanidin can also be explained by considering a role for tyrosine-betaxanthin and dopaxanthin. The first is obtained by the condensation of L-tyrosine with betalamic acid (Figure 2, reaction 13). This pigment is a substrate for the monophenolase activity of tyrosinase, which catalyzes its conversion to the second pigment dopaxanthin (Figure 2, reaction 14) [35]. Dopaxanthin itself has also been demonstrated to be a substrate for tyrosinase, which converts it to dopaxanthin-quinone (Figure 2, reaction 15). As happens with free L-DOPA, if dopaxanthin is to be maintained in the presence of tyrosinase, a reducing agent is necessary to revert the *o*-quinone to the initial pigment (Figure 2, reaction 16). In plants, flowers containing dopaxanthin have high levels of ascorbic acid to protect it from oxidation [35]. In the absence of a reducing agent, dopaxanthin-quinone reactivity promotes an intramolecular nucleophilic attack, causing its cyclization to molecules analogous to betanidin, with identical molecular weights and properties, in line with the reactions experienced by L-DOPA [52]. In contrast to the unique leuko-DOPA-chrome derived from free L-DOPA (Figure 2, reaction 7), the dopaxanthin additional structure extends the nucleophilic power along the resonance

system and multiple cycled molecules are possible. Conditions for the formation of betanidin from dopaxanthin-quinone (Figure 2, reaction 17) have not been determined, but the pathway is plausible and the formation of betanidin may be favored *in vivo*. Another way to join this branch implies the condensation of betalamic acid with L-DOPA to form dopaxanthin (Figure 2, reaction 18). Condensation of betalamic acid with *o*-DOPA-quinone in a short cut to the same branch has not been proposed. Although the amine group is still available to react with the aldehyde group of betalamic acid, the DOPA-quinone chemical instability might avoid this reaction.

Reactions on betacyanins

Betanidin can be transformed into betanin by the enzyme betanidin-5-*O*-glucosyltransferase, which incorporates a glucose residue to the hydroxyl group in position 5 (Figure 2, reaction 19) [56]. A betanidin-6-*O*-glucosyltransferase has also been purified and characterized from the plant *Dorotheanthus bellidiformis* [57] that catalyzes the incorporation of glucose to the other hydroxyl group of betanidin, forming the pigment gomphrenin I. In both cases, further glycosylations and acylations lead to the wide variety of betacyanins identified in natural extracts (summarized in [21]). However, the enzymes or the conditions involved in these processes have not been identified. The generated pigments maintain spectroscopic properties analogous to those of betanidin, preventing its lability [36,58]. Transformation of betanin back to betanidin is possible owing to the activity of β-glucosidase (Figure 2, reaction 20) [59]. Although the physiological relevance of the process is unknown, a role in the catabolism of pigments is probable. In contrast to the general acceptance of betanidin glucosylation, it has also been proposed that betanin is instead formed by the action of a 5-*O*-*cyclo*-DOPA glucosyltransferase that catalyzes the sugar transfer to *cyclo*-DOPA (Figure 2, reaction 21) and subsequent condensation of the derived glucoside with betalamic acid (Figure 2, reaction 22) [60]. This activity has been found in *M. jalapa* and preliminary tests have been performed in other betalain-producing plants [61]. By additional acylation of *cyclo*-DOPA-glucoside it is possible to yield more complex betacyanins after condensation with betalamic acid [62].

Regardless of the role played by tyrosinase in the formation of betanidin, the pigment is a substrate for the enzyme (Figure 2, reaction 23) [36]. This implies the formation of betanidin-quinone, which experiences no further evolution owing to its cycled and condensed nature. Ascorbic acid has been described in betanidin-containing flowers and reverts the quinone to the original pigment (Figure 2, reaction 24) [36]. Although glucosylation protects betanin from the lability described for betanidin, the remaining hydroxyl group can still be oxidized by the enzyme peroxidase (Figure 2, reaction 25). Betanin is transformed into a betanin phenoxy radical that can experience further evolution involving the hydrolysis of the molecule [63]. The oxidation products of betanin are also able to rearrange and yield dehydrogenated and decarboxylated betacyanins [64]. Peroxidase can also act on betanidin to catalyze its conversion to betanidin-quinone through a radical mechanism. Depending on the pH, this may lead to the generation of dehydrogenated and decarboxylated derivatives of betanidin-quinone [64,65].

Formation of decarboxylated betalains

Decarboxylated betacyanins have been described in extracts from *Carpobrotus acinaciformis* [66] and *Celosia* sp. [67] and in hairy root cultures of *B. vulgaris* [43,68]. These betacyanins contain a leuko-dopamine-chrome moiety instead of the leuko-DOPA-chrome present in the more common compounds. The exact point in the route for decarboxylation is currently unknown and two alternatives are possible. Formation of decarboxy-betacyanins from betaxanthins may occur in the same way as mentioned for the carboxylated pigments through tyrosinase-mediated reactions (Figure 2, equivalent reactions 14 and 15) [69]. Decarboxylated analogs of tyrosine-betaxanthin and DOPA-betaxanthin are tyramine-betaxanthin (miraxanthin III) and dopamine-betaxanthin (miraxanthin V), respectively. This possibility is supported by the joint presence of dopamine-betaxanthin and decarboxylated betacyanins in *B. vulgaris* [43] and *Celosia* sp. [67]. Decarboxylation may also occur at the free L-tyrosine or L-DOPA level, to form tyramine or dopamine, respectively. Tyrosinase-mediated and spontaneous reactions can lead to dopamine-chrome (Figure 2, equivalent reactions 5 and 9), which could be transformed to leuko-dopamine-chrome (Figure 2, equivalent reaction 11) by a reducing agent. Condensation with betalamic acid could then occur to form decarboxylated betacyanins [68]. In addition, the peroxidase-mediated reactions mentioned above [64] should be taken into account in further studies on the formation of decarboxylated betalains both *in planta* and as artifacts during pigment extraction.

Key enzymes

The biosynthetic pathway of betalains includes three main enzymes. Two are oxidases that depend on molecular oxygen for the catalysis: tyrosinase and 4,5-DOPA-extradiol-dioxygenase. The other enzyme decorates the structural unit of betacyanins by transferring a sugar residue: betanidin-glucosyltransferase. The key enzymes involved in the route are regulated at the transcription level in plants [40,70,71].

4,5-DOPA-extradiol-dioxygenase

4,5-DOPA-extradiol-dioxygenase catalyzes the transformation of L-DOPA to 4,5-seco-DOPA (Figure 2, reaction 2), which spontaneously yields betalamic acid, the structural and chromophoric unit of betalains. This activity has been described only in plants belonging to the order Caryophyllales [39] and can be considered the characteristic enzyme of the biosynthetic pathway.

Dioxygenases are non-heme iron-containing proteins that catalyze the ring cleavage of catechol derivatives, incorporating both atoms of molecular oxygen [72]. In the case of plant enzymes involved in the formation of betalains, the limited number of sequences available is shown in Figure 3, with only three proteins partially characterized. The first experimental identification of a 4,5-DOPA-extradiol-dioxygenase responsible for betalain

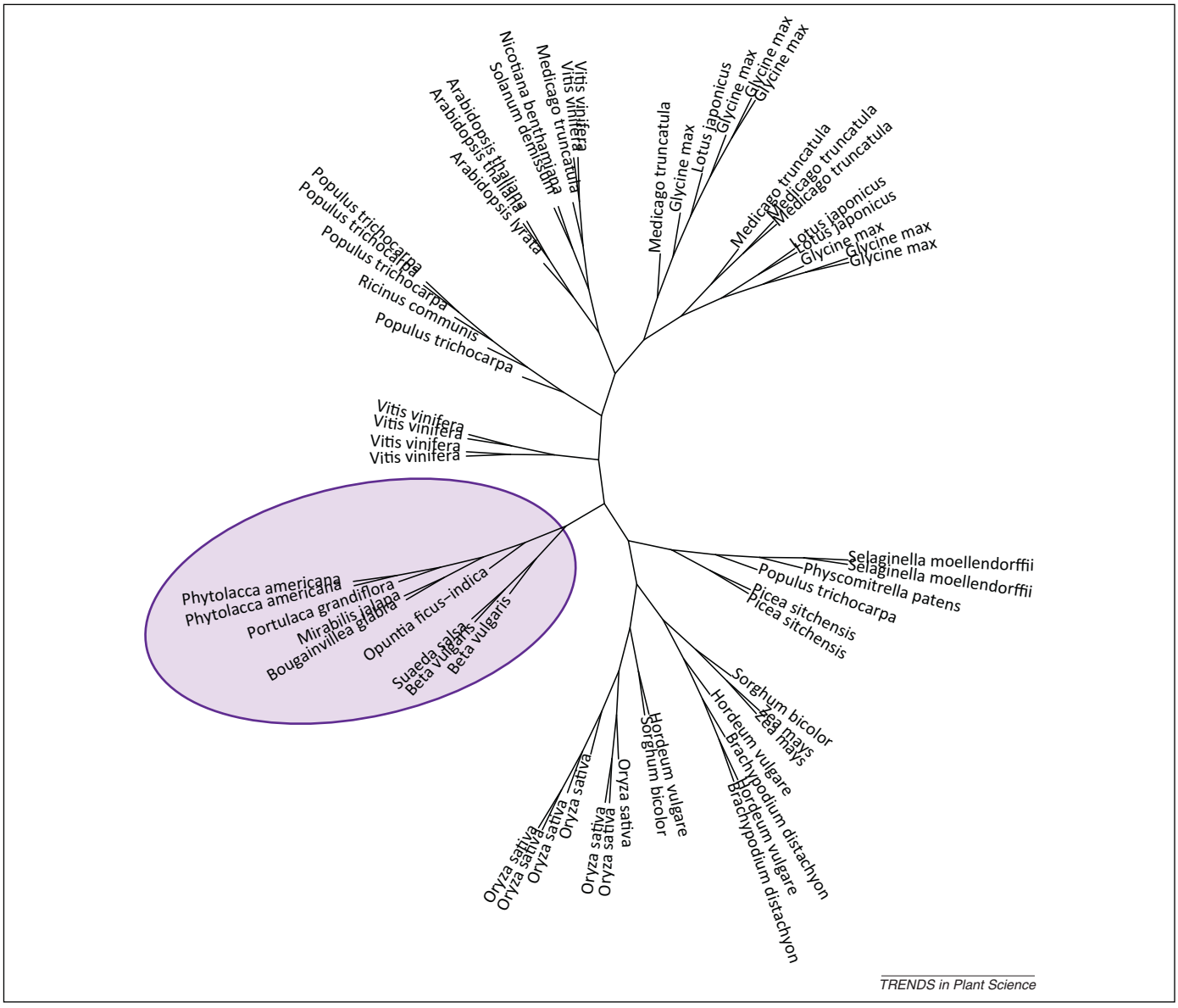

Figure 3. Phylogenetic analysis of plant 4,5-dihydroxyphenylalanine (DOPA)-dioxygenases. Sequences were retrieved from a basic local alignment search (BLAST) (National Center for Biotechnology Information, NCBI). The 4,5-DODA sequence of *Beta vulgaris* was used as a query selecting results with total score >100 (*E* value < 1e-25). Multiple sequence alignment was performed using ClustalW2. The unrooted tree was obtained with the Phylogeny inference package PHYLIP using the Neighbor-Joining algorithm. Accession codes (GenBank) for sequences from betalain-producing plants (highlighted in purple) are: *Beta vulgaris* (gi:34850080, 356968414, 356968424, 356968412), *Portulaca grandiflora* (gi:42558922), *Opuntia ficus-indica* (gi:158714015, 158714117), *Phytolacca americana* (gi:238767594, 238767592), *Mirabilis jalapa* (gi:209976918), *Bougainvillea glabra* (gi:209976920), and *Suaeda salsa* (gi:226510654) [39,40,49,97,101–103]. Accession codes for the non-betalain-producing plants: *Arabidopsis lyrata* (gi:297804756), *A. thaliana* (gi:18414376, 2244866), *Brachypodium distachyon* (gi:357126179, 357126177), *Glycine max* (gi:356559959, 356559963, 255644965, 363807760, 356576585, 356530981, 255646644), *Hordeum vulgare* (gi:326510245, 326512764, 326494650), *Lotus japonicus* (gi:388501808, 388514737, 388491700), *Medicago truncatula* (gi:357498833, 217075713, 388491364, 357504155, 357497583), *Nicotiana benthamiana* (gi:156254540), *Oryza sativa* (gi:218189470, 115441407, 21952792, 115441405, 125572849, 125528585, 125572848), *Physcomitrella patens* (gi:168065783), *Picea sitchensis* (gi:116784335, 116792508), *Populus trichocarpa* (gi:224080093, 224080091, 224145632, 224110828, 222870216), *Ricinus communis* (gi:255553957), *Selaginella moellendorffii* (gi:302806409, 302808895), *Solanum demissum* (gi:47900738), *Sorghum bicolor* (gi:242059425, 242059427), *Vitis vinifera* (gi:359480904, 147844961, 359480925, 296084145, 296089737), and *Zea mays* (gi:226506364, 194703282).

formation in plants was obtained from *P. grandiflora* [39]. White petal cells were transformed by biolistic genetic complementation to pigmented ones in a neat demonstration of the protein function. Previously the 4,5-dioxygenase from *A. muscaria*, showing additional 2,3-dioxygenase activity, was also used to develop color in a transient expression study in *P. grandiflora* [73] and more recently with constitutive expression in *Arabidopsis thaliana* [74]. Recombinant protein expression in bacteria was first achieved for the 4,5-DOPA-dioxygenase from *M. jalapa* [40], demonstrating *in vitro* the formation of betalamic acid. The first betalamic acid-forming enzyme to be purified was from *B. vulgaris* [41]. This enabled the characterization of a monomeric active form of 32 kDa and the determination of the kinetic parameters on the relevant substrate L-DOPA. Regulation of betalain-producing 4,5-dioxygenase in plants has been studied for flowering *M. jalapa* [40]. Enzyme transcripts were detected in developing colored flowers, particularly at the final stages, but not in other parts of the plant. Analogously, northern-blot analyses performed on the enzyme, which had been obtained from *A. muscaria*, revealed regulation at the transcription level, with mRNA accumulated mainly in the colored tissues in the cap [75].

Intramolecular cyclization of the enzyme product 4,5-secodopa to betalamic acid (Figure 2, reaction 3) is assumed to be spontaneous. However, the secodopas can be found in nature without further evolution [76]. Furthermore, only the 4,5-DOPA-dioxygenases of plants belonging to the Caryophyllales are involved in the formation of betalains. This suggests that the betalamic acid-forming activity may be favored by the appropriate enzymes by providing a suitable environment for the condensation of the intermediate. Sequence analysis reveals that those 4,5-DOPA-dioxygenases that are involved in the formation of betalains share a group of conserved amino acids close to the active site that is not present in other plant dioxygenases [39]. This could help in the condensation to betalamic acid. Phylogenetic analysis of currently sequenced dioxygenases clearly shows that those belonging to the betalain-forming plants form a group apart from the other plant homologs (Figure 3).

Tyrosinase

Tyrosinase has been described in five different steps in the biosynthetic route of betalains (Figure 2, reactions 1, 5, 14, 15, and 23). It is widely distributed in nature and its mechanism of action is well known, catalyzing the hydroxylation of monophenols to *o*-diphenols and the oxidation of the *o*-diphenols to *o*-quinones. There is a wealth of literature devoted to the enzyme that analyzes its mechanism and describes the general features of the tyrosinase family [77,78]. However, its role in the formation of low molecular weight metabolites was ignored for a long time in favor of its implicated involvement in the production of melanin-type compounds (Figure 2, reactions 1 and 5–10).

Regardless of the nature of the tyrosinase-catalyzed reactions in the biosynthetic pathway of betalains, the activity of tyrosinase could be highly regulated to contribute to the formation of pigments at a precise time and in the precise tissues. Tyrosinase transcripts have been detected in fruits of *Phytolacca americana* correlating with the accumulation of betacyanins [70]. Tyrosinase activity has also been related to the accumulation of pigments in *B. vulgaris* and *S. salsa* [28,31]. In addition to transcriptional control, regulation of the *B. vulgaris* enzyme has been described according to the presence of activating agents and proteases that are able to transform a latent form into the fully active enzyme [79,80].

In contrast to 4,5-DOPA-extradiol-dioxygenases, a sequence signature characteristic for tyrosinases from betalain-producing plants has not been identified. This is probably due to the low number of tyrosinase sequences available from plants of the Caryophyllales: spinach (*Spinacia oleracea*) and *P. americana* (gi:642023, 1052516, 984206, 1752723, 1741861) [70,81,82]. However, a suitable environment provided by the enzyme that is able to promote the cyclization of the product dopaxanthin-quinone to betanidin (Figure 2, reaction 17) has been proposed in accordance with the results obtained for the tyrosinase involved in the biosynthesis of the plant pigments known as aurones [35,36,83]. The reactions ascribed to tyrosinase in the biosynthetic pathway of betalains could also be catalyzed by separate enzymes. In this sense, a cytochrome P450 has been shown to convert L-DOPA into *cyclo*-DOPA (Figure 2, reaction 8) by genetic complementation and gene silencing in *B. vulgaris* [49]. This enzyme (gi:356968415) is the first member of a new cytochrome P450 subfamily with homologs identified in other betalain-forming plants: *Amaranthus cruentus* (gi:356968419) and *M. jalapa* (gi:356968421).

Betanidin glucosyltransferases

Glycosyltransferases catalyze the transfer of sugar moieties to specific acceptors as environmental toxic compounds, hormones, and secondary metabolites, including pigments [84]. The addition of glucose to betanidin is specifically directed at one of the two hydroxyl groups to generate betanin (Figure 2, reaction 19) or gomphrenin I. Betanidin glucosyltransferases were first purified and characterized from *Dorotheanthus bellidiformis* (gi:5918023, 18033791) [57,85] and the active site was characterized by homology modeling and site-directed mutagenesis, successfully explaining the transformation of betanidin into betanin [56]. Despite the structural analogies with betacyanins, no evidence for glycosylation in betaxanthins has been reported *in vivo* or *in vitro*.

There are 31 glucosyltransferase sequences deposited to date in the databases belonging to betalain-producing plants. However, just a few have been demonstrated to be active towards substrates in the pathway. A glucosyltransferase from *B. vulgaris* (gi:29692096) has been shown to be involved in the formation of betanin [71] and other sequences from the same plant (gi:46430997, 46430995) glucosylate betanidin to a lesser extent than other substrates [86]. In *M. jalapa*, a glucosyltransferase involved in the metabolism of betalains has also been characterized (gi:62086401) [60,61], although activity was detected on *cyclo*-DOPA instead of on betanidin (Figure 2, reaction 21). The same activity has been reported for *Celosia cristata* glucosyltransferase (gi:62086403) [61]. Glucosylation regulation has been shown in *B. vulgaris* at the transcriptional level, with the correlation between transcript accumulation and pigment formation demonstrated with antisense constructs [71]. Glucosyltransferase activity and changes in the amount of transcripts of the enzyme also correlated positively with betanin accumulation in developing flowers of *M. jalapa* [60,61].

Concluding remarks

The biosynthetic pathway of the betalains remains an open question. Multiple reactions have been experimentally demonstrated to be plausible in recent years. The use of molecular tools that were unavailable at the early conception of the pathway has demonstrated the pivotal role of the specific 4,5-DOPA-extradiol-dioxygenase enzyme. The derived product betalamic acid is the starting point for pigment biosynthesis. Its condensation with amines and amino acids to form yellow pigments seems to be spontaneous. However, the reason specific pigments are found in extracts instead of a wide variety of pigments reproducing the amino acid profile of the plants has not been explained and suggests some sort of direction. Less clear is the biosynthesis of the violet pigments, the betacyanins, and their structural unit betanidin. Formation from two units of L-tyrosine implies the activity of oxidase enzymes such

as tyrosinase or the recently described cytochrome P450, before glucosyltransferases may act on the available hydroxyl groups. Given the different levels at which these enzyme-catalyzed processes may occur, and the existence of chemical side reactions, the biosynthesis of betacyanins could occur in alternative ways that have yet to be determined. Further investigations should be able to elucidate the biochemical steps involved in the formation of the pigments, which should transform the biosynthetic net of betalains into a more linear scheme.

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