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The evolution of betalain biosynthesis in Caryophyllales

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Summary

Within the angiosperm order Caryophyllales, an unusual class of pigments known as betalains can replace the otherwise ubiquitous anthocyanins. In contrast to the phenylalanine-derived anthocyanins, betalains are tyrosine-derived pigments which contain the chromophore betalamic acid. The origin of betalain pigments within Caryophyllales and their mutual exclusion with anthocyanin pigments have been the subject of considerable research. In recent years, numerous discoveries, accelerated by -omic scale data, phylogenetics and synthetic biology, have shed light on the evolution of the betalain biosynthetic pathway in Caryophyllales. These advances include the elucidation of the biosynthetic steps in the betalain pathway, identification of transcriptional regulators of betalain synthesis, resolution of the phylogenetic history of key genes, and insight into a role for modulation of primary metabolism in betalain synthesis. Here we review how molecular genetics have advanced our understanding of the betalain biosynthetic pathway, and discuss the impact of phylogenetics in revealing its evolutionary history. In light of these insights, we explore our new understanding of the origin of betalains, the mutual exclusion of betalains and anthocyanins, and the homoplastic distribution of betalain pigmentation within Caryophyllales. We conclude with a speculative conceptual model for the stepwise emergence of betalain pigmentation.

I. Introduction

Betalains are water-soluble, tyrosine-derived pigments which can be biochemically defined by their inclusion of betalamic acid as the central chromophore (Stafford, 1994; Tanaka *et al.*, 2008). Betalains comprise two classes of compounds, betaxanthins and betacyanins. Betaxanthins are derived from betalamic acid via conjugation with different amines and amino acids, and betacyanins are derived by condensation of betalamic acid with cyclo-dihydroxyphenylalanine (*cyclo*-DOPA) (Stafford, 1994; Tanaka *et al.*, 2008). Betaxanthins are yellow with an absorbance spectrum that has a maximal wavelength centred at 480 nm, while betacyanins are violet, and have an absorbance spectrum with a maximal wavelength centred at 536 nm (Gandía-Herrero & García-Carmona, 2013). Betalains function in the attraction of animal pollinators and dispersers, in photoprotection, and confer tolerance to drought and salinity stress (Jain & Gould, 2015). Betalains possess high antioxidant and free radical scavenging activities (Escribano *et al.*, 1998; Cai *et al.*, 2003), are utilized as commercial food colourants and additives (Gandía-Herrero *et al.*, 2013), are being explored for their use as pigments in solar panels (Oprea *et al.*, 2012), and exhibit preventative properties with respect to several types of cancer (Lu *et al.*, 2009; Khan *et al.*, 2012; Krajka-Kuźniak *et al.*, 2012). Furthermore, intermediates in the betalain pathway are important pharmaceuticals (e.g. L-3,4-dihydroxyphenylalanine (L-DOPA) for the treatment of Parkinson's disease) or are substrates for other pharmaceutical agents (e.g. the production of dopamine and isoquinoline alkaloids such as morphine) (Galanie *et al.*, 2015). Given the clear technological applications of betalains, and the benefits of betalains and their intermediates for human health and nutrition, there is considerable interest in the heterologous expression of the betalain biosynthesis pathway in a range of platforms, including food crops (Harris *et al.*, 2012; Hatlestad *et al.*, 2012) and microbial hosts such as *Saccharomyces cerevisiae* (DeLoache *et al.*, 2015; Grewal *et al.*, 2018).

Betalains are known to be produced in three divergent lineages of organisms: the fungal lineage, Basidiomycota; the flowering plant lineage, Caryophyllales (Brockington *et al.*, 2011); and the bacterial species, *Gluconacetobacter diazotrophicus* (Contreras-Llano *et al.*, 2019). The core Caryophyllales is a well-defined clade of angiosperms comprising *c.* 29 families and *c.* 9000 species (Bremer *et al.*, 2003). Anthocyanins have never been detected within betalain-producing species (Bate-Smith, 1962; Clement & Mabry, 1996); however, other flavonoid-derived compounds, such as proanthocyanidins, can be found in the seed coat of some betalain-pigmented species (e.g. *Spinacia*) (Shimada *et al.*, 2004, 2005). These observations imply that betalain pigmentation can substitute for the otherwise ubiquitous anthocyanic pigmentation (Bischoff, 1876; Clement & Mabry, 1996), which is the dominant form of pigmentation across land plants (Campanella *et al.*, 2014) (Fig. 1). Most families within the core Caryophyllales are betalain-pigmented, with the exception of Caryophyllaceae, Molluginaceae *sensu strictu*, Kewaceae, Limeaceae, Macarthuriaceae and Simmondsiaceae, which have been reported to produce anthocyanins (Clement & Mabry, 1996; Thulin *et al.*, 2016). These six anthocyanic lineages are scattered across the core Caryophyllales, either sister to or nested within betalain-pigmented lineages resulting in a homoplastic distribution of these two pigments (Brockington *et al.*, 2011, 2015) (Fig. 2). Betalains have never been detected or reported in any of these six anthocyanic lineages, drawing a clear pattern of mutual exclusion between the two pigment types (Stafford, 1994; Clement & Mabry, 1996).

In recent years, numerous developments have increased our understanding of betalain biosynthesis, shedding light on the evolution of the betalain biosynthesis pathway and the distinctive evolutionary patterns that typify betalain pigmentation. Here, we begin by reviewing how molecular genetics have advanced our current understanding of the betalain biosynthetic pathway, and discuss the impact of phylogenetics in revealing the evolutionary history of key genes encoding the enzymes of the pathway. In light of these insights, we revisit our understanding of the three key evolutionary patterns that characterize the betalain phenomenon in Caryophyllales, namely: the origin of betalains in Caryophyllales; the mutual exclusion of betalains and anthocyanin pigments; and the homoplastic distribution of betalain pigmentation within Caryophyllales. Finally, we conclude by synthesizing these data into a conceptual stepwise model of how betalain pigmentation has evolved within Caryophyllales.

II. The betalain biosynthesis pathway

Betalains are synthesized from tyrosine, which is derived from the shikimate pathway. In most plants, the precursor arogenate is decarboxylated into tyrosine, by the action of arogenate dehydrogenase (ADH; Rippert *et al.*, 2009). Following tyrosine synthesis, the betalain pathway comprises three main steps requiring enzymatic catalysis (Fig. 3). Tyrosine is first converted to L-DOPA through a tyrosine hydroxylation reaction, catalysed by cytochrome P450 enzymes. The cyclic ring within L-DOPA is then cleaved in a ring-opening oxidation reaction by the enzyme L-DOPA 4,5-dioxygenase (DODA) to produce the intermediate 4,5-seco-DOPA, which then undergoes spontaneous intramolecular condensation to produce betalamic acid. Alternatively, L-DOPA can be oxidized to dopaquinone after which it cyclizes to form *cyclo*-DOPA, a step that is also catalysed by a cytochrome P450 enzyme. Betalamic acid can spontaneously conjugate with the imino group of *cyclo*-DOPA (Delgado-Vargas & Paredes-López, 2003), ultimately leading to the formation of red-violet betacyanins. The alternative fate of betalamic acid is to spontaneously condense with the imino or amino group of amino acids to give yellow betaxanthins. In addition to these central enzymatic steps, additional moieties such as glucosyl or acyl groups can be enzymatically added to betacyanins. Glycosylation can occur either on *cyclo*-DOPA before condensation with betalamic acid, or on betanidin after the condensation of *cyclo*-DOPA and betalamic acid, catalysed by *cyclo*-DOPA 5-*O*-glucosyltransferase (*c*DOPA5GT) or betanidin glucosyl-transferases, respectively (Fig. 3). In addition to glycosylation, betacyanins can undergo

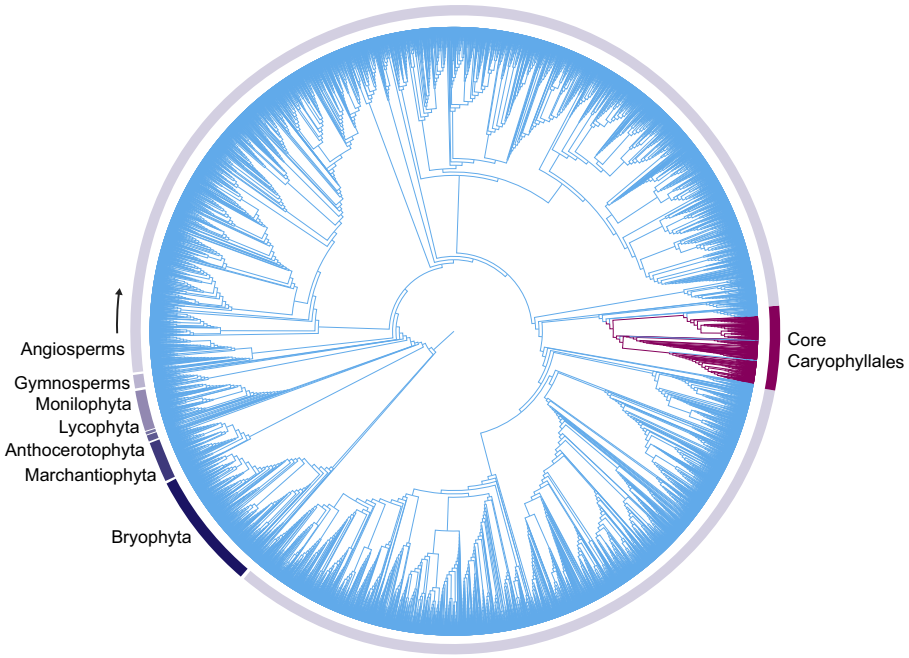

Fig. 1 A generic-level phylogenetic tree of the land plants, illustrating the predominant flavonoid-based pigments (blue) and the origin of betalains to Caryophyllales.

other enzymatically catalysed modifications, which can add a range of moieties, contributing to the structural diversity of betalains. In the following sections, we sequentially discuss the molecular genetic characterization of the enzymes implicated in betalain biosynthesis, and, for each enzyme, outline the phylogenetic context and evolutionary history of the encoding loci.

III. Arogenate dehydrogenase

1. Characterization of a deregulated ADH

Recent research has emphasized a role for modulation of primary metabolism in the evolution of the betalain biosynthesis pathway (Lopez-Nieves *et al.*, 2018). In plants, the pathway to tyrosine synthesis is usually highly regulated at ADH, which is strongly feedback-inhibited by tyrosine (Maeda & Dudareva, 2012). A recent study recognized that many species in Caryophyllales possess a canonical form of ADH (termed ADHβ) and an additional paralogue of the ADH enzyme (termed ADHα) (Fig. 3). Functional characterization of the novel ADHα isoform using both *in vitro* and heterologous transient assays in *Nicotiana benthamiana* established that, in contrast to ADHβ, ADHα has relaxed sensitivity to the negative feedback inhibition by tyrosine (Lopez-Nieves *et al.*, 2018). As a result, these deregulated ADHα enzymes are able to synthesize higher concentrations of tyrosine in *in vitro* assays relative to the canonical tyrosine-sensitive ADHβ, with 10-fold increases in tyrosine reported on transient assay in *N. benthamiana* (Lopez-Nieves *et al.*, 2018). A subsequent study coupled the deregulated ADHα to a module comprising the complete betalain biosynthesis pathway to demonstrate that increased availability of tyrosine results in as much as a seven-fold increase in betalain pigmentation on transient assay in *N. benthamiana* (Timoneda *et al.*, 2018). Together these studies imply an intimate link between the evolution of Caryophyllales-specific deregulated ADHα, and Caryophyllales-specific betalain biosynthesis.

2. Evolutionary history of ADH in Caryophyllales

The deregulated ADHα isoform is the product of a gene duplication event specific to core Caryophyllales (Lopez-Nieves *et al.*, 2018). The duplication event occurred at the base of the core Caryophyllales and gave rise to two clades, termed ADHα (which are deregulated and have reduced tyrosine-mediated feedback) and ADHβ (which exhibit canonical sensitivity) (Fig. 4a). Consequently, most species in Caryophyllales possess at least two copies of ADH, the deregulated ADHα and the canonical tyrosine-sensitive ADHβ (Lopez-Nieves *et al.*, 2018). Interestingly, the duplication giving rise to the deregulated ADHα lineage occurred long before the inferred origin of betalain pigmentation, suggesting an adaptive significance to higher concentrations of tyrosine long before the evolution of betalain pigmentation. It is an interesting hypothesis, therefore, that betalain pigmentation may have emerged in a metabolic context that was already generally enriched for tyrosine (Lopez-Nieves *et al.*, 2018). Moreover, ADHα appears to be lost, downregulated or under relaxed selection in anthocyanic lineages in Caryophyllales (Lopez-Nieves *et al.*, 2018) (Fig. 4a). These observations imply that the inferred loss or absence of betalain pigmentation in anthocyanic lineages in Caryophyllales is also coupled to more global changes in tyrosine metabolism represented by ADH activity.

IV. Tyrosine hydroxylation and L-DOPA oxidase

The conversion of tyrosine to L-DOPA (tyrosine hydroxylase) and L-DOPA to *cyclo*-DOPA (L-DOPA oxidase) has long been ascribed

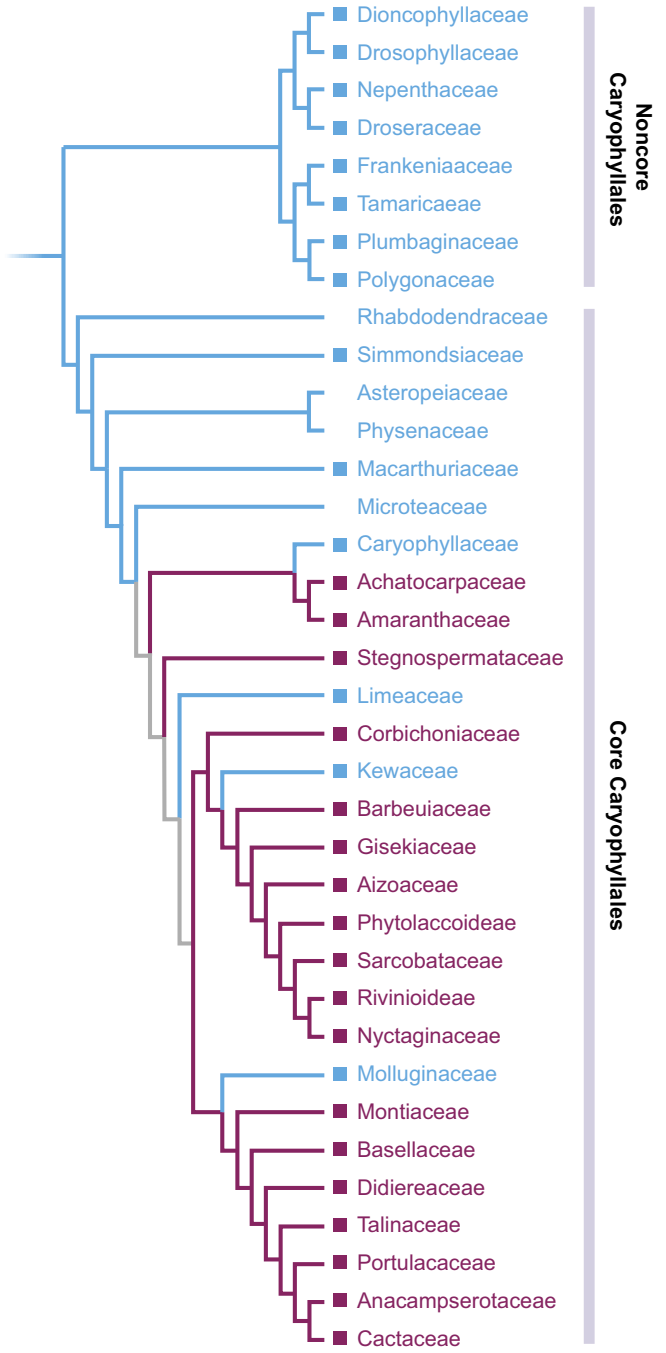

Fig. 2 A family-level tree of the Caryophyllales *sensu lato*, depicting the homoplastic distribution of betalains (purple) and anthocyanins (blue) within core Caryophyllales and illustrating potential reversals to anthocyanin pigmentation in Caryophyllaceae, Limeaceae, Kewaceae and Molluginaceae. Coloured squares indicate known pigment states (modified from Brockington *et al*., 2015).

to a putative polyphenol oxidase (PPO) (Steiner *et al*., 1999; Gandía-Herrero & García-Carmona, 2012). However, although PPO enzymes have been isolated from betalain-pigmented taxa (Steiner *et al*., 1999; Gandia-Herrero *et al*., 2005; Gao *et al*., 2009), there is no direct evidence regarding their role in betalain biosynthesis *in vivo*. Hatlestad *et al*. (2012) reasoned that distinct enzymes catalyse the tyrosine hydroxylase and L-DOPA oxidase steps, because of the widespread existence of yellow betaxanthin morphotypes, as well as violet betacyanic morphotypes among many betalain-pigmented taxa. It was argued that in these betaxanthin-pigmented taxa, the tyrosine hydroxylase step must still be intact, with the L-DOPA oxidase step disrupted, preventing betacyanin formation. As outlined in the following, the prediction that PPOs could be involved in betalain biosynthesis has been borne out, and on the basis of comparative transcriptomic analyses, cytochrome P450-type enzymes are responsible for the catalysis of these two steps (Hatlestad *et al*., 2012; Polturak *et al*., 2016; Sunnadeniya *et al*., 2016).

1. Characterization of L-DOPA oxidase (CYP76AD1/3)

Initially, *BvCYP76AD1* from *Beta vulgaris* was detected as a highly expressed gene in betalain-producing tissues (Hatlestad *et al*., 2012). On silencing of the *BvCYP76AD1* locus in *B. vulgaris*, a loss of betacyanins was observed, while transgenic expression of *BvCYP76AD1* recovered betacyanin pigmentation in '*Golden Globe*', a commercial yellow variant of *B. vulgaris*. In the same study, analysis of a *B. vulgaris* cv C8689 that segregates for yellow and red hypocotyls found that a recessive insertional mutant allele of *BvCYP76AD1* leading to an inactive protein segregated with the yellow phenotype (Hatlestad *et al*., 2012). Together these results confirm that *BvCYP76AD1* possesses L-DOPA oxidase activity, providing the *cyclo*-DOPA moiety for betacyanin production (Hatlestad *et al*., 2012). Transposon-mediated mutation in *MjCYP76AD3*, an orthologue of *BvCYP76AD1* derived from *Mirabilis jalapa*, results in loss of betacyanin production, consistent with a role of MjCYP76AD3 in *cyclo*-DOPA production (Suzuki *et al*., 2014). Most recently, a mutant screen in *Chenopodium quinoa* has led to the identification of a CYP76AD1 orthologue which restores betalain pigmentation in a mutant deficient for betalains in its hypocotyl (Imamura *et al*., 2018). Taken together, these data provide evidence of a class of cytochrome P450 enzymes with L-DOPA oxidase activity that are conserved across divergent lineages in Caryophyllales.

2. Characterization of tyrosine hydroxylase (CYP76AD1/5/6/15)

Identification of *BvCYP76AD1* and its role in L-DOPA oxidation also led to discovery of the genes encoding the enzymes for the preceding tyrosine hydroxylase step. Heterologous expression of *BvCYP76AD1* in yeast demonstrated its capacity not only to oxidize L-DOPA, but also to catalyse the conversion of tyrosine to L-DOPA, the first step of the betalain pathway (DeLoache *et al*., 2015). Tyrosine hydroxylase activity of BvCYP76AD1 *in planta* was then confirmed by gene silencing in *B. vulgaris* and recombinant expression assays in *N. benthamiana* (Polturak *et al*., 2016). Further to the discovery of the tyrosine hydroxylase activity of BvCYP76AD1 (DeLoache *et al*., 2015; Polturak *et al*., 2016), subsequent efforts uncovered additional enzymes responsible for catalysing the first step of the betalain pathway. Polturak *et al*. (2016) showed that tyrosine hydroxylation is also catalysed by a

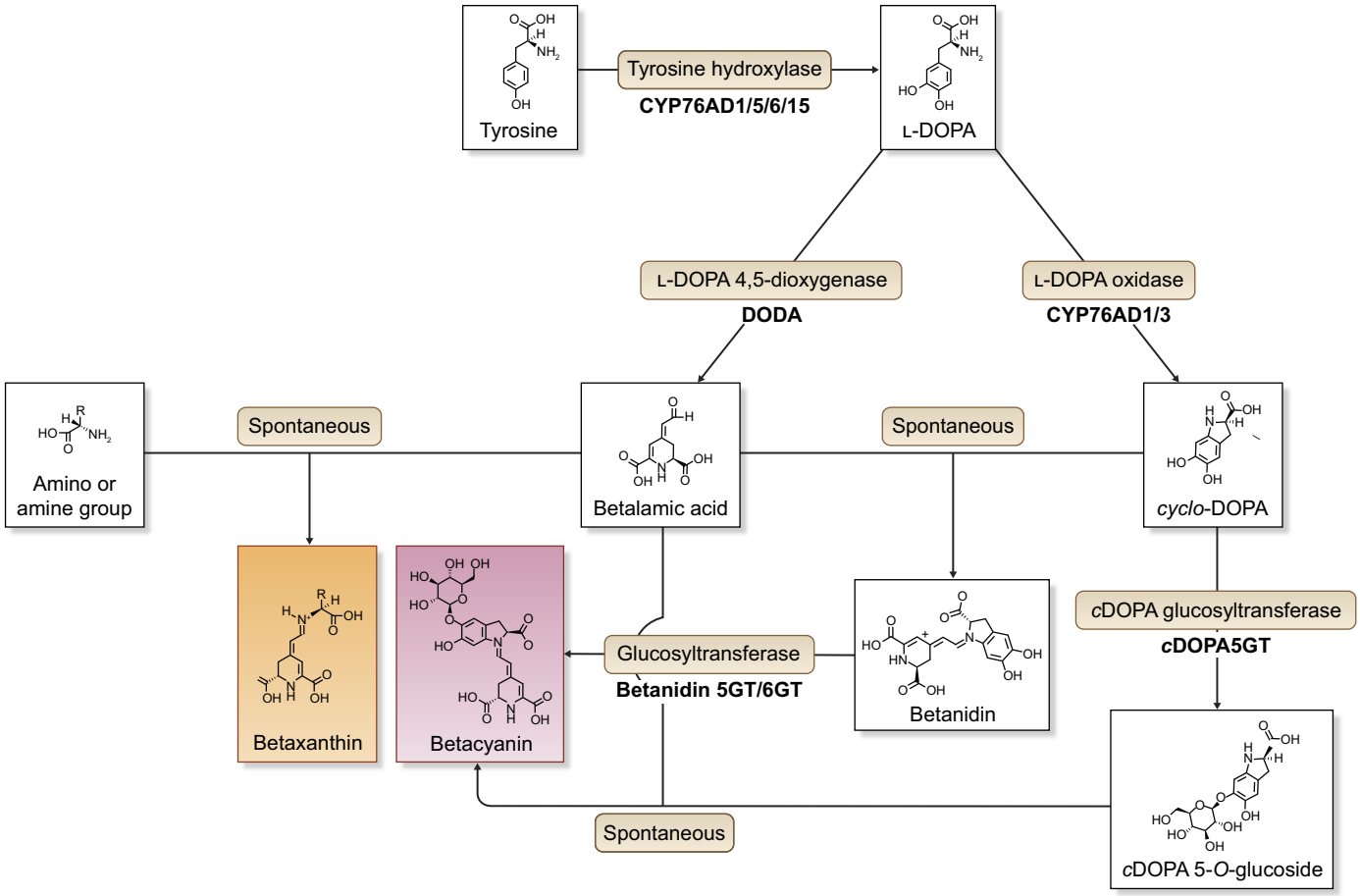

Fig. 3 The betalain biosynthesis pathway including the noncommitted step of oxidative decarboxylation of arogenate catalysed by arogenate dehydrogenase (ADH), tyrosine hydroxylation catalysed by cytochrome P450 enzymes (CYP76AD1/5/6/15) to form L-3,4-dihydroxyphenylalanine (L-DOPA), the formation of betalamic acid from L-DOPA through the action of L-DOPA 4,5-dioxygenase (DODA), the conversion of L-DOPA to *cyclo*-DOPA by cytochrome P450 (CYP76AD1/3), glucosylation of *cyclo*-DOPA via the action of *cyclo*-DOPA 5-*O*-glucosyltransferase (*c*DOPA5GT), glucosylation of betanidin to form betanin by betanidin 5/6 glucosyltransferase (betanidin 5GT/6GT), and the spontaneous condensation of betalamic acid with *cyclo*-DOPA 5-*O*-glucoside to form purple betacyanins, or with amino or amine groups to form yellow betaxanthins. The insert box depicts the two forms of ADH found in Caryophyllales.

closely related CYP76AD homologue, *BvCYP76AD6*, identified in *B. vulgaris*. Sunnadeniya *et al.* (2016) confirmed these findings and identified yet another CYP76AD homologue, *BvCYP76AD5* from *B. vulgaris*, also with tyrosine hydroxylase activity. Expression of both *BvCYP76AD5* and *BvCYP76AD6* homologues in yeast led to the production of L-DOPA (Sunnadeniya *et al.*, 2016). Unlike *BvCYP76AD1*, the newly discovered enzymes *BvCYP76AD5* and *BvCYP76AD6* only exhibit tyrosine hydroxylase activity and cannot perform the L-DOPA oxidase step. However, betanidin was detected by LC-MS at very low intensities, suggesting that these enzymes may have a weak ability to produce *cyclo*-DOPA (Sunnadeniya *et al.*, 2016). The differential ability to produce *cyclo*-DOPA between *BvCYP76AD1*, *BvCYP76AD5* and *BvCYP76AD6* provides the genetic architecture for selective production of yellow or red betalains, as is commonly seen in agricultural cultivars (Sunnadeniya *et al.*, 2016). In fact, coexpression of *BvCYP76AD1* and *BvCYP76AD6* in tobacco has been shown to produce a combination of betaxanthins and betacyanins, resulting in an orange-pink coloration and suggesting that the ratio of expression of these enzymes may be responsible for the variety of hues observed in nature (Polturak *et al.*, 2017). However, yellow beet cultivars containing a defective *BvCYP76AD1* allele were also shown to display overall limited pigment production and increased tyrosine accumulation *in planta*, indicating that the lack of an active BvCYP76AD1 can substantially reduce the plant's capacity to oxidize tyrosine into L-DOPA (Wang *et al.*, 2017). Although these tyrosine-hydroxylase-specific CYP76AD enzymes were initially identified in *B. vulgaris*, recent analyses have identified a homologue in *M. jalapa*, *MjCYP76AD15* (Sunnadeniya *et al.*, 2016; Polturak *et al.*, 2018), which exhibits similar tyrosine hydroxylase activity. Together these data confirm the existence of a conserved class of cytochrome P450 with tyrosine hydroxylase activity in lineages of the Caryophyllales.

3. Evolutionary history of the CYP76AD lineage in Caryophyllales

The cytochrome P450 gene subfamily CYP76AD lineage is closely related to the CYP76T and CYP76C families of CYP P450 genes (Hatlestad *et al.*, 2012). Phylogenetic analysis of the CYP76AD

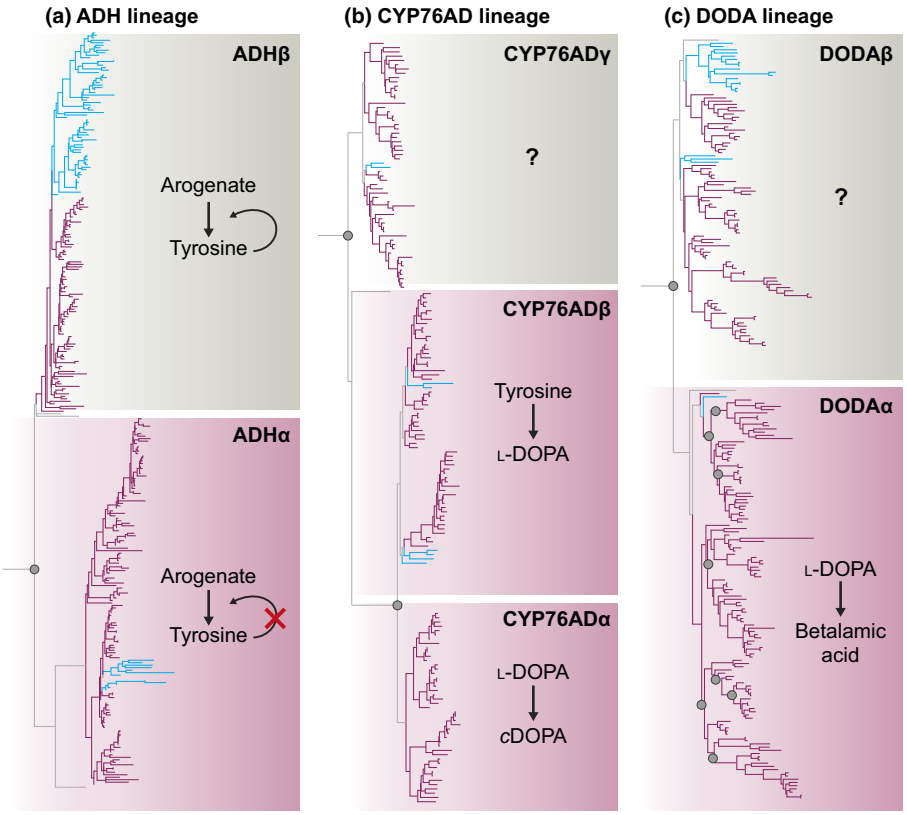

Fig. 4 The phylogenetic history of major genes implicated in the betalain biosynthesis pathway that exhibit a Caryophyllales-specific gene duplication: (a) arogenate dehydrogenase (ADH) (modified from Lopez-Nieves *et al*., 2018); (b) cytochrome P450 subfamily CYP76AD (modified from Brockington *et al*., 2015); (c) L-3,4-dihydroxyphenylalanine (L-DOPA) 4,5-dioxygenase (DODA) (modified from Brockington *et al*., 2015). Purple lines are genes derived from betalain-pigmented species, and blue lines are genes derived from anthocyanin taxa. Grey circles highlight inferred gene duplication events. *c*DOPA, *cyclo*-dihydroxyphenylalanine.

lineage indicates that this lineage underwent two relatively deep gene duplications within Caryophyllales, giving rise to three paralogous lineages: CYP76AD alpha (α), beta (β) and gamma (γ) (Brockington *et al*., 2015) (Fig. 4b). The *BvCYP76AD1* and *MjCYP76AD3* genes, which possess both tyrosine hydroxylase and L-DOPA oxidase activity, fall into the CYP76ADα lineage. The *BvCYP76AD5*, *BvCYP76AD6* and *MjCYP76AD15* homologues, which possess only tyrosine hydroxylase activity, are paralogues of *BvCYP76AD1* and *MjCYP76AD3* and belong to the β clade (Brockington *et al*., 2015; Sunnadeniya *et al*., 2016) (Fig. 4b). No function has been ascribed to any homologues within the CYP76ADγ clade. Assuming that the functional annotations conferred by *BvCYP76AD1* and *MjCYP76AD3* and *BvCYP76AD5*/*BvCYP76AD6*/*MjCYP76AD15* extend to all members of their respective clades, it is tantalizing to conclude that the phylogenetic history of the CYP76AD lineage is recapitulating the sequential biosynthetic steps in the betalain pathway (Sunnadeniya *et al*., 2016). In other words, the first function to have evolved within the CYP76AD lineage was the first step in the betalain synthesis pathway, tyrosine-hydroxylation, leading to L-DOPA formation (represented by the CYP76ADβ clade). Following gene duplication, a CYP76AD paralogue evolved the novel additional activity of L-DOPA oxidase (represented by CYP76ADα clade) allowing for the evolution of betacyanins. Interestingly, in contrast to CYP76ADα, homologues in the CYP76ADβ lineage are retained in anthocyanic lineages, perhaps indicating that L-DOPA has additional metabolic fates in Caryophyllales, other than their incorporation into betalain pigments (Brockington *et al*., 2015).

V. Production of betalamic acid by DODA

1. Characterization of DODA

In plants, betalamic acid is formed via the extradiol cleavage of L-DOPA between positions 4 and 5 of the aromatic ring. A LigB enzyme with DODA activity was first identified and purified in the betalain-pigmented fungi *Amanita muscaria* (Girod & Zrÿd, 1991; Terradas & Wyler, 1991). Cloning and heterologous expression of the *A. muscaria DODA* gene in white petals of *Portulaca grandiflora* (Caryophyllales) and *Antirrhinum majus* (Lamiales) result in the formation of yellow and purple spots containing betalains (Hinz *et al*., 1997; Mueller *et al*., 1997; Harris *et al*., 2012). Informed by earlier classical genetic work, Christinet *et al*. (2004) isolated the transcript of a *LigB* candidate gene encoding an enzyme with DODA activity within Caryophyllales, by constructing subtractive cDNA libraries between differently coloured phenotypes of isogenic lines of *P. grandiflora*. Biolistic transformation and genetic complementation in white petals of *P. grandiflora* confirmed the

role of this *LigB* gene in betalain production. Following characterization of this *LigB* homologue in *P. grandiflora*, DODA activity has been either characterized or implicated in multiple *LigB* homologues across betalain-pigmented Caryophyllales, including *Amaranthus hypochondriacus* (Casique-Arroyo *et al.*, 2014), *Hylocereus polyrhizus* (Qingzhu *et al.*, 2016), *Suaeda salsa* (Zhao *et al.*, 2011), *B. vulgaris* (Gandía-Herrero & García-Carmona, 2012; Hatlestad *et al.*, 2012), *C. quinoa* (Imamura *et al.*, 2018), *Parakeelya mirabilis* (Chung *et al.*, 2015), *M. jalapa* and *Bougainvillea glabra* (Sasaki *et al.*, 2009). The introduction of *P. grandiflora LigB* in nonbetalain backgrounds and feeding with its substrate, L-DOPA, were also sufficient to trigger betalain production (Harris *et al.*, 2012).

2. Evolutionary history of the DODA lineage in Caryophyllales

Currently all characterized enzymes exhibiting DODA activity fall into the LigB gene family, which is present across all major kingdoms of life and occurs in all land plants, from bryophytes to angiosperms (Christinet *et al.*, 2004; Chung *et al.*, 2015). Phylogenetic analysis of the LigB gene lineage in Caryophyllales identified that a gene duplication occurred early in the evolution of the order, giving rise to two major clades of LigB genes, termed DODAα and DODAβ (Brockington *et al.*, 2015). Consequently, all betalain-pigmented lineages of Caryophyllales contain at least two *LigB* genes, including one paralogue from the *DODA*α lineage and one paralogue from the *DODA*β lineage. The function of DODAβ is unknown, but a number of lines of evidence suggest that, following this duplication, neofunctionalization occurred within the DODAα lineage leading to the evolution of DODA activity: the gene duplication giving rise to the DODAα lineage occurred just before the earliest inferred origin of betalain pigmentation; all functionally characterized LigB homologues known to possess DODA activity fall into the DODAα lineage (as opposed to the DODAβ lineage) (Fig. 4c); and anthocyanic lineages that arose after the origin of the DODAα lineage have generally lost or downregulated their representative of the DODAα lineage, emphasizing that DODAα is associated with betalain-specific activity (Brockington *et al.*, 2015) (Fig. 4c). Intriguingly, one of the betalain-specific DODAα paralogues sits in close proximity to *CYP76AD1* on chromosome 2 of the *B. vulgaris* genome, indicating the possibility of a metabolic operon (Osbourn, 2010), and implicating genomic rearrangement in the evolution of the betalain biosynthesis pathway, at least in Amaranthaceae (Brockington *et al.*, 2015).

The evolution of DODA activity within the context of the DODAα lineage is complex. Numerous further duplications have occurred within this clade, and in addition to DODAα homologues that produce betalamic acid, several betalain-pigmented species possess DODAα paralogues which alone have no or little apparent ability to produce betalamic acid. These are thus unlikely to have a role in betalain production *in planta* but their actual function is unknown (Chung *et al.*, 2015; Bean *et al.*, 2018). Nonetheless, the existence of these DODAα paralogues without DODA activity has been used to identify residues that are critical for DODA function. Bean *et al.* (2018) made an intraspecific comparison between a pair of these paralogues in *B. vulgaris*: BvDODA1, the DODA characterized to be involved in betalain production in beet (Hatlestad *et al.*, 2012); and BvDODA2, a DODA that has only shown minor DODA activity *in planta* and *in vitro*, and no activity in yeast (Chung *et al.*, 2015; Gandía-Herrero & García-Carmona, 2012; Bean *et al.*, 2018). Seven residues were identified that are necessary and sufficient to confer DODA activity on BvDODA2, when heterologously expressed in yeast.

VI. Further structural modification of betalains (transferases)

1. Betanidin glucosyltransferases (5GT and 6GT)

All betacyanins are composed of betanidin conjugated to different glycosyl moieties (Strack *et al.*, 2003; Sasaki *et al.*, 2005). By contrast, betaxanthins do not possess a glucose moiety and are not found to be glycosylated in nature (Strack *et al.*, 2003; Sasaki *et al.*, 2004). Betacyanins can be glycosylated at the *cyclo*-DOPA (Wyler *et al.*, 1984; Sasaki *et al.*, 2004, 2005) or betanidin intermediates of the pathway (Vogt *et al.*, 1999; Vogt, 2002). Two betanidin glucosyltransferases have been identified from *Dorotheanthus bellidiformis*, which vary in the position of the aromatic ring to which they add the glucose. Betanidin 5-*O*-glucosyltransferase (betanidin 5GT) catalyses the transfer of glucose to the 5-hydroxyl group of betanidin (Vogt *et al.*, 1999), whereas betanidin 6-*O*-glucosyltransferase (betanidin 6GT) acts on the 6-hydroxyl group (Vogt, 2002). A UDP-glucosyltransferase with high similarity to the betanidin 5GT has also been isolated from *B. vulgaris*, *BvGT*, whose expression correlates with betalain pigmentation induced by abiotic and biotic stresses (Sepúlveda-Jiménez *et al.*, 2005). Antisense knockdown of *BvGT* led to a reduction in betalain pigmentation, supporting its role in betanidin modification, although substrate specificity of *BvGT* has not yet been confirmed (Sepúlveda-Jiménez *et al.*, 2005).

2. *cyclo*-DOPA 5-*O*-glucosyltransferases (*c*DOPA5GT)

An early publication showed high amounts of *cyclo*-DOPA glucoside in red beet plants (Wyler *et al.*, 1984), with glycosylation at the *cyclo*-DOPA step first demonstrated in *M. jalapa* crude petal extracts (Sasaki *et al.*, 2004). Thus, an alternative glycosylation scenario was proposed in which betanin and other glucosylated betacyanins can be synthesized via *cyclo*-DOPA modification. Later, cDNA encoding the enzyme cDOPA5GT was isolated and characterized (Sasaki *et al.*, 2005). Increased expression of *cDOPA5GT* correlates with the accumulation of betanin during flower development in *M. jalapa*, supporting its role in betalain biosynthesis (Sasaki *et al.*, 2005). An orthologue of *cDOPA5GT* was also characterized from the distantly related *Celosia cristata* (Sasaki *et al.*, 2005), indicating that this class of enzyme is broadly conserved across Caryophyllales. Furthermore, *M. jalapa cDOPA5GT* has been included in multigene constructs containing the other betalain synthesis genes designed for heterologous

accumulation of stable betacyanins, suggesting the importance of *cDOPA5GT* for betacyanin pigmentation (Polturak *et al.*, 2016, 2017; Timoneda *et al.*, 2018).

3. Betalain acylation

In addition to the structural complexity attained through glycosylation, betacyanins can undergo additional acylation reactions. Acylated betalains have been reported from at least four different families within Caryophyllales. Betacyanins are further decorated with a variety of groups, including acyl, malonyl, apiosyl, feruloyl, glucosyl, hydroxyl-cinnamoyl and other moieties. Much less is understood about the enzymes responsible for these additional moieties; however, betacyanin acylation would probably be catalysed by either acyl-coenzyme A-dependent acyltransferases from the BAHD superfamily or serine-carboxy-peptidase-like acyltransferases (Tanaka *et al.*, 2008). Hydroxycinnamoyl D-glucoses have been shown to serve as acyl donors for betalain acylation in cell cultures derived from betalain species. Consistent with these observations, a recent comparative transcriptomics study identified a candidate acyltransferase from *M. jalapa*, a hydroxycinnamate gluosyltransferase (*MjHGCT*) (Polturak *et al.*, 2018). Subsequent coexpression of *MjHGCT* in *N. benthamiana* in conjunction with the betalain biosynthesis genes reportedly resulted in cinnamoyl-betanin, coumaroyl-betanin, caffeoyl-betanin and feruloyl-betanin (Polturak *et al.*, 2018).

4. Evolutionary history of betalain-related transferases in Caryophyllales

Little is known about the evolutionary history of the recently discovered MjHGCT, responsible for betalain acylation, but the phylogenetic context of betalain-implicated gluosyltransferases has been well studied. The betanidin 5GT from *D. bellidiformis* revealed a sequence with high similarity to glucosyltransferases in Solanaceae as well as unknown sequences of *Arabidopsis thaliana*. However, the two betanidin 5GT and 6GT genes show only 15% amino acid sequence identity with each other and are grouped into two different clusters within the GT superfamily (Vogt, 2002). These data imply that 5GT and 6GT were derived from different ancestors and have been independently recruited to a role in betalain pigmentation (Fig. 5a). Similar to betanidin 5GT and 6GT, phylogenetic analysis of the betalain-active *c*DOPA5GT indicates that it forms a distinct subclade within a broader context of glucosyltransferases associated with flavonoid and phenylpropanoid substrate specificity (Fig. 5b; Sasaki *et al.*, 2005). In many instances of glucosyltransferases implicated in betalain pigmentation, there is evidence of broader substrate specificity not only for betanin, but also for other flavonoids, supporting the concept that betalain glucosyltransferases could have arisen as an adaptation of the already existing flavonoid enzymes (Vogt *et al.*, 1999; Vogt, 2002). Indeed, the production of betalains by transformation of DODA in the white petals of *A. majus* led to the detection of betanin, indicating again that endogenous glucosyltransferases in anthocyanic taxa could act on the novel betanidin substrates (Harris *et al.*, 2012). Together these data imply that glucosyltransferases implicated in betalain pigmentation repeatedly arose from flavonoid-related glucosyltransferases.

VII. Transcriptional regulation of the betalain pathway (BvMYB1)

1. The role of MYB transcription factors

In addition to resolving the structural genes of the betalain biosynthesis pathway, progress has been made in understanding their transcriptional regulation. MYB and basic helix–loop–helix (bHLH) transcription factors are found in all eukaryotes and are among the largest transcription factor families in plants (Xu *et al.*, 2015). Plant MYB transcription factors are characterized by two or three imperfect repeats of the MYB DNA-0binding motifs (R1, R2 and R3). Most plant MYBs belong to the R2R3 family, and normally interact with WD40 and bHLH proteins, forming what is called the MBW complex to regulate gene expression. Members of the R2R3 MYB group control anthocyanin biosynthesis as well as a number of other plant traits such as trichome and root hair formation (Ramsay & Glover, 2005; Xu *et al.*, 2015). Several putative binding sites for bHLH and MYB transcription factors were identified in two DODA genes in *Phytolacca americana* (Takahashi *et al.*, 2009). In light of this, and given the fact that betalains and anthocyanins assume similar physiological roles *in planta*, it was hypothesized that the same MBW complex containing MYB and bHLH transcription factors could also be participating in the regulation of betalain biosynthesis (Hatlestad *et al.*, 2014). A BLAST search for homologues of *R2R3 MYB* genes implicated in anthocyanin pigmentation in a beet RNAseq database identified a highly expressed *R2R3 MYB* gene, *BvMYB1*, which was also underexpressed in unpigmented varieties of *B. vulgaris*. Overexpression of *BvMYB1* in white beet roots resulted in the emergence of red coloration, indicating that *BvMYB1* can regulate betalain synthesis (Hatlestad *et al.*, 2014). Consistent with this, virus-induced gene silencing of *BvMYB1* in red varieties of *B. vulgaris* produced plants with betalain-free sectors. Finally, yeast-one-hybrid analysis showed that BvMYB1 could bind the promoters of both *BvDODA* and *BvCYP76AD1*, and that BvMYB1 can upregulate *BvDODA in vivo* under an inducible system. These results confirm that betalain biosynthesis is controlled by a MYB R2R3 transcription factor, *BvMYB1* (Hatlestad *et al.*, 2014).

2. Potential additional regulatory partners

The involvement of a R2R3 MYB as a regulator of betalain structural genes is consistent with the MBW model of regulation of anthocyanin pigmentation, but BvMYB1 is actually unable to interact with known bHLH partners derived from anthocyanic model organisms. Furthermore, it lacks five of the seven conserved amino acids previously determined to be important for bHLH interaction (Grotewold *et al.*, 2000; Zimmermann *et al.*, 2004; Hatlestad *et al.*, 2014). Resurrection of these missing bHLH interaction residues enables interaction of BvMYB1 with standard anthocyanic bHLH partners (Hatlestad *et al.*, 2014). Unlike R2R3

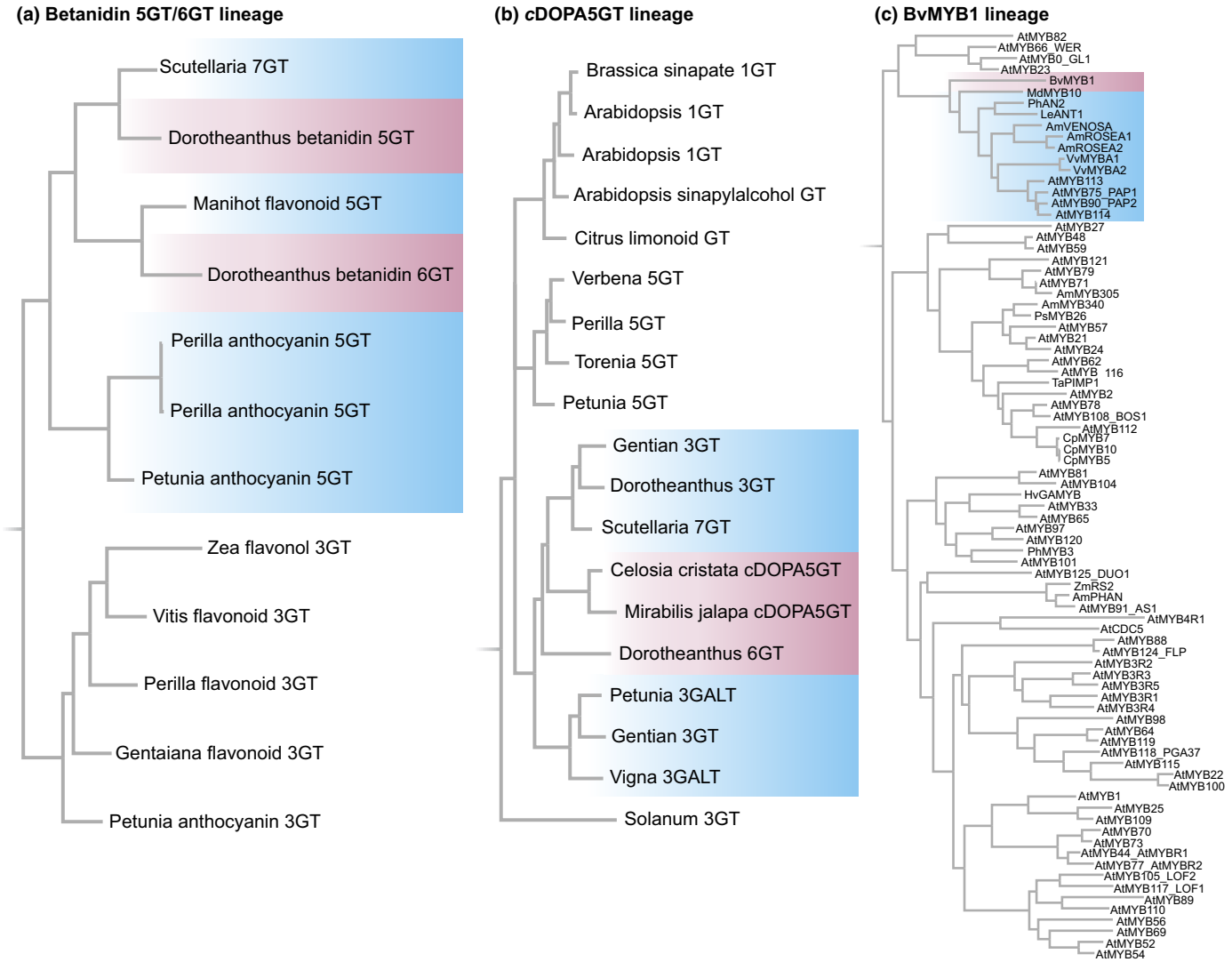

Fig. 5 The phylogenetic history of major genes implicated in the betalain biosynthesis pathway that were coopted from flavonoid or anthocyanin pathways: (a) betanidin 5 glucosyltransferase and 6 glucosyltransferase (betanidin 5GT/6GT) (phylogeny re-estimated based on data from Vogt, 2002); (b) *cyclo*-DOPA 5-*O*-gluosyltransferase (cDOPA5GT) (phylogeny re-estimated based on data from Sasaki *et al*., 2005); (c) MYB1 gene lineages (phylogeny re-estimated based on data from Hatlestad *et al*., 2014). Purple indicates betalain implicated genes, and blue indicates genes with anthocyanin/flavonoid-specific activity.

MYB transcription factors controlling anthocyanic pigmentation, it is possible that BvMYB1 does not interact with bHLH partners in the regulation of betalain synthesis. However, it is equally possible that BvMYB1 interacts with an unknown bHLH protein that possesses compensatory mutations which circumvent the requirement for the canonical R2R3 MYB interaction residues (Hatlestad *et al.*, 2014). In light of this possibility, it is interesting that a genetic linkage map for *Spinacia oleracea* constructed to determine quantitative trait loci (QTLs) controlling leaf colour identified at least two bHLH transcription factors in the vicinity of a major QTL that accounts for 69.3% of colour variation (Cai *et al.*, 2018). At least one of these bHLH genes, *ORF11*, is highly expressed in betacyanic tissue in *S. oleracea*, consistent with a role in betalain pigmentation (Cai *et al.*, 2018). Finally, a recent study has shown that a WRKY transcription factor, *HpWRKY*, identified by a RNAseq screen in the *Hylocereus polyrhizus*, can bind a W-box motif present in the promoter of a *CYP76AD1* homologue from the same species (Cheng *et al.*, 2017). WRKY transcription factors have more recently emerged as putative interacting partners with MBW complexes, controlling anthocyanin synthesis in both *A. thaliana* and *Petunia hybrida* (reviewed in Lloyd *et al.*, 2017). Despite the absence of canonical bHLH interacting residues in BvMYB1 therefore, these data may hint that a more canonical-type MBW complex is at work in the regulation of betalain biosynthesis.

3. Evolution of the BvMYB1 lineage in Caryophylalles

Analysis of the phylogenetic context of *BvMYB1* indicates that it probably belongs to the subgroup 6 clade of R2R3 MYB genes, which also contains the *AtPAP1* and *AtMYB114* gene controlling anthocyanin production in anthocyanic species (Fig. 5c). However, it was demonstrated that betalain-specific *BvMYB1* and

anthocyanin *PAP1* homologues are not interchangeable, and *BvMYB1* cannot regulate the anthocyanic biosynthetic pathway, nor can *AtMYB114* regulate the betalain biosynthesis pathway (Hatlestad *et al.*, 2014). These data imply that *BvMYB1* has been coopted from anthocyanin MYBs but has diverged in its ability to regulate anthocyanin production, and instead evolved to regulate betalain biosynthesis. Cooption of an ancestral pigment regulation pathway from anthocyanins is an attractive hypothesis, because both betalains and anthocyanins exhibit similar expression patterns and responses to environmental and developmental stimuli, implying a similar regulatory network for both betalains and anthocyanins (Jain & Gould, 2015; Lloyd *et al.*, 2017).

VIII. The origin of betalain pigmentation in Caryophyllales

1. Caryophyllales-specific gene duplication and neofunctionalization

The origin of betalains in the plant kingdom can now clearly be attributed to a number of lineage-specific duplications in Caryophyllales (Brockington *et al.*, 2015; Yang *et al.*, 2015). First, duplications within the CYP76AD lineage followed by neofunctionalization have been clearly shown to give rise to two key enzymatic steps in the betalain pathway – tyrosine hydroxylase and L-DOPA oxidase – both from the cytochrome P450 family (Brockington *et al.*, 2015; Polturak *et al.*, 2016; Sunnadeniya *et al.*, 2016). Furthermore, within a similar evolutionary time-frame, a duplication within the LigB lineage gave rise to an additional LigB paralogue which probably neofunctionalized to gain DODA activity (Brockington *et al.*, 2015; Bean *et al.*, 2018). All central enzymatic steps in the committed betalain pathway therefore arose by lineage-specific duplication in only two gene lineages within the core Caryophyllales before the earliest inferred origin of betalain pigmentation (Fig. 6). But although the proximal mechanisms underlying the evolution of the betalain biosynthetic pathway are clarified by the detection of these lineage-specific duplications, the discovery of a Caryophyllales-specific ADH now provides a deeper causal explanation (Lopez-Nieves *et al.*, 2018).

The additional Caryophyllales-specific paralogue, ADHα, acquired relaxed sensitivity to feedback inhibition by tyrosine, and consequently was more efficient in the production of tyrosine. In this context, the newly increased availability of tyrosine served as a precondition that facilitated the subsequent radiation of tyrosine-derived metabolic pathways. Within this new metabolic adaptive landscape, duplication and neofunctionalization within the CYP76AD lineage gave rise to tyrosine hydroxylase activity, leading to an increased availability of L-DOPA, facilitating the later radiation of L-DOPA-derived metabolic pathways (Brockington *et al.*, 2015). Subsequent duplication and neofunctionalization within the same CYP76AD lineage gave rise to L-DOPA oxidase activity, while a gene duplication and neofunctionalization in the LigB lineage gave rise to DODA activity (Brockington *et al.*, 2015), leading to the production of betalains (Fig. 6).

2. The adaptive significance of betalains

The concept of tyrosine-rich metabolism is compelling but incomplete, as numerous diverse lineages of angiosperms exhibit the metabolic signature of tyrosine-rich metabolism (e.g. the presence of high concentrations of L-DOPA in species within Fabaceae) (Soares *et al.*, 2014). The example of hyperaccumulated L-DOPA from Fabaceae is particularly instructive, as L-DOPA is the key precursor to betalamic acid, yet to our knowledge betalains are not reported in Fabaceae. Clearly, similar metabolic preconditions for the evolution of betalain biosynthesis exist outside of Caryophyllales, which begs the question as to the selective advantage of betalains. However, emphasis in the literature has tended to be on the functional equivalence of betalains and anthocyanins, rather than the selective advantages of betalains (Jain & Gould, 2015). A recent review examined the evidence for the functional equivalence of anthocyanins and betalains and concluded that there is considerable empirical evidence to support the functional equivalence of anthocyanins and betalains with respect to photoprotection, tolerance to drought and salinity stress, and as scavengers of reactive oxygen species in plants facing a variety of abiotic stressors (Jain & Gould, 2015). However, Jain & Gould (2015) also examine the unique properties of betalains relative to anthocyanins, as key differences that may underlie the selection for betalains in Caryophyllales. Given that betalains are stable through a wider pH range, they propose that betalains may be more effective pigments in the context of CAM and C4 species, owing to acidification of the vacuole, which may occur as a consequence of CAM and C4 physiology. Furthermore, Jain & Gould (2015) highlight a correlation between betacyanin accumulation and V-ATPase activity which is associated with the transport of sodium in halophyte species, suggesting that betalains may enhance salt sequestration. Although the evidence for both these hypotheses is weak, they are intriguing in the context of Caryophyllales, which represent one of the largest concentrations of CAM, C4 and halophyte species in the plant kingdom. Such hypotheses are needed and represent an important conceptual advance in tackling the question of the constrained distribution of betalains to Caryophyllales.

IX. The mutual exclusion of betalains and anthocyanins

1. ADHα-driven depletion of phenylalanine and phenylalanine-derived pathways

The identification of the Caryophyllales-specific deregulated ADH also provides an underlying mechanism for the eventual mutual exclusion of betalains and anthocyanins. In most plants, phenylalanine-derived anthocyanin pathways and tyrosine-derived betalain pathways are both derived from, and compete for, the common substrate arogenate (Maeda & Dudareva, 2012). Lopez-Nieves *et al.* (2018) demonstrated that overexpression of the Caryophyllales-specific deregulated ADH in *N. benthamiana* results in increased concentrations of tyrosine, but also depleted concentrations of phenylalanine. Therefore, in the context of a more efficient

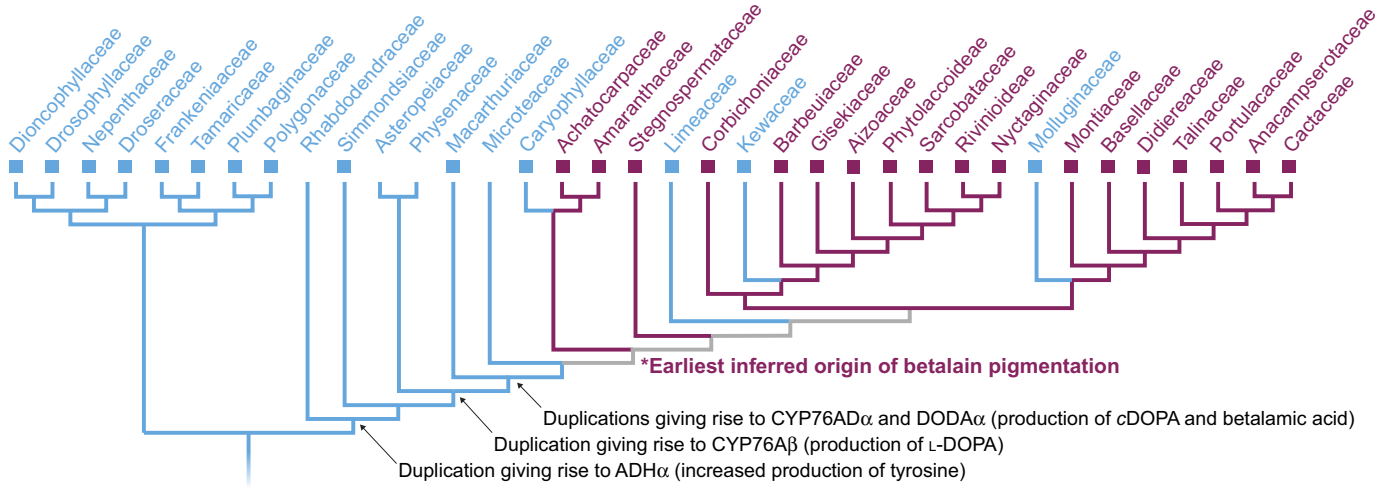

Fig. 6 The relative timing of gene duplications in the arogenate dehydrogenase (ADH), cytochrome P450 subfamily CYP76AD and L-3,4-dihydroxyphenylalanine (L-DOPA) 4,5-dioxygenase (DODA) lineages relative to the earliest inferred origin of betalain pigments as mapped to a family-level organismal tree (modified and inferred from Brockington *et al*., 2015; Lopez-Nieves *et al*., 2018). *c*DOPA, *cyclo*-dihydroxyphenylalanine.

deregulated ADH, a greater proportion of tyrosine is produced from the common substrate arogenate, potentially at the expense of phenylalanine and phenylalanine-derived metabolites. Depletion of phenylalanine-derived metabolites has yet to be tested, but this hypothesis is supported by the observation that the deregulated ADH is lost in anthocyanic lineages in Caryophyllales, implying a connection between transitions in pigment type and the balance of available tyrosine and phenylalanine (Lopez-Nieves *et al*., 2018). However, the fundamental imbalance between tyrosine and phenylalanine-derived pathways in Caryophyllales seems to be reinforced by additional mechanisms, as discussed in the following.

2. A role for the coopted BvMYB1 transcription factor in mutual exclusion

Initially it is conceivable that anthocyanins and betalains co-occurred with distinct expression patterns within a now extinct ancestor, but later the cooption of the MYB-bHLH-WD40 transcriptional complex from the anthocyanin regulatory pathway led to the tight coexpression of the two pigments. In a scenario in which the two pigments were tightly coexpressed, tyrosine-rich metabolism driven by deregulated ADH would ensure that competition between the two pigments for the common arogenate substrate favoured the dominance of betalain pigmentation. However, the apparent loss of the bHLH interacting residues from the BvMYB1 is intriguing and provides a potential evolutionary mechanism to reinforce the mutual exclusion of the two pigment types. BvMYB1 is actually unable to interact with known bHLH partners derived from anthocyanic model organisms (Grotewold *et al*., 2000; Zimmermann *et al*., 2004; Hatlestad *et al*., 2014). Consequently, cooption of BvMYB1 to betalain pigmentation, followed by loss of the bHLH-interacting residues, could have deprived the anthocyanin pathway of a key transcriptional regulator and contributed to transcriptional downregulation of anthocyanin genes.

3. Transcriptional downregulation of anthocyanin genes

The transcriptional downregulation of anthocyanin synthesis in betalain-pigmented species is well documented (Shimada *et al*., 2004, 2005, 2007). Two sequential studies have looked for the presence of the late-acting anthocyanin biosynthesis enzymes, dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS), in two betalain-pigmented species, *S. oleracea* and *P. americana* (Shimada *et al*., 2004, 2005). It was found that both *DFR* and *ANS* are present as apparently intact copies, and functional characterization in recombinant *Escherichia coli* confirmed that the isolated genes possessed dihydroflavonol 4-reductase and anthocyanidin synthase activity (Shimada *et al*., 2004, 2005). However, transcriptional expression of *DFR* and *ANS* was only found in the seed in *S. oleracea* and was not found to be expressed in leaf, stem or root tissue. On the basis of this observation, it was inferred that fully functional copies were maintained for the production of proanthocyanidins that are present in the seed coat of betalain-pigmented Caryophyllales, but that transcriptional downregulation in other tissues prevented the production of anthocyanins in those tissues (Shimada *et al*., 2004, 2005). A subsequent analysis of the promoter regions of *DFR* and *ANS* from betalain-pigmented species found that canonical MYB and bHLH trans-acting factors from the anthocyanic model *P. hybrida* could bind the *DFR* and *ANS* promoters of *S. oleracea* in a yeast one-hybrid assay but could not activate expression in the context of *P. americana* cell cultures or *S. oleracea* leaf assays, indicating some promoter-specific effects in the context of betalain-pigmented plant assays (Shimada *et al*., 2007). On the basis of these data, it was proposed that changes to the promoter regions of these key late-acting anthocyanin synthesis genes may limit their expression in betalain-pigmented plants.

In contrast to this perspective, Polturak *et al*. (2018) studied the expression of anthocyanin pathway genes in betalain-accumulating petals of *M. jalapa* and found that a homologue of *ANS* was highly expressed. However, this copy exhibits a 69 amino acid truncation

and is unable to restore the wild-type phenotype in an *A. thaliana ans* mutant. Additionally, multiple genes in the core phenylpropanoid and anthocyanin biosynthetic pathways were expressed over the course of floral development, leading the authors to suggest that the truncated ANS may explain the loss of anthocyanins in *M. jalapa*. The sequence level of homology of the truncated copy of *MjANS* is otherwise well conserved, and the truncation is not shared with the previously investigated ANS homologue from *P. americana* (Shimada *et al.*, 2005). Polturak *et al.* (2018) suggest that the truncation of *MjANS* is another mechanism that may explain the mutual exclusion of anthocyanins and betalains more broadly, or may simply be restricted only to *M. jalapa* or Nyctaginaceae.

X. Homoplastic distribution of betalains and anthocyanins

Phylogenetic reconstruction analyses using the known pigment status of extant species in Caryophyllales have been used to explore the homoplastic patterns of anthocyanin and betalain pigmentation (Brockington *et al.*, 2011, 2015). The analyses are inevitably inconclusive but emphasize that, depending on the evolutionary model, pigment distribution patterns are consistent with a single origin of betalains with reversals to anthocyanin, as well as allowing for multiple origins of betalain pigmentation with reversals to anthocyanin (Brockington *et al.*, 2011). However, the fact that the anthocyanin pathway is maintained but transcriptionally downregulated in betalain-pigmented species (Shimada *et al.*, 2004, 2005, 2007) provides a potentially simple mechanism for the resurrection of the anthocyanin pathway, and favours a scenario with multiple reversals to anthocyanins. With the elucidation of the betalain biosynthesis pathway, it has become possible to explore the fate of the betalain pathway in lineages that are inferred to have undergone reversals to anthocyanic pigmentation.

In large-scale phylotranscriptomic approaches, it was observed that putative betalain-specific lineages of DODAα and CYP76ADα were present at low abundance in transcriptomes derived from anthocyanic species in the core Caryophyllales, relative to their betalain-pigmented relatives (Brockington *et al.*, 2015). Furthermore, in the only sequenced genome from a Caryophyllales anthocyanic species, *Dianthus caryophyllus* (Caryophyllaceae), representatives of DODAα and CYP76ADα appear to have been lost. On the basis of these data, it was inferred that betalain pigmentation must have evolved before the divergence of anthocyanic Caryophyllaceae and anthocyanic Molluginaceae, and perhaps indicated a single origin of betalain pigmentation (Fig. 6) (Brockington *et al.*, 2015). Interpreted strictly, however, Brockington *et al.* (2015) speaks only to the likelihood of reversals to anthocyanin pigmentation and cannot rule out multiple origins of betalains together with multiple losses. It is also intriguing that the ADHα genes show similar patterns of gene loss in anthocyanic lineages, indicating that reversals to anthocyanin pigmentation may be underpinned by broader shifts from tyrosine-rich metabolism back to phenylalanine-rich metabolism.

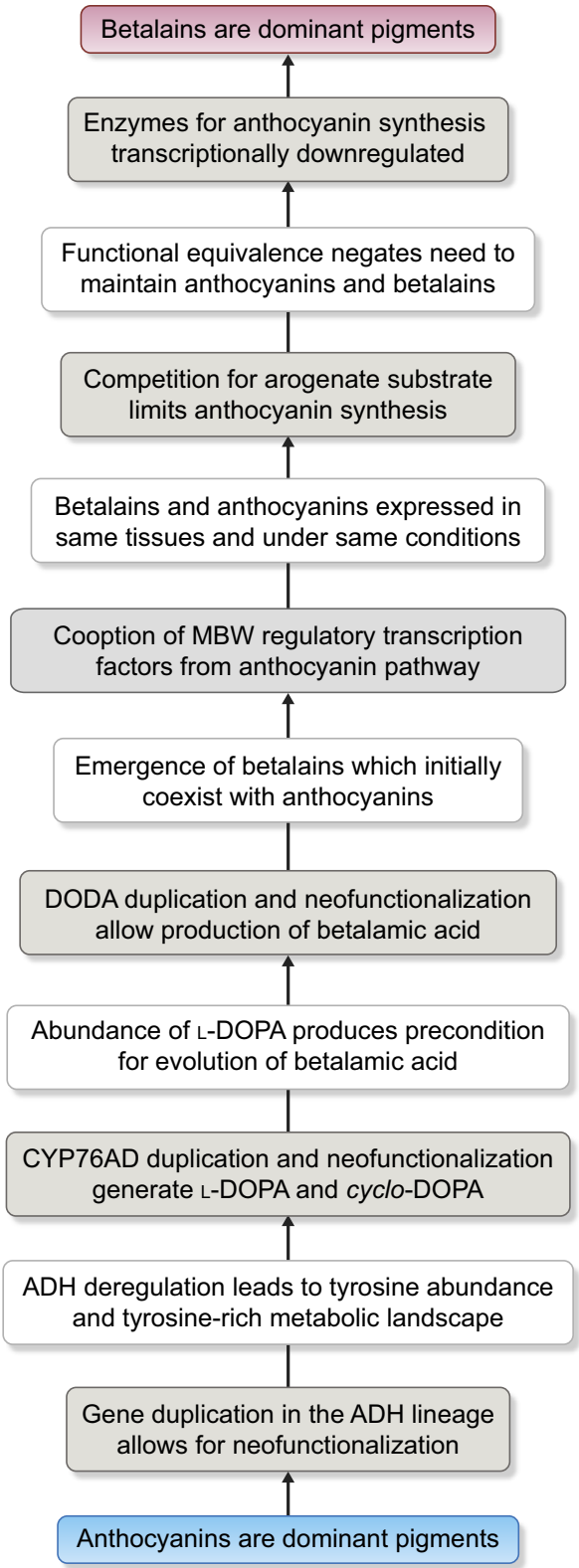

Fig. 7 A conceptual stepwise model for the evolution of betalain biosynthesis and the emergence of betalains as a dominant pigment. ADH, arogenate dehydrogenase; DODA, L-3,4-dihydroxyphenylalanine (L-DOPA) 4,5-dioxygenase; CYP76AD, cytochrome P450 subfamily CYP76AD.

XI. Conceptual stepwise model for the emergence of betalain pigmentation

We conclude by assembling the observations discussed earlier into a speculative stepwise model for the evolution of betalains as dominant pigments in Caryophyllales (Fig. 7). We suggest that a first and fundamental step in the evolution of betalain pigmentation was a gene duplication event in the ADH gene lineage at the base of the core Caryophyllales that occurred long before the evolution of the committed betalain biosynthesis pathway. The additional paralogue of ADH acquired relaxed sensitivity to feedback inhibition by tyrosine, which led to an enhanced ability to produce tyrosine and a consequent decrease in phenylalanine availability. The high availability of free tyrosine served as a precondition that facilitated the subsequent radiation of tyrosine-derived metabolic pathways (i.e. betalains). Within this tyrosine-rich metabolic adaptive landscape, duplication and neofunctionalization in the CYP76AD lineage gave rise to tyrosine hydroxylase activity, leading to an increased availability of L-DOPA. Subsequent duplication and neofunctionalization within the same CYP76AD lineage gave rise to L-DOPA oxidase activity while a gene duplication in the LigB lineage gave rise to DODA activity, leading to the production of betalains: betaxanthins and betacyanins. The availability of high concentrations of tyrosine may have ensured that betalains were produced in large amounts and subject to selection, but it is unclear what the selection pressures were that ensured the early maintenance of betalains. It seems likely that betalains and anthocyanins co-occurred at some point in evolutionary history, and the cooption of the MYB transcription from the anthocyanin regulatory pathway may ultimately have led to the tight coexpression of the two pigments, even though we have no evidence of co-occurring pigments from extant species. Nevertheless, in the scenario of coexpressed anthocyanin and betalains, tyrosine-rich metabolism driven by a deregulated ADH could have ensured that competition for the common arogenate substrate favoured the prevalence of betalains. Possibly through cooption of key components of the anthocyanin MBW complex, the functionally equivalent anthocyanin pigments were eventually transcriptionally downregulated, leading to the dominance of betalain pigmentation (Fig. 7).

XII. Conclusions

The past few years have been an exciting time for betalain research. The central committed steps in the biosynthesis of betalains are now elucidated, and identification of the underlying genes, coupled with dense genomic and transcriptomic resources, has resolved the evolutionary emergence of the betalain biosynthesis pathway. The discovery of a modulated interface of primary and specialized tyrosine metabolism in Caryophyllales holds much promise in explaining the fundamental evolutionary patterns of pigmentation in Caryophyllales, especially with respect to the mutual exclusion of anthocyanins and betalains. However, there is much still to discover. Fundamental aspects to the pathway are still unclear, such as the nature of the full transcriptional complex involved in regulating betalain synthesis, and how this complex was recruited and evolutionarily assembled. The identification of a putative metabolic operon hints at further layers of regulation and will probably facilitate further gene discovery with respect to unknown entities involved in betalain metabolism and catabolism. Likewise, the concept of Caryophyllales as a clade of organisms with generally enriched tyrosine metabolism will drive our understanding not just of the betalain biosynthesis pathway, but also of the biosynthesis of tyrosine-derived metabolites more generally. We know little about the transport of betalains from their site of synthesis in the cytoplasm to the vacuole where they are stored. Furthermore, the extent of the decorating enzymatic toolkit and its contribution to the evolution of the immense structural diversity of betalains is a clear area of future research. The adaptive significance of betalains in the context of Caryophyllales remains a key uncertainty, but the ability to heterologously synthesize betalains should now drive this forward. Undoubtedly, advances on all of these topics, coupled with the distinctive evolutionary patterns of betalain pigmentation, will ensure that the betalain biosynthesis pathway in Caryophyllales continues to develop as a compelling system to explore the emergence and evolution of novel specialized metabolic pathways.

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