Commentary

The push and pull of plant specialized metabolism underlies a long-standing, colorful mystery

O my Luve is like a red, red rose
That's newly sprung in June;
O my Luve is like the melody
That's sweetly played in tune.

These lines from 'The Red, Red Rose', a poem composed by the great Scottish poet Robert Burns, eloquently describe the feelings a bright red rose stirs in the heart. From the golden daffodils of William Wordsworth to rebellions associated with the trade of indigo, plant pigments have influenced human civilization throughout its history. One such pigment – the red color of beets – has intrigued researchers for over a century, and a report in this issue of *New Phytologist* (pp. 896–908) by Lopez-Nieves *et al.* provides new insights on this pigment's enigmatic origins.

'From a biochemical and mechanistic perspective, the mutual exclusion of anthocyanins and betalains is an enduring puzzle – why have no species been found that produce both?'

Three types of compounds – chlorophylls, carotenoids and anthocyanins – are responsible for generating the vast majority of colors in the plant world. However, in 1918, a PhD student working with Richard Willstätter – the 1915 Chemistry Nobel Prize Winner who studied chlorophyll – recognized that the red color in beets is different (Ainley & Robinson, 1937). The red, purple and blue colors in many plants are a result of accumulation of anthocyanins, which lack nitrogen. However, the red pigment in beets – composed of the compound betanin – was found to contain nitrogen. Betanin soon came to be annotated (Ainley & Robinson, 1937) – with some suspicion (Pucher *et al.*, 1938) – as a 'nitrogenous anthocyanin', with one or more amino acids conjugated to an anthocyanin backbone. However, this inference was proved incorrect when, in 1957, it was demonstrated that the nitrogen in betanin was actually contained in a pyrrole ring and not in a conjugated amino acid (Peterson & Joslyn, 1958). This intriguing difference between anthocyanin and betalains, both imparting red/purple color, however, was only part of the mystery.

Betalains, distinguished into the red–violet betacyanins and yellow betaxanthins by the 1960s, were found to be restricted to multiple families in the order Caryophyllales and were used as chemotaxonomic markers for phylogenetic inference. However, molecular systematic studies revealed that families producing betalains instead of anthocyanins do not form a monophyletic group, raising questions about whether betalain production evolved several times or was independently lost in multiple lineages of Caryophyllales (Clement & Mabry, 1996). Subsequent phylogenetic studies supported the latter hypothesis, that several clades within the Caryophyllales have reverted from betalain production back to the ancestral state of anthocyanin production (Brockington *et al.*, 2011).

The unique origin of betalain production in the Caryophyllales was reinforced through the elucidation of the underlying biochemical pathway and the evolutionary history of its component enzymes. Betalains are produced from the amino acid tyrosine (Tyr) through the action of two types of enzymes, CYP76AD1 (a cytochrome P450) and DODA (an L-DOPA dioxygenase) (Christinet *et al.*, 2004; Hatlestad *et al.*, 2012). Through phylogenetic and transcriptomic analyses, Brockington *et al.* (2015) showed that these two tightly-linked enzymes arose via duplication after the origin of the core Caryophyllales and are shared among the betalain-producing lineages. Moreover, the sequences of these enzymes were undetectable in the transcriptomes of closely related anthocyanin-producing taxa, suggesting that gene loss or down-regulation led to the reversion to anthocyanin accumulation.

Despite the rapid progress in understanding the origin and function of the betalain pathway, many key questions remain unanswered. For example, it is unclear what evolutionary or ecological factors could have driven the initial transition from anthocyanins to betalains or the later reversions to anthocyanins, especially given that both pigment types result in superficially similar coloration. Also, from a biochemical and mechanistic perspective, the mutual exclusion of anthocyanins and betalains is an enduring puzzle – why have no species been found that produce both? Through an elegant series of phylogenetically-informed biochemical experiments, Lopez-Nieves *et al.* demonstrate that the answers to these questions lie, in part, outside of the betalain pathway, in the steps that control amino acid synthesis.

Like betalains, anthocyanins are derived from aromatic amino acids arising from the shikimate pathway, although in the case of anthocyanins, the precursor is phenylalanine (Phe) as opposed to Tyr. Moreover, both Phe and Tyr in plants are commonly made from the precursor arogenate by the action of arogenate dehydratase (ADT) and arogenate dehydrogenase (ADH), respectively (Maeda *et al.*, 2010; Fig. 1a). Thus, changes in the relative conversion of

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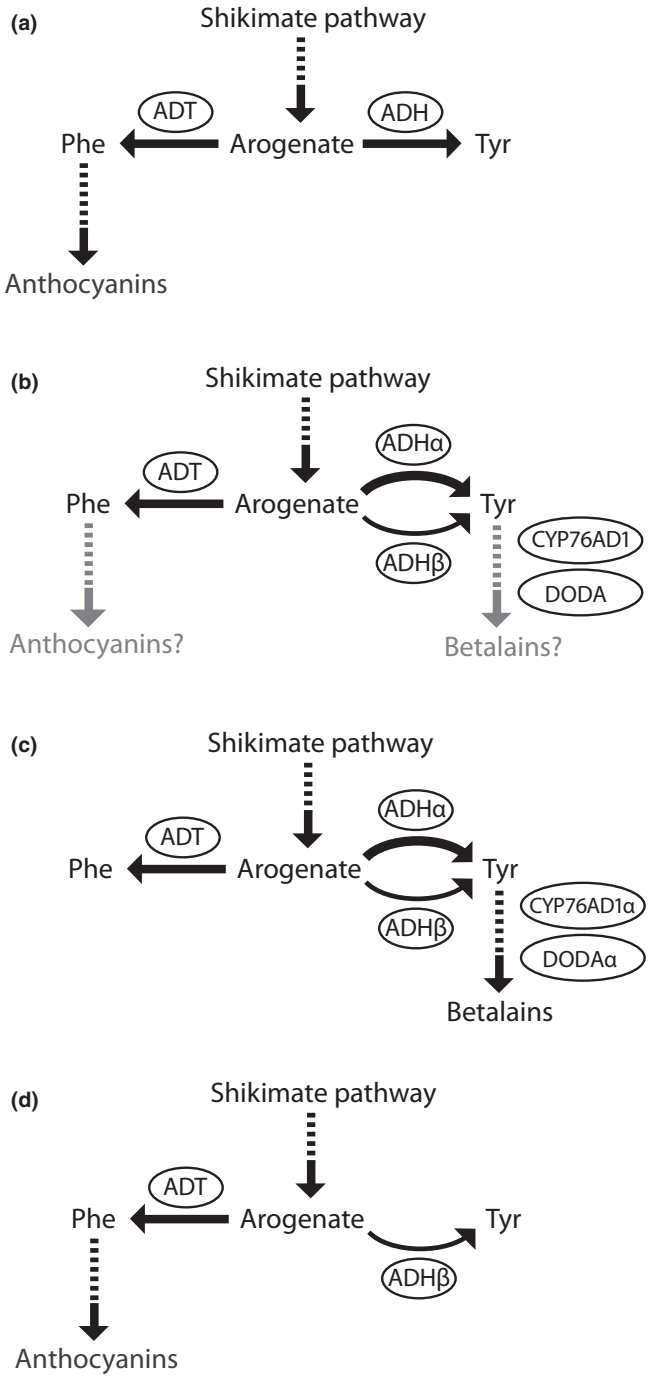

Fig. 1 Hypothetical steps involved in the transitions between anthocyanin and betalain production. (a) In the ancestral lineage of Caryophyllales, anthocyanin pigments are accumulated through flux from the shikimate pathway via phenylalanine (Phe) produced by arogenate dehydratase (ADT). (b) Duplication of arogenate dehydrogenase (ADH) and loss of feedback inhibition in ADHα allows build-up of tyrosine (Tyr). This might lead to small amounts of betalain production depending on the activity of CYP76AD, DODA or other enzymes on Tyr and other betalain precursors. Given that many of the pathway steps are spontaneous, it is possible that only one of these enzymes would be required for some betalain production (Harris *et al.*, 2012). (c) Duplication and functional specialization of CYP76AD1α and DODAα leads to complete transition to betalain production. (d) The regain of anthocyanin production is accompanied by loss of expression and/or degeneration of the gene copies (ADHα, CYP76AD1α and DODAα) that were specialized for betalain production.

arogenate to Phe versus Tyr have the potential to influence the accumulation of their downstream products, including anthocyanins and betalains. Lopez-Nieves *et al.* uncovered a major evolutionary event that appears to favor betalain production in this tug of war – the duplication of ADH before the origin of the core Caryophyllales. Following the duplication event, one of the two copies, ADHα, lost the feedback inhibition that typically limits accumulation of Tyr. The betalain-producing lineages have retained the ADHα copy and show up to 85-fold higher Tyr concentrations, while lineages that have reverted to anthocyanin production exhibit relaxed selection or degeneration at the ADHα locus.

These findings paint a new picture for how betalain production may have evolved in the Caryophyllales some 100 million years before the present. In the ancestral lineage, Tyr accumulation was restricted due to feedback inhibition, and anthocyanin pigmentation resulted from flux via Phe (Fig. 1a). The duplication of ADH allowed for functional divergence and loss of Tyr sensitivity in the α copy, resulting in novel Tyr accumulation (Fig. 1b). If the pre-duplication copies of CYP76AD and DODA had some functional promiscuity, betalain production may have been possible. In support of this notion, several members of the CYP76AD clade of P450s can complete the first necessary step toward betalain production, the hydroxylation of Tyr (Sunnadeniya *et al.*, 2016). However, it is not known if dioxygenases related to DODA also have activity on betalain precursors. Alternately, the Tyr accumulated by ADHα may have originally had a different metabolic destination. Regardless, the eventual duplication of CYP76AD and DODA allowed for the present-day specialization of particular copies for function in the betalain pathway, and a complete shift away from anthocyanin production (Fig. 1c). Assuming the structural genes of the anthocyanin pathway remain intact, anthocyanin production could later be restored, possibly via loss of expression or loss of function mutations in ADHα or the betalain pathway genes (CYP76AD1α, DODAα). Nonetheless, one copy of ADH (presumably ADHβ, which retains the ancestral activity) would be retained for Tyr production (Fig. 1d).

The study by Lopez-Nieves *et al.* captures several emerging themes in the evolution of plant specialized metabolic pathways. First, it illustrates the significant role played by gene duplication in the emergence of biochemical novelty. Enzyme families such as cytochrome P450s and dioxygenases repeatedly participate in building novel metabolic pathways because of frequent duplication events, neo/sub-functionalization of activities, and enzyme promiscuity. Second, it highlights the role of primary metabolic pathways as the fountainhead of enzymes for specialized metabolism. Novel metabolic classes such as pyrrolizidine alkaloids, glucosinolates, acylsugars, acridone alkaloids and now, betalains, have emerged due to duplication of primary metabolic enzymes and subsequent neo-functionalization of duplicates (reviewed in Moghe & Last, 2015). These duplicates catalyze novel reactions with primary metabolites and divert the metabolic flux away from primary metabolism, which may impose strong selective pressure to revert to the original equilibrium or utilize the accumulating novel metabolite, triggering additional functional evolution. Finally, this study illustrates the complex interplay of connected metabolic pathways, where the production of specialized metabolites can be

favored by 'pushing' the accumulation of precursors from primary metabolism as well as 'pulling' precursors through functional specialization of downstream enzymes. Understanding how plants have evolved their tremendous diversity of specialized pathways, while meeting the primary metabolic needs for development and other functions, is a crucial gap for ongoing research in evolutionary biochemistry.

The growing knowledge of plant metabolic pathways provides new opportunities for interrogating evolutionary questions with experimental approaches. In the case of betalains, the results of Lopez-Nieves *et al.* raise a new suite of tractable questions about the mechanisms underlying biochemical innovation. For example, what alterations in protein sequence and structure were required for the loss of Tyr sensitivity? Would the accumulation of Tyr alone be sufficient to trigger even a small amount of betalain production with the pre-duplication copies of CYP76AD and DODA? Additionally, how did the emergence of ADHα influence the flux to Phe through the upstream shikimate pathway? Addressing this question may be important, given efforts to engineer this pathway in crop plants (Polturak *et al.*, 2017).

Even as we gain greater understanding of the steps leading to the evolution of betalain production, explaining the regain of anthocyanin production millions of years after its initial loss in the Caryophyllales remains challenging. The production of anthocyanins from Phe requires at least nine enzymatic steps, and if not expressed, these enzymes would be expected to quickly degenerate (Marshall *et al.*, 1994), as observed with the elements of the betalain pathway in anthocyanin-producing Caryophyllales. The retention of the anthocyanin pathway genes may be related to the continued production of nonanthocyanin flavonoids (such as flavonols in leaves and proanthocyanidins in seeds) in betalain-producing taxa (Shimada *et al.*, 2005). In this case, the re-appearance of anthocyanin production in core Caryophyllales may be related to changes in *cis*- or *trans*-regulation, as opposed to any structural evolution of the pathway. In this context, it is notable that anthocyanins and betalains are regulated by two closely related members of the MYB family of transcription factors (Hatlestad *et al.*, 2015) and the similarity in patterns of anthocyanin and betalain production in flowers and other tissues is likely due to shared ancestry of the MYBs. While the evolutionary history of these transcription factors has not been explored across the Caryophyllales, this similarity in regulation is yet another link between these two colorful and historically intertwined classes of pigments. Ultimately, dissecting these connections will be essential for constructing a complete evolutionary model for the emergence of novelty in plant pigments, and more broadly, in plant specialized metabolism.

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