

Commentary

To betalains and back again: a tale of two pigments

Plants produce myriad chemical compounds beyond those required for basic cellular function. Among these diverse molecules the floral pigments are certainly the most visible to nature lovers and the best-studied by plant biologists. Nearly all flowering plants color their petals with the red, blue, or purple products of the anthocyanin biosynthetic pathway (ABP; Rausher, 2006). Anthocyanin pigments play additional roles in vegetative tissues, providing protection against ultraviolet (UV), herbivores, and pathogens (Gould, 2004). A second group of colorful pigments, the betalains, also attract pollinators to flowers and likely also have a role in vegetative stress resistance (Hatlestad & Lloyd, 2015). Interestingly, these two classes of pigments have never been found in the same plant species (Stafford, 1994). For nearly a century, plant biologists have debated why some plants abandoned anthocyanins in their flowers, how many times betalain pigments evolved, and how betalains are functionally related to anthocyanins (Mabry, 1964). The paper by Brockington *et al.* (2015) in this issue of *New Phytologist* (pp. 1170–1180) brings some of this debate to a close, and opens the field to exciting new questions as to how and why evolution tinkers with molecular pathways during the process of adaptation.

‘For an evolutionary biologist, the fun with betalains has just now begun.’

Like anthocyanins, betalains are derived from the products of primary metabolism: biochemically the similarities between the two classes of pigments ends there (Brockington *et al.*, 2011; Hatlestad & Lloyd, 2015). Anthocyanins are synthesized from 3-malonyl Co-A and a derivative of phenylalanine, while betalains are synthesized from tyrosine and, in the case of the yellow betaxanthin, additional amino acids or amines. Betalains were once believed to be nitrogen-containing variants of anthocyanins and, indeed, for several decades in the 1900s they were often called ‘nitrogenous anthocyanins’ (Mabry, 1964).

Betalains are likely familiar to most people as the reds and yellows in chard and table beets, both cultivars of *Beta vulgaris*. Taxonomic surveys in the 1960s and 1970s revealed that betalain pigments are found in lineages throughout the Caryophyllales, an order of flowering plants that also contains spinach (*Spinacia oleracea*), four o’clocks (*Mirabilis jalapa*), quinoa (*Chenopodium quinoa*),

pokeweed (*Phytolacca americana*), and cacti (Clement & Mabry, 1996). Interestingly, several prominent lineages of Caryophyllales *do not* express betalains and, instead, produce anthocyanins. These anthocyanic lineages include the Caryophyllaceae (carnations, campions), the Kewaceae, and the Molluginaceae (Fig. 1). This spotty taxonomic distribution, along with uncertainty regarding phylogenetic relationships within the Caryophyllales, led to protracted debates on the number of origins, or losses, of betalain biosynthesis, as well as speculation on the potential adaptive value of one class of pigments over the other (Clement & Mabry, 1996). Anthocyanin production in flowers is clearly the ancestral condition – and the core ABP is highly conserved across flowering plants (Rausher, 2006) – suggesting several possible evolutionary scenarios: were betalains gained once in the Caryophyllales, with independent loss of betalain biosynthesis in anthocyanic lineages? Did betalain biosynthesis evolve multiple times independently in the Caryophyllales? Or perhaps the betalain-producing lineages are monophyletic, with the anthocyanic lineages sister to them?

In addressing these and other questions, Brockington *et al.* (2015) built on three key developments. First was the recent identification and characterization of the genes responsible for two of the three central enzymatic steps in betalain biosynthesis (Christinet *et al.*, 2004; Hatlestad *et al.*, 2012). Interestingly, in both cases these genes are members of gene families found throughout flowering plants. The second development was clarification of the phylogenetic relationships within the Caryophyllales and its sister lineages (Brockington *et al.*, 2011). Finally, the third critical development was the tremendous collaborative effort to generate transcriptome sequences for phylogenetically diverse plant species (Matasci *et al.*, 2014), along with improved analytical methods for comparative transcriptomics (Yang *et al.*, 2015).

Brockington *et al.* (2015) performed phylogenetic analyses that suggest a single origin of betalain production, early in the evolution of the Caryophyllales, and at least two subsequent independent losses of betalain production in conjunction with reversion to anthocyanin production. They further demonstrate that the two betalain-producing enzymes, DODA- α and CYP76AD1- α , are products of gene duplication events that occurred at, or near, the origin of the Caryophyllales (Fig. 1). A phylogenetic analysis of the sequence signature of natural selection on these two genes reveals candidate amino acid substitutions that may have facilitated the enzymatic shift to betalain substrates. And finally, Brockington *et al.* (2015) show that the genes encoding these two novel enzymes are closely linked on the same chromosome in table beet, suggesting that they arose from a single duplication event and may be co-regulated in a manner analogous to the famous bacterial operons.

For an evolutionary biologist, the fun with betalains has just now begun. Our new perspective afforded by these recent discoveries sets the stage for several fascinating questions: what evolutionary

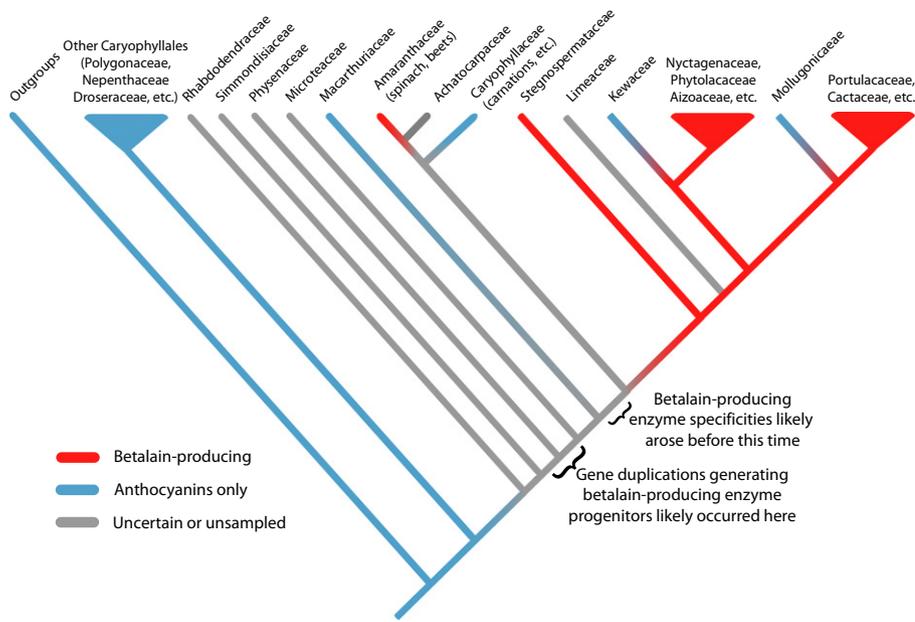


Fig. 1 Phylogenetic relationships in the Caryophyllales, with character reconstructions of anthocyanin or betalain production as inferred by Brockington *et al.* (2015) in this issue of *New Phytologist* (pp. 1170–1180). For clarity, not all lineages are shown. (Figure derived from Brockington *et al.* (2015), Fig. 4.)

forces caused those earliest lineages of Caryophyllales to abandon synthesizing anthocyanins in their floral tissues in favor of a seemingly equivalent alternate pigment? Why have some lineages reverted to anthocyanins? And how many molecular changes were required to assemble this novel adaptation (and how many were required for its subsequent loss)?

In part, these new research directions depend on our rich knowledge of the ABP. The biochemistry, genetics, and regulation of the ABP are exceptionally well understood, making the ABP a model system for studying metabolic pathway function and evolution (Rausher, 2006). Five core ABP reactions are catalyzed by enzymes encoded by known genes found across flowering plants. Several branchpoints along the ABP lead to a large number of molecules – collectively, the flavonoids – whose phylogenetic distribution are more restricted than anthocyanins and many of whose functions are not understood. The transcriptional activity of the five core genes is controlled by a suite of transcription factors: this is where the Caryophyllales becomes a fascinating evolutionary system.

All evidence to date suggests that betalain-producing members of the Caryophyllales retain a functional ABP. Many of the flavonoids are found in the vegetative tissues of betalain-producing Caryophyllales. In fact, all five core ABP enzymes are functional in betalain-producing *S. oleracea* and *P. americana* (Shimada *et al.*, 2005). So the evolutionary ‘loss’ of anthocyanins in betalain-producing plants may be as simple as transcriptional downregulation of one or two key steps of the ABP, and then only in flowers or other specific tissues. Moreover, recent molecular work in beets demonstrates that transcriptional regulation of the betalain biosynthetic genes is controlled by a MYB-family transcription factor that is closely related to a MYB which regulates the ABP (Hatlestad *et al.*, 2015).

How many mutations does it take to transition from an anthocyanin-only plant to one producing betalains? This is now a very tractable question. At least three novel enzyme specificities are

required, two of which we now know arose via duplication of existing genes followed by point mutations in those duplicates that altered substrate specificity. A transcription factor must evolve preference for the promoter regions of these new genes. And at least one enzyme in the ABP must be downregulated in tissues which express betalains: the late-acting anthocyanidin synthase (ANS) is the most likely candidate in at least some betalain producing lineages (Shimada *et al.*, 2005). As is often found to be the case, the reverse transition back to anthocyanin production requires fewer mutations: loss of at least one part of betalain biosynthesis and gain of ANS expression in the target tissue. Brockington *et al.* (2015) demonstrate the loss of DODA and/or CYP76AD1 in several lineages of anthocyanin-producing Caryophyllales. Regaining ANS expression in floral tissue could be driven by mutation in a single promoter motif, or a well-placed transposable element insertion. Further understanding of the precise sequence of events will come from careful functional analysis in plant lineages which bracket the key evolutionary transitions identified by Brockington *et al.* (2015). Particular attention might be afforded the lineages which branch from the core Caryophyllales near the inferred origin of DODA- α and CYP76AD1- α : the Neotropical Rhabdodendraceae and the Simmondsiaceae of the North American Sonoran desert may represent tractable candidates.

We are then left to address why betalains evolved in the first place, and why they were subsequently lost in some lineages. Brockington *et al.* (2011) dismiss an old hypothesis that the earliest Caryophyllales lacked all pigment in their flowers – perhaps because they were not pollinated by color-seeking animals – as inconsistent with floral character state reconstructions. Under that model, betalains were inferred to have evolved to fill the void left by anthocyanins in flowers, perhaps following an ecological shift that brought animal pollinators back into the equation. Other authors have speculated on the relative metabolic costs of producing betalains and anthocyanins – perhaps one is ‘cheaper’ under some environmental conditions or preferable if their metabolic

precursors are limiting for some reason (Clement & Mabry, 1996). One striking finding is that betalains and anthocyanins are never produced in the same plant (Stafford, 1994): is there some cost to producing both? It is possible to produce both pigments *in planta* through transgenic manipulation and substrate-feeding (Harris *et al.*, 2012). Such an approach could represent an interesting way to explore biochemical constraints on expressing the two pathways concurrently.

Biologists have long been fascinated by unusual and taxonomically-restricted adaptations as a means to understand the evolutionary mechanisms that shape organismal fitness in the natural environment. Our increasingly interdisciplinary field now permits us to make meaningful progress on many of these long-standing hypotheses.

David L. Des Marais

Arnold Arboretum and Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138-2902, USA
(tel +1 617 384 5495; email desmarais@fas.harvard.edu)

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