Review

Almost 200 Years of Anthocyanin Research: What We Know, What We Assume, and What Remains Unknown

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Almost 200 Years of Anthocyanin Research: What We Know, What We Assume, and What Remains Unknown

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Abstract

Flavonoids are frequently presented as health-beneficial compounds of plants, and derived anthocyanins are famous for striking flower colors. The biosynthesis of flavonoids has been studied for decades and developed into a model system. Despite thousands of publications about this pathway, there are still open questions about fundamental parts of the anthocyanin biosynthesis. Here we review the evolution of the anthocyanin biosynthesis and highlight open questions - some of which are sitting in plain sight.

Keywords: flavonoids; pigmentation; evolution; coloration; MYB; transcriptional regulation

Introduction

Few plant traits have captivated scientists for as long, or as productively, as the color of plants. When Robert Boyle described the pH sensitivity of 'syrup of violets' in *Experiments and Considerations Touching Colours* (1664), and when Gregor Mendel reported the dominance of purple over white pea flowers two centuries later, neither knew they were observing what we now call anthocyanins (Boyle, 1664; Mendel, 1865). Boyle discovered a pigment responsive to acids and bases, while Mendel recognized a heritable trait. Only much later were these chemical and genetic observations unified (Winkel-Shirley, 2001; Hellens *et al*., 2010; Feng *et al*., 2025; Grünig *et al*., 2025). In 1835, Ludwig Clamor Marquart introduced the name 'Anthokyan' for the blue flower pigment of cornflower described in his treatise 'Die Farben der Bluthen' (Marquart, 1835). As chemical nomenclature standardized during the late nineteenth century, the term evolved into "anthocyanin". In the early 20th century, Richard Willstätter demonstrated that these pigments are glycosides of flavylium-based aglycones, which he termed anthocyanidins (Robinson, 1935). From that point onwards, anthocyanins were no longer merely colors, but chemically defined metabolites.

After decades of classical biochemical and genetic research, the first sequences of genes underlying anthocyanin biosynthesis were identified and cloned (Holton & Cornish, 1995), establishing anthocyanins as one of the best-understood specialized metabolic pathways in plants (**Figure 1**). Today, a wide range of ecological and physiological functions is attributed to anthocyanins, including pollinator and seed disperser attractions (Davies *et al*., 2012; Renoult *et al*., 2014), sunscreen (Gould *et al*., 1995; Agati *et al*., 2021), camouflage (Givnish, 1990), and mimicry (Lev-Yadun & Inbar, 2002). In biotechnology, anthocyanins are valued as strong antioxidants (Butelli *et al*., 2008, 2021) and food colorants (Appelhagen *et al*., 2018). Due to the visible phenotypes of mutations in the anthocyanin biosynthesis, the pathway and its regulation have also been studied as a model system in evolutionary biology (Smith & Rausher, 2011; Smith *et al*., 2013; Marin-Recinos & Pucker, 2024).

The anthocyanin biosynthesis pathway branches from the flavonoid biosynthesis (Winkel-Shirley, 2001; Grünig *et al*., 2025). Enzymes for the biosynthesis include chalcone synthase (CHS),

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chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), anthocyanin-related glutathione S-transferase (arGST), UDP-dependent anthocyanidin 3-O-glucosyltransferase (U3GT), and additional modifying enzymes (**Figure 2a**) (Grünig *et al*., 2025). Following F3H, the two optional enzymes flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) can add one or two hydroxyl groups, respectively, which alter the biochemical properties of the intermediate, ultimately leading to different anthocyanin colors. The simplest pathway leads to orange pelargonin, one additional hydroxylation yields pink cyanin, and two additional hydroxylations yield blue delphinin (Schwinn *et al*., 2014).

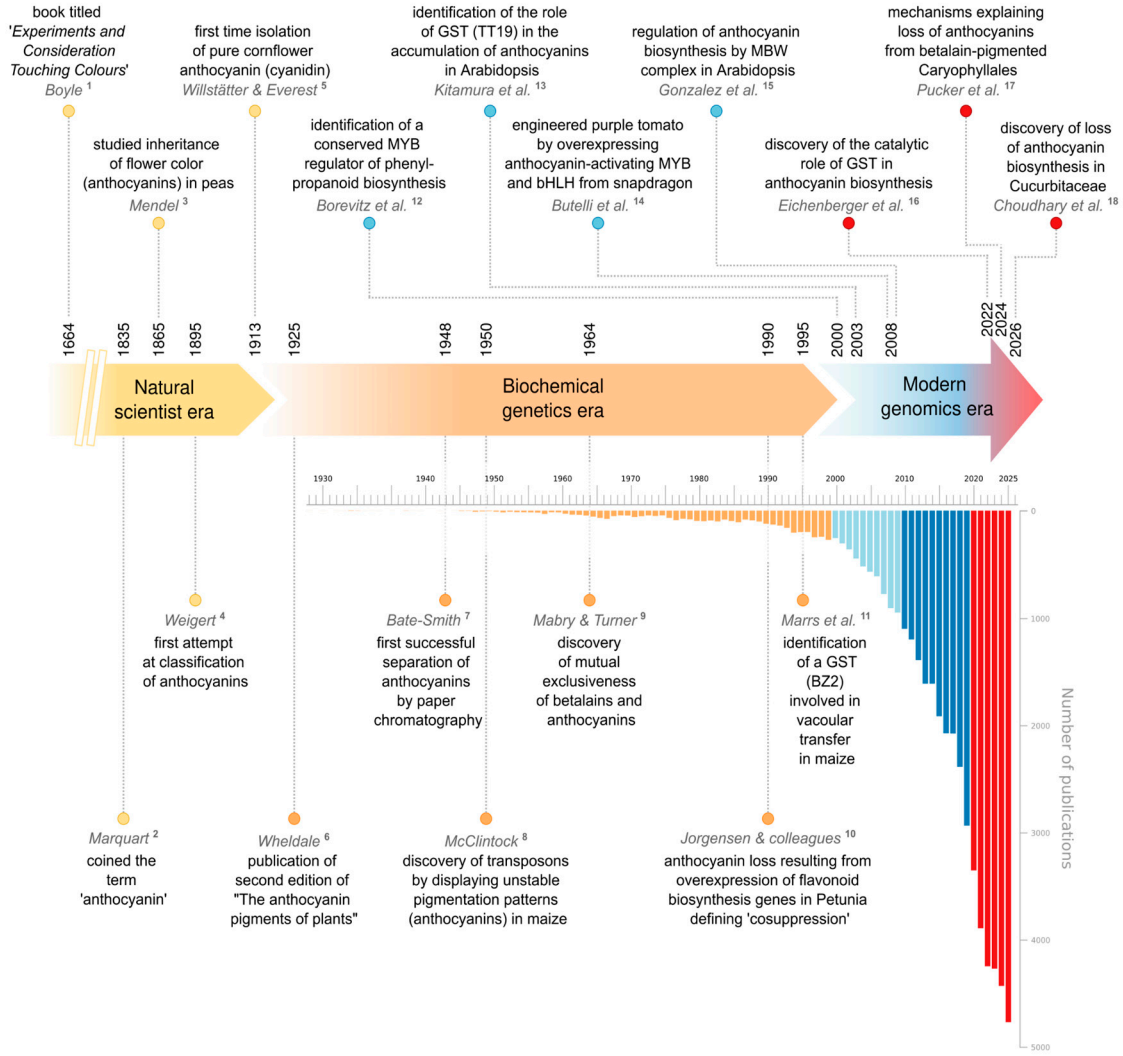

Figure 1. Timeline of fundamental discoveries and milestones in the anthocyanin biosynthesis research. The number of publications per year (from 1920 onwards) was retrieved from PubMed and Web of Science using the query ‘anthocyanin OR anthocyanidin OR leucoanthocyanidin OR cyanidin OR delphinidin OR pelargonidin OR malvidin OR petunidin OR peonidin’. References: 1 (Boyle, 1664); 2 (Marquart, 1835); 3 (Mendel, 1865); 4 (Weigert, 1895); 5 (Willstätter & Everest, 1913); 6 (Wheldale Onslow, 1925); 7 (Bate-Smith, 1948); 8 (McClintock, 1950); 9 (Mabry & Turner, 1964); 10 (Napoli *et al*., 1990; Jorgensen, 1995); 11 (Marrs *et al*., 1995); 12 (Borevitz *et al*., 2000); 13 (Kitamura *et al*., 2004); 14 (Butelli *et al*., 2008); 15 (Gonzalez *et al*., 2008); 16 (Eichenberger *et al*., 2023); 17 (Pucker *et al*., 2024); 18 (Choudhary *et al*., 2026).

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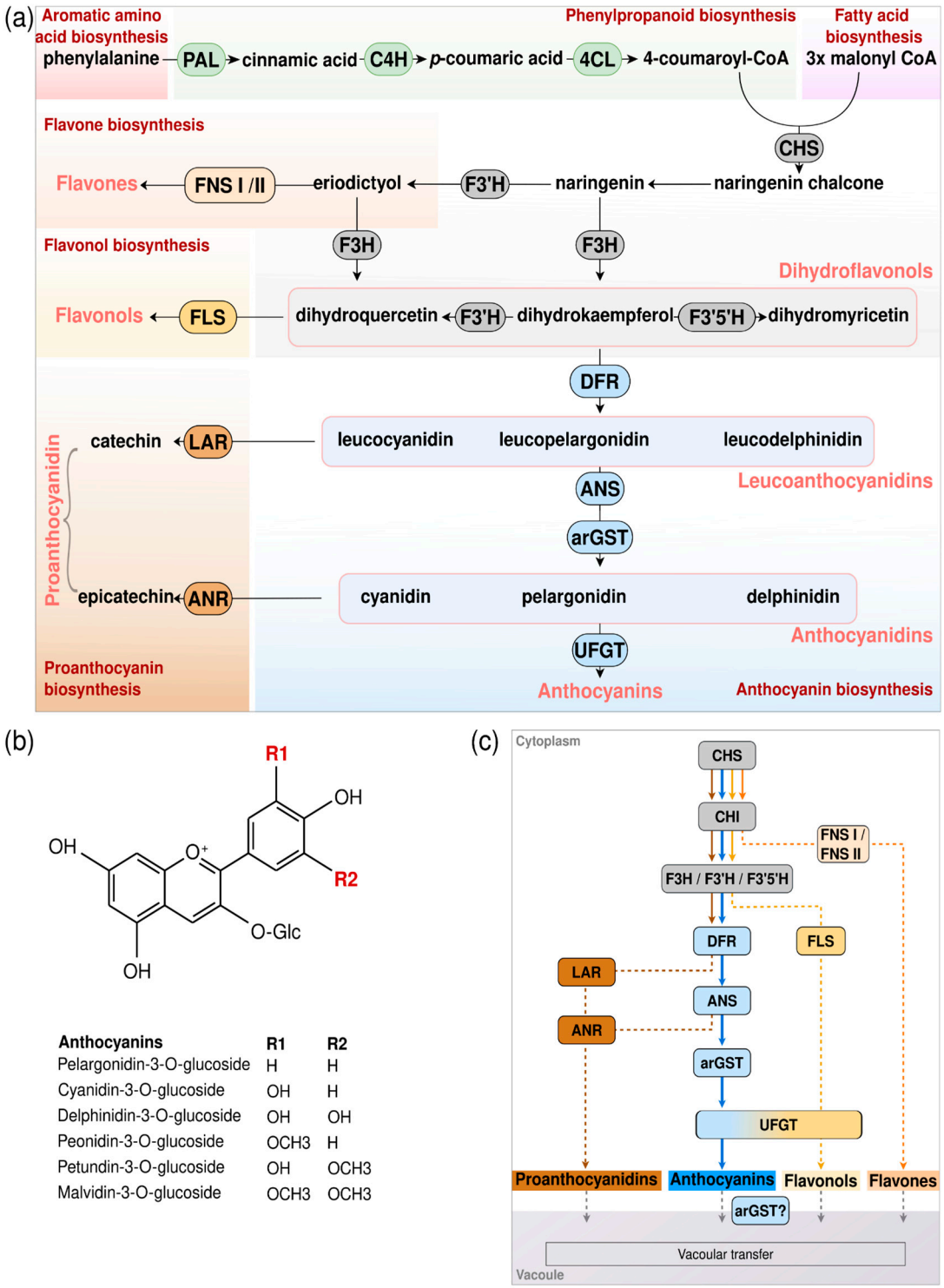

Figure 2. The core pathway for anthocyanin biosynthesis. (a) Simplified illustration of the flavonoid biosynthesis pathway in plants, highlighting the major branching pathways. The anthocyanin biosynthesis pathway and genes are shown in blue. Gene abbreviation not given in text: PAL, Phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; FNS, flavone synthase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase. (b) Overlap of different branches in the flavonoid biosynthesis. The presence of the function of the enzyme in any flavonoid branch is indicated by colored circles. Flavone (orange), flavonol (yellow), anthocyanin (blue), and proanthocyanidin (brown) branches are shown. (c) Basic structure of anthocyanin and representative anthocyanins.

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The transcriptional activation of the anthocyanin biosynthesis involves three transcription factors from different protein families that form a complex (MBW): MYB, bHLH, and WD40 (Gonzalez *et al*., 2008; Grünig *et al*., 2025). The MYB component is considered to be the most specific part, because it appears to be committed to the anthocyanin biosynthesis (Wheeler *et al*., 2022; Marin-Recinos & Pucker, 2024). The bHLH and WD40 components have a range of functions in plant development and physiology beyond regulating anthocyanin biosynthesis. Several other transcriptional regulators have been reported (Grünig *et al*., 2025), including a number of transcriptional repressors (LaFountain & Yuan, 2021).

Despite decades of intensive study of anthocyanin biosynthesis as a model system, major discoveries have emerged in the last few years. This includes arGST as an additional biosynthesis enzyme (Eichenberger *et al*., 2023) and the complete loss of the anthocyanin biosynthesis in Cucurbitaceae (Choudhary *et al*., 2026). Patterns of duplication, subfunctionalization, loss, and convergent evolution across angiosperms suggest that this seemingly 'textbook' pathway continues to yield unexpected insights.

Here, we revisit nearly two centuries of anthocyanin research to distinguish what is firmly established from what is inferred, assumed, or still unknown. By highlighting overlooked observations, unresolved evolutionary questions, and modern methodologies, we aim to outline a research agenda for the next phase of anthocyanin research.

Main

Is There a Dedicated Anthocyanin Biosynthesis?

The set of genes reported as "anthocyanin biosynthesis" varies considerably across studies. In some cases, the entire upstream core flavonoid pathway is implicitly treated as committed to anthocyanin biosynthesis, overlooking the multiple competing branches that lead to other end products. In others, DFR and ANS are identified as core components of the pathway, while additional steps such as arGST, glycosyltransferases, and other modifying enzymes are often neglected.

The flavonoid biosynthesis is best understood as a highly complex interconnected network with branches leading to chemically distinct products, including flavones, flavonols, anthocyanins, and proanthocyanidins (Winkel-Shirley, 2001; Grünig *et al*., 2025). The early steps are largely shared, and metabolic flux is directed into specific branches only at later steps. There is a substantial overlap in participating enzymes involved in anthocyanin and proanthocyanidin biosynthesis (**Figure 2b**). Both pathways require DFR and ANS, indicating that these enzymes are not uniquely committed to anthocyanin biosynthesis. Recently, arGST was characterized as an enzyme in the anthocyanin biosynthesis (Eichenberger *et al*., 2023), after a role in anthocyanin transport was postulated and widely propagated for over two decades (Mueller *et al*., 2000, p. 200; Pucker & Selmar, 2022). The *Arabidopsis thaliana* arGST (*transparent testa 19, tt19*) mutant was originally identified as defective in seed coat pigmentation, i.e., proanthocyanidin biosynthesis (Kitamura *et al*., 2004; Sun *et al*., 2012), which raises the question of whether this enzyme might be involved in both anthocyanin and proanthocyanidin biosynthesis. Plant lineages without arGST orthologs provide first hints for the dispensability of arGST in the context of proanthocyanidin biosynthesis and also offer an opportunity to explore this in greater detail (Pucker *et al*., 2024; Khatun *et al*., 2025). While grasses (Poaceae) possess BZ2 as a functional replacement of otherwise canonical arGSTs (TT19/An9 orthologs) (Khatun *et al*., 2025), several Caryophyllales families appear to lack a functional equivalent, but still harbour proanthocyanidin biosynthesis genes (Pucker *et al*., 2024). This suggests that arGST is dispensable for proanthocyanidin production - at least in some plant lineages. Meanwhile, the long-standing model of arGST-mediated anthocyanin transport has not been revisited in light of its discovery as an enzyme. Vesicle-based anthocyanin transport mechanisms have also been postulated and supported by experimental evidence (Poustka *et al*., 2007; Pucker & Selmar, 2022). Since functional redundancy is unlikely to be maintained over long evolutionary timescales, it seems improbable that two fully redundant transport systems coexist. In summary, these observations

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suggest that arGST might fulfill a more specific role as an enzyme in the anthocyanin biosynthesis rather than acting as a general requirement for flavonoid biosynthesis. The step following arGST in the anthocyanin biosynthesis is catalyzed by a glucosyltransferase. In *Arabidopsis thaliana*, UGT78D2 (At5g17050) converts unstable colorless anthocyanidins into anthocyanins, but also appears to modify flavonols (Tohge *et al*., 2005). In summary, these observations suggest that there might be no set of enzymes exclusively dedicated to anthocyanin biosynthesis. Instead, a coordinated activation of genes needed for the biosynthesis, combined with selective regulation and suppression of genes for competing branches, might form the anthocyanin biosynthesis. This would align with observations from evolutionary studies that reported the anthocyanin-specific MYB activator as the most often implicated component in the anthocyanin biosynthesis block (Marin-Recinos & Pucker, 2024). While there are numerous enzymes for further modifications of anthocyanins (Grünig *et al*., 2025), these extend beyond the core biosynthetic steps. The multifunctionality observed in the anthocyanin biosynthesis genes aligns with the established role of enzyme promiscuity shaping the diversity of the (specialized) metabolism in plants (Leong & Last, 2017; Yokoyama, 2024). For practical reasons, however, it remains useful to define a minimal functional module for anthocyanin production. This core set, comprising the DFR, ANS, arGST, and UFGT, enables the synthesis of stable anthocyanin pigments and provides a working definition of "anthocyanin biosynthesis".

Why Is There Independent Evolution of Certain Steps in the Anthocyanin Biosynthesis?

Anthocyanin biosynthesis has generally been considered to be well conserved across land plants (Falcone Ferreyra *et al*., 2012; Xu *et al*., 2015; Tohge *et al*., 2017). Most documented exceptions involve lineage-specific losses of anthocyanins, but these are often restricted to the species level (Marin-Recinos & Pucker, 2024). For a long time, the replacement of anthocyanins by betalains in several families of the Caryophyllales represented the only known scenario of large-scale anthocyanin loss (Mabry, 1964; Mabry & Turner, 1964; Brockington *et al*., 2011; Timoneda *et al*., 2019). More recent studies, however, have covered additional cases of pathway gene loss and rewiring at the family level in Cucurbitaceae (Choudhary *et al*., 2026) and Poaceae (Khatun *et al*., 2025), respectively, suggesting a more dynamic evolutionary history of anthocyanin biosynthesis than previously assumed. However, most enzymes of the biosynthesis pathway appear to be monophyletic and broadly conserved across anthocyanin-producing plant lineages.

An exception is the arGST encoding gene. The two classical arGSTs, BZ2 in maize (McLaughlin & Walbot, 1987; Alfenito *et al*., 1998) and An9 in petunia (Alfenito *et al*., 1998), were discovered decades ago and share low sequence similarity between them. However, a reciprocal complementation experiment demonstrated that they apparently perform the same function in the anthocyanin biosynthesis (Alfenito *et al*., 1998). Comprehensive phylogenetic analysis has shown that they are members of different GST lineages, with the BZ2 lineage being restricted to Poaceae, and the An9 lineage is found in other anthocyanin-pigmented plants (**Figure 3**) (Khatun *et al*., 2025). It still remains an open question why the An9 lineage was lost in the grasses and why it was replaced by the BZ2 lineage. This might be associated with changes in the transcriptional regulation of the anthocyanin biosynthesis in Poaceae.

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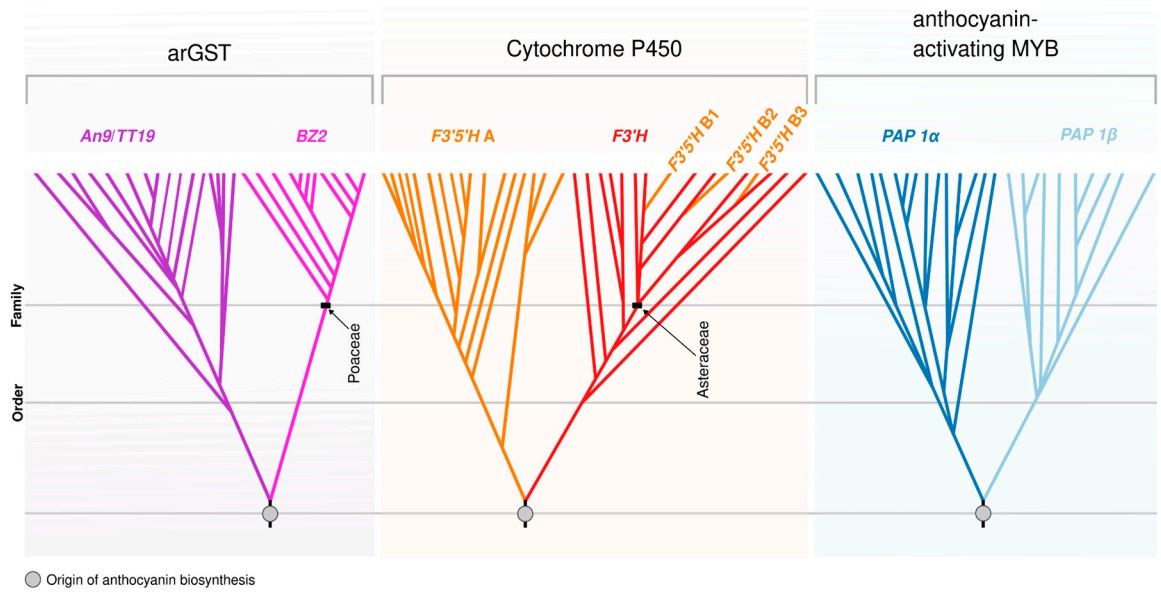

Figure 3. Simplified schematic illustration of some known duplication events affecting anthocyanin biosynthesis genes. From left to right: independent arGST lineages, independent F3′5′H evolution events, and complex MYB relationships. An9, Anthocyanin9; TT19, Transparent Testa 19; PAP1α, Promoter of Anthocyanin Production 1α; PAP1β, Promoter of Anthocyanin Production 1β; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase.

In general, the regulatory components of anthocyanin biosynthesis appear to be more evolutionarily flexible than the structural genes. In Poaceae, the canonical anthocyanin-activating MYB lineage appears to be lost and has been functionally replaced by a closely related MYB lineage (Khatun *et al*., 2025). It is possible that such a change in transcriptional regulation facilitated the recruitment of a different structural component, such as the distinct GST lineage. Broadly, a deep duplication event of the anthocyanin-activating MYB in anthocyanin-pigmented plants, followed by secondary loss of one MYB lineage in many plant lineages, has resulted in a complex evolutionary relationship between the anthocyanin regulators in different plants (Pucker *et al*., 2024). Therefore, insights from one species cannot always be directly transferred to other species if anthocyanin biosynthesis in these species is not controlled by an orthologous MYB.

The optional hydroxylation step of anthocyanidin precursors catalyzed by F3′5′H is another well-characterized example of independent evolution. F3′H (CYP75B) and F3′5′H (CYP75A) are sister lineages within the large cytochrome P450 family (Tanaka & Brugliera, 2013). While both lineages split at the basis of anthocyanin-pigmented plants (Tanaka & Brugliera, 2013), there are several cases of independent evolution of F3′5′H activity from F3′H genes (Seitz *et al*., 2006, 2015; Lui *et al*., 2020). This repeated emergence is facilitated by the fact that only a small number of amino acid substitutions are required to shift enzyme specificity (Seitz *et al*., 2007). Therefore, it seems plausible that mutations at the required sites happened multiple times during evolution. Since the gene expression pattern of F3′H already aligns with other anthocyanin biosynthesis genes, derived gene copies would already show the gene activity needed to form anthocyanins.

In summary, the evolution of the anthocyanin biosynthesis appears to be more complex than previously appreciated, including multiple events of gene loss or gene gain. A key challenge would be to understand the molecular and ecological drivers of this flexibility. Shifts in pollination strategies, changes in transcriptional regulation, and competition for shared substrates are some factors that may contribute in generating evolutionary novelty.

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Does Substrate Hydroxylation Pattern Influence Acceptance by ANS, arGST, and Downstream Enzymes?

Dihydroflavonols, which differ in their B-ring hydroxylation pattern, represent a central branching point in the flavonoid pathway. These intermediates can be utilized by flavonol synthase (FLS) and DFR to form flavonols or to form anthocyanins and proanthocyanidins, respectively. FLS and DFR show differential substrate preferences, which could serve as a mechanism to mitigate direct competition between these enzymes (Choudhary & Pucker, 2024). For example, FLS in many species prefers the single hydroxylated substrate dihydrokaempferol, while DFR prefers the double hydroxylated substrate dihydroquercetin (**Figure 4**). The activity of F3'H, which converts dihydrokaempferol into dihydroquercetin, therefore could be an important control point for directing metabolic flux between competing branches. A single amino acid residue in DFR has been reported as the decisive factor regarding substrate preference (Johnson *et al*., 2001). Based on this residue, which is position 133 in *A. thaliana*, the DFRs discovered in different plant species can be broadly classified into three types: the common DFR$_N$ type, which mostly produces cyanidin, the DFR$_D$ type, which accepts double/triple hydroxylated substrates and can produce cyanidin/delphinidin, and the rarer DFR$_A$ type, which favors single hydroxylate substrates and produces mostly pelargonidin (Johnson *et al*., 2001; Miosic *et al*., 2014).

These different substrates of DFRs result in the formation of leucoanthocyanidins with distinct hydroxylation patterns, which then serve as substrates for downstream enzymes. In the anthocyanin branch, these intermediates are further processed by ANS, arGST, glucosyltransferases, and other decoration enzymes. This raises the question, "Do downstream enzymes in anthocyanin biosynthesis also exhibit substrate preferences linked to hydroxylation patterns?" If ANS, arGST, or UFGTs show similar substrate-specificity constraints as DFR, substrate hydroxylation could be the unifying principle governing pathway flux and product composition. Identifying such key residues, if any, would not only deepen our understanding of the pathway but could also provide opportunities in crop improvement and for the horticulture industry.

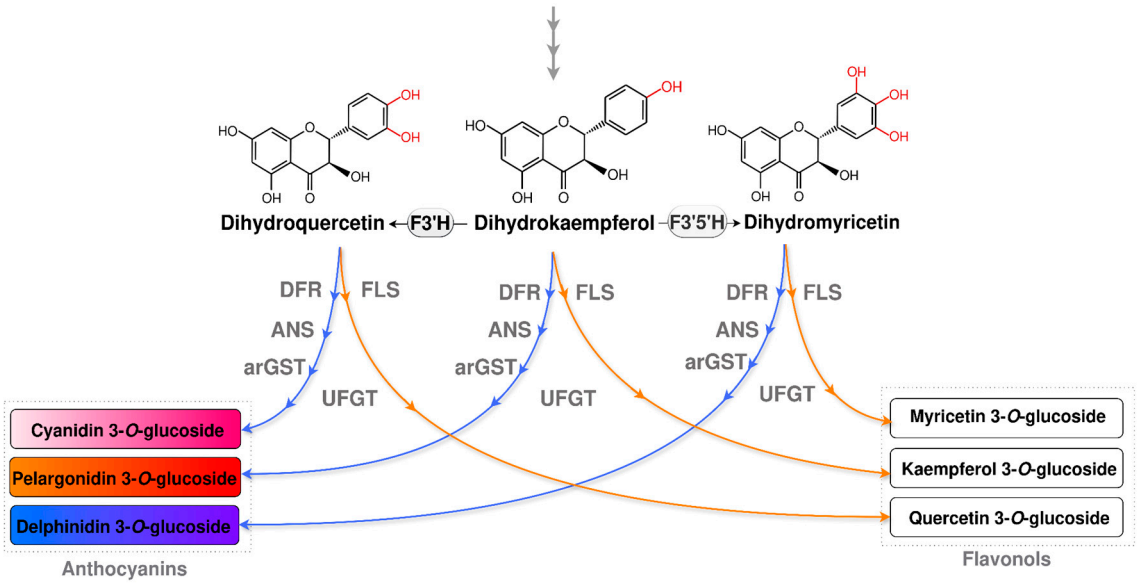

Figure 4. The major branching point in the flavonoid biosynthesis leading to anthocyanins (left) and flavonols (right). The hydroxylation pattern of dihydroflavonols is an important factor for the anthocyanin and competing flavonol biosynthesis. Dihydrokaempferol, with a single hydroxylation of the B ring, leads to orange pelargonidin derivatives; dihydroquercetin, with two hydroxylations at the B ring, results in pink cyanidin derivatives, and dihydromyricetin, with three hydroxyl groups at the B-ring is converted into blue delphinidin derivatives. Different DFR types have preferences for substrates with a specific hydroxylation pattern. Competition between DFR and FLS is mitigated through differential substrate preferences and gene expression profiles.

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Which Factors Control Transcription of arGST, F3'H, and F3'5'H?

Transcriptional regulation of the anthocyanin biosynthesis appears to be the most flexible element and the most important factor during evolutionary change. Although the regulation of the anthocyanin biosynthesis through the MBW complex is considered a model system for transcriptional control in eukaryotes, there are still significant knowledge gaps in plain sight. The MBW complex is a central component, but numerous additional factors have been reported that might fine-tune the activation of different anthocyanin biosynthesis genes under specific conditions (Jaakola, 2013; Liu *et al*., 2019; LaFountain & Yuan, 2021; Grünig *et al*., 2025). Comparing the transcriptional regulation of arGSTs in the BZ2 and An9 lineage could help to understand fundamental differences, which might have provided an evolutionary advantage that facilitated the replacement of An9 by BZ2 in the Poaceae. Simultaneously, this would also contribute to answering the question of whether arGST is a committed player in the anthocyanin biosynthesis.

Several hints suggest that the F3'H and F3'5'H encoding genes are not strongly coexpressed with other genes of the anthocyanin biosynthesis. For example, flavonol regulators of the MYB subgroup 7 lineage triggering *CHS*, *CHI*, and *F3H* do not seem to activate *F3'H* in *A. thaliana* (Stracke *et al*., 2007). In our experience, the coexpression of *F3'H* with *DFR* and *ANS* in *A. thaliana* is rather weak. A precise regulatory control of *F3'H* and *F3'5'H* might be a mechanism to mediate the flux into the competing pathways flavonol and anthocyanin biosynthesis (Choudhary & Pucker, 2024).

Is arGST Only an Enzyme or Also Involved in Anthocyanin Transport?

The evidence for the arGST role as an enzyme in the anthocyanin biosynthesis (Eichenberger *et al*., 2023) questions the previous hypothesis that the protein would function in anthocyanin transport (Mueller *et al*., 2000). Originally, there was enzymatic activity reported for BZ2 (Marrs *et al*., 1995), but numerous subsequent studies explained the association of this protein with the anthocyanin metabolism with a non-enzymatic role as a 'ligandin' protecting anthocyanins during transport to the central vacuole (Mueller *et al*., 2000; Kitamura *et al*., 2004; Pucker & Selmar, 2022). While there is no clear evidence for an arGST role in anthocyanin transport, changing this well-established narrative is difficult. Ultimately, it is impossible to completely rule out a function in anthocyanin transport, but gathering evidence for a purely enzymatic role would be an important contribution to an accurate picture of the anthocyanin metabolism.

Do anthocyanins have an indispensable function?

Anthocyanins are best known for their role as visible pigments, contributing red to blue to purple coloration to plants. This pigmentation is directly linked to well-established ecological functions, including the attraction of pollinators and seed dispersers (Winkel-Shirley, 2001; Grünig *et al*., 2025), as well as roles in camouflage and mimicry (Givnish, 1990; Lev-Yadun & Inbar, 2002). These functions provide a clear explanation for the widespread occurrence of anthocyanins in angiosperms, i.e., the flowering plants. However, a broader range of physiological roles, beyond pigmentation, has been attributed to anthocyanins, including photoprotection (Gould *et al*., 1995; Agati *et al*., 2021), antioxidant activity (Butelli *et al*., 2008), and tolerance to abiotic stresses (Chalker-Scott, 1999; Pietrini *et al*., 2002). Many of these functions are also described for other flavonoid classes from the branches of the flavonoid pathway. This raises the question whether these roles are specific to anthocyanins or instead properties of flavonoids as a class. From an evolutionary perspective, this distinction is particularly relevant. The core flavonoid pathway, including flavone and flavonol biosynthesis, is deeply conserved and likely originated early in land plant evolution (Davies *et al*., 2024). The complete suite of enzymes required for canonical anthocyanin biosynthesis appears to have originated first in gymnosperms (Piatkowski *et al*., 2020) and is most consistently observed in angiosperms, suggesting it might have evolved as a reproduction strategy. Anthocyanin loss provides further insights. In Caryophyllales and Cucurbitaceae, betalains and carotenoids, replacing anthocyanins, perform similar functions, hinting that the pigmentation function of anthocyanins is replaceable (Timoneda *et al*., 2019; Choudhary *et al*., 2026). Whether the proposed physiological functions of anthocyanins are replaceable is less clear. However, if they were necessary for

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photoprotection or stress tolerance, their large-scale loss would have been difficult to reconcile over large evolutionary times. In this context, anthocyanins may represent one of the interchangeable solutions from the flavonoid pathway rather than a necessarily required component.

Conclusion

Recent studies suggest that there is still potential for fundamental discoveries in anthocyanin biosynthesis. The picture of a well-conserved pathway is challenged by species-specific differences and multiple cases of anthocyanin loss that have been revealed in recent years. The importance of transcriptional regulation emphasizes questions about the fine-tuning of different steps and control mechanisms that can prevent competition between branches of the flavonoid biosynthesis. All these findings support the anthocyanin biosynthesis as an excellent model system, as it also reflects the complexity, diversity, and evolutionary flexibility of the specialized plant metabolism.

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