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Conservation and diversification of flavonoid metabolism in the plant kingdom

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Flavonoids are by far the largest class of polyphenols with huge structural and functional diversity. However, the mystery regarding the exact evolutionary pressures which lead to the amazing diversity in plant flavonoids has yet to be completely uncovered. Here we review recent advances in understanding the conservation and diversification of flavonoid pathway from algae and early land plants to vascular plants including the model plant Arabidopsis and economically important species such as cereals, legumes, and medicinal plants. Studies on the origin and evolution of R2R3-MYB regulatory system demonstrated its highly conserved function of regulating flavonoid production in land plants and this innovation appears to have been crucial in boosting the overall levels of these compounds in land plants. Convergent evolution has occurred as different flavonoids independently which emerged in distant taxa resulting in similar defense and tolerance characteristics against environmental stresses. Future studies on an increasing number of plant species taking advantage of newly developed genomic and metabolite profiling technologies are envisaged to provide comprehensive insight into flavonoid biosynthesis as well as pathway diversification and the underlying evolutionary mechanisms.

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Introduction

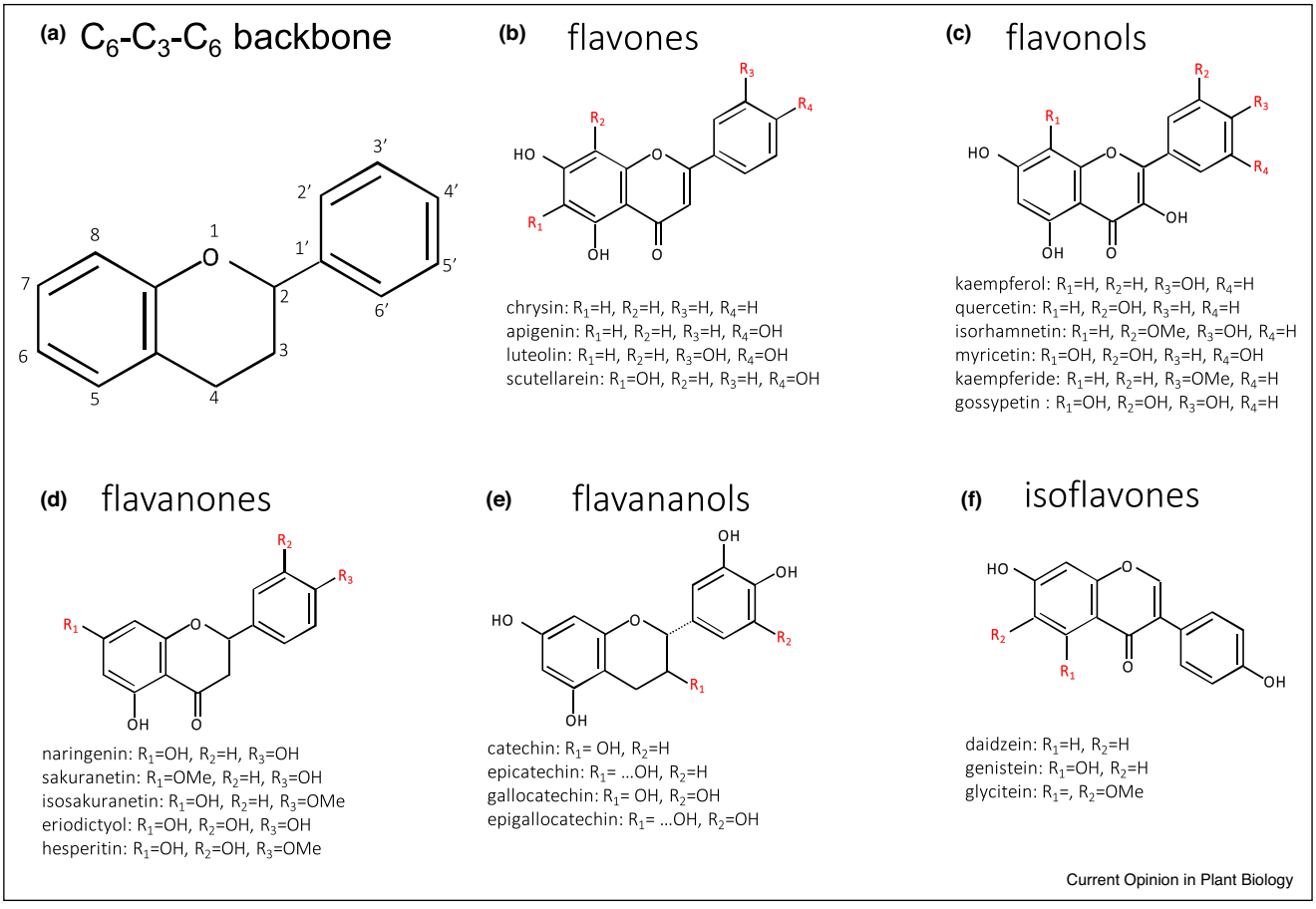
Flavonoids are by far the largest class of polyphenols and have been estimated to comprise over 8000 metabolites — they bear a common diphenylpropane (C6-C3-C6) backbone in which two aromatic rings are linked via a three-carbon chain (Figure 1). The A ring is normally formed from a molecule of resorcinol or phloroglucinol synthesized via the acetate pathway [1] and displays a characteristic hydroxylation pattern at the C5 and C7 positions [2••]. By contrast, the B ring comes from the far more comprehensively characterized shikimate pathway and is often 4′-hydroxlated, 3′4′-hydroxlated, or 3′4′5′-hydroxlated [3]. Flavonoids have been further subdivided into six major subclasses and in excess of 5000 minor subclasses, in the seminal phytochemical work of Harbone (see for example [4]), on the basis of the oxygenation pattern of the heterocyclic C ring; namely flavones, flavonols, flavanones, flavanols, anthocyanidins, and isoflavones. We will, largely, not discuss anthocyanidins here since they are covered in detail in another article in this issue (Tohge article this issue), although for the discussion on transcriptional control we do additionally cover this class of flavonoids.

Before discussing the natural variation in flavonoid composition and content we feel it is prudent to briefly outline what is known concerning their biological function(s). Flavonoids play a variety of roles in extant plants such as protectant functions against biotic and abiotic stresses, visual signals for attracting pollinators, and the regulation of plant hormonal activity. For instance, the levels of phenylpropanoids fluctuate greatly in response to extracellular circumstance with numerous factors including light irradiation [5] as well as other abiotic stresses including nitrogen deficiency, cold and drought [6]. In addition, they have been demonstrated to be very important with regard to biotic stresses with evidence accumulating for the roles of flavones and isoflavones in the response of medicago to fungal pathogens [7], while the presence of maysin in maize confers resistance against the major pest *Helicoverpea zea* [8•]. The function of flavonoids has recently been comprehensively reviewed [2••], so we will not cover this here.

Flavonoid biosynthesis in early diverging extant plant lineages

It is considered that defense against solar irradiation and regulation of plant hormonal activity were the original functions of flavonoids in the earliest flavonoid producing plants, and these functions have been greatly diversified in the long evolutionary process [9,10]. Although some algal species can produce mycosporin-like amino acids as light tolerance compounds, generating novel mechanisms or compounds to deal with the increased stress from lack

Figure 1



Structure of major flavonoid aglycones **(a)**. Flavones **(b)**, Flavonols **(c)**, Flavanones **(d)**, Flavananols **(e)** and Isoflavones **(f)**.

of light protection by water are postulated as key evolutionary innovations that occurred during land colonization [9–12]. That said the capacity of flavonoids to scavenge ROS (generated by high irradiance but essentially all types of biotic and abiotic stresses), has also been suggested to be a driver for the fixation of flavonoid chemistry with the capacity to absorb UV-B radiation perhaps being, at least initially, ancillary [9–12]. Furthermore, in angiosperms anthocyanin pigments function as visual signals for attracting pollinators and fruit dispersal agents, but these functions were acquired late in the evolutionary diversification of flavonoids [9,10].

Genome surveys carried out in 2013 refuted early claims [13] of the presence of flavonoid biosynthesis in the unicellular green alga *Chlamydomonas eugametos* [2••]. However, in marine brown algae specialized polyphenolic derivatives for UV-B protection such as phlorotannins and fucols derived via the polyketide pathway have been found (reviewed in Ref. [2••]). There is furthermore convincing evidence that phenylpropanoid production occurs in charophytic algae – the likely precursors of land plants – including the presence of both orthologs of phenylpropanoid biosynthetic genes [14] and downstream lignin and lignin-like structures [15]. The suggestion by Stebbins and Hill that Charophyceae secondarily returned to an aquatic habitat, after adaptation to terrestrial or amphibian life may, however, offer an explanation for the above-mentioned observations [16]. Wolf *et al.* found the existence of UV-B-dependent induction of flavonol biosynthesis in *Physcomitrella patens* which supports the hypothesis that enzymes involved in the early flavonoid secondary metabolism and corresponding signal transduction pathways evolved with the water-to-land transition due to higher UV-B radiation exposure, as extant algal species are not able to synthesize flavonoids [17]. Gene duplication and cis-regulatory evolution are proposed as the major driving forces for evolution of phenolics in land plants. For instance, chalcone synthase (CHS), the first enzymatic gene of flavonoid biosynthesis,

like many genes involved in secondary metabolism, has been derived via gene duplication from genes coding for enzymes of primary metabolism and undergone cis-regulatory evolution [2••].

Moreover, knowledge concerning biochemical plant-host responses to microbial invasion, which has until recently been poorly characterized, has recently advanced considerably. Indeed, Carella *et al.* [18••], recently studied the response of the early divergent liverwort *Marchantia polymorpha* to infection with the oomycete pathogen *Phytophthora pakmivora* uncovering a robust response utilizing the same conserved pathways observed in the model angiosperm *Nicotiana benthiamiana*. Another study on liverwort by Albert *et al.* supports the hypothesis that an inducible flavonoid pathway was an early evolutionary adaption to the abiotic stresses of life on land [19•]. As such these data suggest that combined pressures from light stress, the need to develop a vertical stand and defense against both biotic and abiotic stress led to the metabolic innovation that results in the core pathway of flavonoid metabolism. In addition, recently Goiris *et al.* found the widespread occurrence of flavonoids (flavones, isoflavones, and flavonols) in microalgae from diff ;erent evolutionary lineages (Cyanobacteria, Rhodophyta, Chlorophyta, Haptophyta, and Ochrophyta), implying that plants may have acquired the ability to produce flavonoids earlier than previously thought [20]. These also suggest that the ability of plants to produce flavonoids, which provide the adaptive mechanisms needed for their survival in changing environments, may have evolved multiple times, or that in some instances the ability was lost during evolution.

In the following sections we will discuss some papers detailing the diversification of flavonoids in angiosperms exemplified by model plant Arabidopsis, and economically important plants including cereals, legumes, and the medicinal plant *Scutellaria baicalensis*. For deeper information concerning research on the evolution of citrus flavonoids the reader is referred to recent studies and reviews [21•,22].

Arabidopsis

As a model species, the flavonoid biosynthetic pathway and genes have been relatively well characterized in Arabidopsis [23–27]. A recent work, which identified flavonol-phenylacyltransferase 2 (FPT2) as being responsible for the production of saiginols and conferring greater UV light tolerance in planta based on the natural variation in Arabidopsis, is noteworthy [28•]. The phenylacylated-flavonols (saiginols) highly accumulated in floral tissues of Arabidopsis were identified in a subset of accessions, especially those deriving from latitudes between 16° and 43° North. The presence of a functional FPT2 seemingly confers a selective advantage in high light growth habits. Moreover, analysis of polymorphism within the FPT duplicated genomic region provides an evolutionary framework of the natural history and current status of this locus in the Brassicaceae. In addition, a recent report revealed that there is phenylacylation of flavonoids at a position distinct from that of the saiginols which is rare but occurs in families spanning the green lineage [6].

Cereals

The top three high produced cereals worldwide, maize (*Zea mays* ssp. mays), rice (*Oryza sativa*), and wheat (*Triticum aestivum*) are of massive importance to the human diet. Several kinds of flavonoids are ubiquitously distributed in these grass crops but almost absent in Brassicaceae for instance glycosylated flavones [29]. A recent study indicated that flavones constitute the majority flavonoids in rice and two UDP-dependent glycosyltransferases (i.e. OsUGT707A2 termed flavone 5-*O*-glucosyltransferase, F5GlcT and OsUGT706D1 termed flavone 7-*O*-glucosyltransferase, F7GlcT) in a new clade of flavonoid glucosyltranferases are the major enzymes controlling the natural variation of flavones in rice [29]). It is also showed that variation of major rice flavonoids is regulated at RNA transcript and enzyme activity level, thus providing an example of multi-layered regulation of natural variation within a single biosynthetic pathway. In accordance with, the results, it was speculated that decorating enzymes, which are responsible for the final steps of metabolite synthesis generally make a greater contribution to the natural variation of metabolite abundance than early pathway enzymes. More interestingly, this study also suggested that *O*-glycosylated flavones play a positive role in plant UV-B protection and that the allelic variation of these two newly characterized genes contributes to UV-B tolerance in nature. However, the exact mechanism by which this superior protection to UV-B is conferred is currently unknown.

Studies on maize flavonoid biosynthesis have been carried out since the 1970s. From these studies a large number of genes including enzymes, and regulatory factors and transporters were identified and characterized [30,31]. Specially, the flavonoid diversity of maize kernels has been repeatedly studied as its kernels make a very large contribution to the diets of humans and animals [32–34]. Flavones which have potent anti-inflammatory and anticarcinogenic activities are present primarily as C-glucosides and *O*-glucosides in maize kernels [32,35]. A C-glycosyl flavone – maysin – confers natural resistance to the maize earworm (*H. zea*) when present in silks. The maize maysin biosynthetic pathway is completed and the Sm1 (UDP-rhamnose synthase RHS1) and Sm2 (rhamnosyl transferase UGT91L1) responsible for the last biosynthetic steps are direct targets of P1 which is an R2R3-MYB transcription factor [32]. P1 is the major regulator for a set of genes involved in flavonoid biosynthesis and a minor modulator of the expression of a much larger gene set that includes genes involved in primary metabolism and production of other specialized compounds [36]. However, P1 does not control

anthocyanin biosynthesis [30]. Studies on metabolic divergence between maize and its wild progenitor teosinte have been reported recently that focused on several metabolic pathways (carbohydrates, amino acids, alkaloids, terpenoids, flavonoids, lipids etc.) and provided important insights into domestication-associated changes in the metabolism [37,38]. In terms of flavonoid pathway, Xu *et al.* [36] found two genes FHT1 (flavanone 3-hydroxylase) and *Pr1* (flavonoid 3′-hydroxylase, F3′H) contributed to the metabolic divergence and the cis-variant at FHT1 might have played an important role in driving the metabolic divergence in flavonoid subgroups, including flavanones, flavones, isoflavones, and flavonols, since maize domestication.

Legumes

Flavonoid metabolism in legumes is of particular interest given the presence of isoflavonoids which play important roles as phytoalexins and as nodulation signals — they are additionally almost entirely restricted to the subfamily Papilionoideae [39]. A total of 690 isoflavonoids have been reported to date [6]. As can be seen in Figure 2, isoflavonoid biosynthesis shares the core pathway with other flavonoids up to CHS where legume-specific type II CHSs convert both naringenin-chalcone and isoliquirtigenin into naringen and liquirtigenin, respectively, before subsequent metabolism by isoflavone synthase and 2-hydroxyflavanone dehydratase which result in genistein and daidzein [40]. These backbones are subjected to further modifications — normally by glycosyltransferases and methyltransferases [41] with their products being stored in the vacuole [5]. Alternatively they can be converted into antimicrobial pterocarpans [42].

This isoflavonoid class of flavonoids are almost certainly a consequence of tandem gene duplications and neo-functionalization (the most prominent route for innovation in plants; [43]). Indeed, isoflavone-related orthologs of chalcone reductase, isoflavone reductase, and 2-hydroxyflavanone dehydratase are only found in leguminous species and are co-incident with high levels of gene duplication within these species [44]. Furthermore, the isoflavones used for sensing root nodule interactions [45] are thought to have co-evolved with the symbiosis of plants and nitrogen fixing bacteria. In addition, non-isoflavonoid flavonoids in bean have been subject to considerable research [39]; however, these are by-and-large similar to those synthesized by non-leguminous species [46], so we will not detail them further here.

The medicinal plant *S. baicalensis*

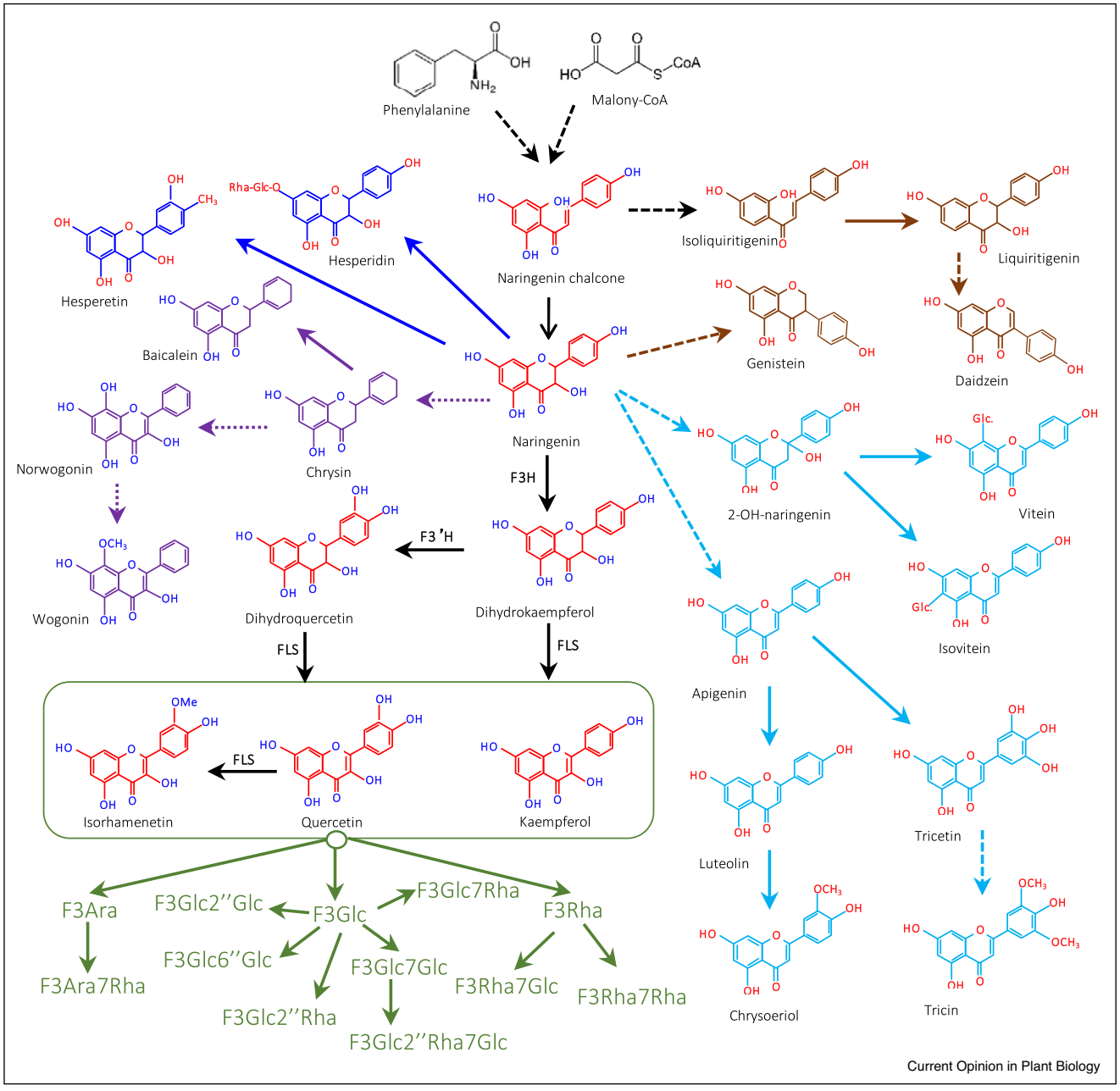
Flavonoids are a major constituent in lists of metabolites responsible for the bioactivities of medicinal plants. We cannot possibly do justice to the diversity of this compound class in a short review, so rather focus on recent insights derived from a single such species. The family Lamiaceae notably contains multiple species such as *S. baicalensis* (Huangqin) and *Salvia miltiorrhiza* (Danshen) which are commonly used in traditional Chinese medicine. For instance, the bioactivities of the root flavonoids of *S. baicalensis* include antibacterial, antiviral, antioxidant, anticancer, hepatoprotective, and neuroprotective properties [47–50]. To date the genomes of *S. baicalensis*, S. *miltiorrhiza and Salvia splendens* of the Lamiaceae family have been reported [51,52•,53]. A high-quality 386.63 Mb (about 94%) reference genome sequence for *S. baicalensis* released recently is the first genome assembly at chromosome-level resolution in the family Lamiaceae [52•].

Scutellaria is rich in flavones and expression of the classic flavone biosynthetic pathway genes leads to the production of the 4 O-hydroxyflavone apigenin, which is hydroxylated and glycosylated to form scutellarein and scutellarin, respectively in the aerial parts of the *S. baicalensis* plant [49]. However, Scutellaria roots accumulate large amounts of specialized root-specific 4′-deoxyflavones biosynthesized from a new pathway which has evolved relatively recently [49]. Root-specific flavones (RSFs) in *S. baicalensis* include chrysin, norwogonin, baicalein, wogonin, together with their glycosides baicalin and wogonoside. RSFs without a 4′−OH on the B-ring are the major bioactive compounds found in S. baicalensis [54,55]. On top of the well-assembled genome of S. baicalensis and comparative genomic analyses, Zhao *et al.* [52•] suggested that the evolutionary path for the biosynthesis of RSFs appears to have arisen by specific recruitment of a gene encoding a CoA ligase, and four of the genes involved in RSF synthesis (CHS-2, FNSII-2, F8H, and PFOMT5) present as tandem repeats in the genome of *S. baicalensis* are the result of relatively recent tandem duplications. The results also revealed that gene duplications, segmental duplication, gene amplification, and point mutations coupled to gene neofunctionalizations and subfunctionalizations were involved in the evolution of 4′-deoxyflavone synthesis in the genus Scutellaria. The high-quality reference genome of *S. baicalensis* thus would facilitate improved assembly of other genomes of members of the mint family, including *S. splendens* and *S. miltiorrhiza* as well as the elucidation of the biosynthetic pathways of specialized metabolites.

Regulatory genes in flavonoid biosynthesis

The flavonoid biosynthetic pathway has been deemed as a model system for understanding gene regulation in plants. In the case of transcriptional regulators MYB, WD40 and bHLH proteins have been extensively characterized in the biosynthesis of flavonoid. The transcriptional control of flavonoid biosynthesis by MYB–bHLH–WDR complexes has been well reviewed elsewhere [56]. While some late biosynthetic genes involved in the flavonoid pathway are activated by the R2R3MYB-bHLH-WD40 (MBW) ternary transcriptional complex here we detail in the recent advances on R2R3-MYBs which are characterized in a wider range of species in the

Figure 2



Representative flavonoid biosynthetic pathways. Different colors of metabolites correspond to known flavonoids in Arabidopsis (green), medicinal plants (purple), legumes (brown), and cereals (light blue). Note: this only covers a small fraction of the diversity of flavonoids known to occur in nature.

past few years. Compared to those of basal plants the R2R3MYB gene families were expanded in angiosperms, which are key for environmental and developmental regulation of flavonoid production.

Compared to the R2R3-MYBs that regulate other flavonoid compounds, anthocyanin-regulating R2R3-MYBs have been more frequently characterized in species such as Arabidopsis, apple, carrot, cherry, citrus, grapevine, potato, pear, strawberry, tea, tomato, and so on (Table 1). The origin and evolution of its regulatory system in flavonoid biosynthesis are intriguing and to what extent this system is conserved remains an open question. The survey of orthologous MYB genes regulating phenolic secondary metabolites indicates that not only phenolic secondary metabolism enzymatic genes but also their

Table 1

Summary of R2R3MYBs characterized in a wide range of species

Species	Genes	Metabolites	Reference
Arabidopsis thaliana	MYB4	Flavonoids	[59]
Vitis vinifera	VvMYBPA1	Flavonol, PA	[60]
	VvMYBC2	Anthocyanin, PA	[61]
Solanum lycopersicum	SlAN2	Anthocyanin	[62]
	SlMYB75	Anthocyanin, flavonoids	[63]
Camellia sinensis	CsMYB75	Anthocyanin	[64]
	CsMYB5b	Catechins, PA	[65]
Citrus sinensis	Ruby1& Ruby2, CsPH4, Noemi	Anthocyanin PA	[65,66] [67]
Daucus carota	DcMYB113, DcMYB7, DcMYB6	Anthocyanin	[68–70]
Marchantia polymorpha	MpMyb14	Flavones, riccionidin A	[19•]
Malus domestica Borkh	MdMYB1	Anthocyanin, Malate	[71]
	MdMYB9, MdMYB11	Anthocyanin, PA	[72]
Malus sieversii f. niedzwetzkyana	MYB12, MYB22	PA, flavonol	[73]
Pyrus bretschneideri Rehd	PyMYB114	Anthocyanin	[74]
	PbMYB10b, PbMYB9	Anthocyanin, PA, flavonol	[75]
Actinidia species	AcMYB110	Anthocyanin	[76]
Medicago truncatula	MtMYB2	Anthocyanin, PA	[77]
Populus spp	MYB182	Anthocyanin	[78]
Solanum tuberosum	AN1, MYBA1	Anthocyanin	[79]
Fragaria vesca	FveMYB10	Anthocyanin	[80]
Prunus avium L.	PavMYB10.1	Anthocyanin	[81]
Picea abies	PaMYB29, PaMYB32	Flavonoids	[57]

PA: proanthocyanin.

transcription factors are absent in algal species [2••]. It is suggested that phenolic secondary metabolites regulating MYB genes co-evolved at a later stage of evolution. A recent study on R2R3-MYBs in liverwort, one of the earliest diverging land plant, found *MpMyb14* activates both flavone glycoside and red pigmentation riccionidin A biosynthesis [19•]. An ancient origin in the plant lineage was also suggested by a recent finding of a conserved MBW complex which activates flavonoid biosynthesis in Norway spruce, a gymnosperm species [57]. The observations indicate that R2R3MYB-activation of flavonoid production which may arise early during land colonization is a conserved characteristic across land plants. The number of MYB and bHLH genes in the genomes of the basal plant groups liverworts [58] and mosses [58] are much smaller compared with those of angiosperms and gymnosperms. The regulatory system in marchantia could be a basal state which has been expanded through duplication and sub-functionalization of R2R3MYB genes during evolution, promoting the diversity of flavonoid functions in angiosperms [19•].

Conclusion and future directions

The increasing number of plant species under investigation coupling with the advances of metabolite profiling techniques enabled the illustration of unprecedented metabolic diversity in the plant kingdom. Flavonoid biosynthesis initiates from phenylalanine which is catalyzed by the core general phenylpropanoid-related biosynthetic genes phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate CoA ligase (4CL), providing precursors for the biosynthesis of all major phenolic secondary metabolites in higher plants. The variation of flavonoids is extensive and exhibits both qualitative and quantitative pattern across species as well as between different genotypes/tissues/developmental stages within a specific species. Despite its huge diversity the core flavonoid biosynthesis pathway is conserved in plants which is ancient and diversity is rather conferred by the reactions that tailor the core.

Plants are sessile organisms and consequently must be extremely adaptive to the environment. As a large group of plant-specialized metabolites flavonoids represent adaptive characteristics that have been subject to natural selection during evolution. Extension of the flavonoid pathway during evolution underpins the emergence of huge plant diversity and confers functional compounds for plant fitness in challenging and changing environmental niches. The examples provided above suggest that convergent evolution has occurred as different kinds of flavonoids that independently evolved in unrelated organisms resulted in similar defense and tolerance characteristics against various environmental stresses. It is thus of great interest to uncover the historical genetic and biochemical basis underlying the independent origins of such similar characteristics.

Because of the availability of multiple resources such as genomic and metabolomic data, genetic populations, and experimental tools for gene function analysis, a great amount of information on model species Arabidopsis and the cereals has been acquired. Species containing particular flavonoids such as the legumes have also been extensively characterized. Our knowledge of the dynamics of metabolic evolution has been considerably boosted recently owing to the availability and exploration of the whole genomes on more species. However, as studies in the metabolic pathways are limited to a subset of plant species so far, we consider that we have only scratched the surface of the diversity of flavonoids. Studies on non-traditional models and non-crop plants for instance the medicinal species and basal plants are at the beginning, which are showing how diverse the flavonoid pathway

may actually be. The novel flavones discovered in medicinally useful species [50] and the recent discovery of the auronidins in basal plants are notable [82]. It is challenging to extend such depth of study across the huge diversity of species of interest. Newly developed genomic and metabolite profiling technologies such as sequencing platforms including the Pacific Biosciences and Oxford Nanopore Technologies, machine learning based bioinformatic approaches and genome editing tools would offer excellent opportunities for us to further boost understanding of flavonoid biosynthesis and pathway diversification as well as their underlying evolutionary mechanisms. As such we are entering a highly exciting era — one in which great insights into the evolution of metabolism will certainly be realized.

Conflict of interest statement

Nothing declared.

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