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The Flavonoid Biosynthetic Pathway in Arabidopsis: Structural and Genetic Diversity

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Abstract

Flavonoids are representative plant secondary products. In the model plant *Arabidopsis thaliana*, at least 54 flavonoid molecules (35 flavonols, 11 anthocyanins and 8 proanthocyanidins) are found. Scaffold structures of flavonoids in *Arabidopsis* are relatively simple. These include kaempferol, quercetin and isorhamnetin for flavonols, cyanidin for anthocyanins and epicatechin for proanthocyanidins. The chemical diversity of flavonoids increases enormously by tailoring reactions which modify these scaffolds, including glycosylation, methylation and acylation. Genes responsible for the formation of flavonoid aglycone structures and their subsequent modification reactions have been extensively characterized by functional genomic efforts - mostly the integration of transcriptomics and metabolic profiling followed by reverse genetic experimentation. This review describes the state-of-art of flavonoid biosynthetic pathway in *Arabidopsis* regarding both structural and genetic diversity, **focusing on the genes encoding enzymes for the biosynthetic reactions and vacuole translocation.**

1. Introduction

Flavonoids are extensively distributed in the plant kingdom and form a group of representative plant secondary products. Given the ubiquitous distribution of flavonoids, they stride the boundary of primary and secondary metabolism. Since these compounds display several physiological functions in response to environmental factors, notwithstanding the fact that they do not play an essential role in plant growth, flavonoid biosynthetic genes have evolved to become one of the most **widely-spread** pathways in plants. Interestingly, this is also reflected in quantitative terms with the total carbon flux through the pathway constitutes approximately 20% of the total carbon flux [1]. As often claimed, flavonoids exhibit a variety of biological activities not only to plants, which produce these compounds, but also to animals, which intake flavonoids in their diet [2-4]. In the model plant *Arabidopsis thaliana*, flavonoids are one of major secondary products. The total number of metabolites (metabolome) in *A. thaliana* is estimated to exceed 5,000 [5, 6]. Linking metabolome to genome is a challenging issue even in *A. thaliana*, in which the genomic resources far outstrip those of other plant species. However, investigations of flavonoid associated gene annotation have proven highly successful in Arabidopsis. In this review, we discuss the structural diversity of flavonoid molecules and their biosynthetic genes **encoding enzymes for the reactions and vacuole translocation but not about transcription factors**, in order to understand the entire genetic basis of biosynthesis of this class of plant natural products. Since this review does not describe all aspects of biosynthesis of flavonoids in plant species besides *A. thaliana*, we refer readers to the recent more comprehensive articles on genetics and biochemistry of plant flavonoid biosynthesis [7-10].

2. Flavonoid molecules and their differential accumulation in Arabidopsis

In general, flavonoids are sub-classified into several families including flavonol, flavone, flavanone, flavan-3-ol, isoflavone and anthocyanidin according to the structure of and the modifications to the A, B and C rings which constitute their structural chemical. In *A. thaliana*, several flavonol- and anthocyanidin-derivatives have additionally been isolated and characterized. Given that *A. thaliana* is the premier model plant for post-genomic biology flavonoid profiling in this species has greatly advanced since the introduction and application of liquid chromatography-mass spectrometry (LC-MS) approaches. Using such approaches renders the elucidation of key physicochemical properties derived from flavonoid structures considerably more facile

than previously [11-13]. Flavonols and anthocyanins are identifiable since they display clear spectral absorbance of wavelengths at around 340 nm and 520 nm due to the presence of flavonol and modified flavilium aglycone (glycosyl and acyl adducts), respectively. Similarly, proanthocyanidins are identifiable since they display absorbance at 280/320 nm due to the presence of more highly condensed flavan-3-ol units. All of these metabolite groups have ether, ester and C4-C8 bonds which are easily cleaved in MS/MS analysis. Subsequently to these analyses deciphering the MS/MS fragmentation patterns obtained using public MS and MS/MS resources from databases and literatures [14-16] readily supports chemical assignment of aglycone structures as well as other modifications. Such LC-MS-based flavonoid profiling has allowed chemical structural assignments as well as a metabolite abundance atlas of flavonoids in *A. thaliana* as shown in Figure 1. *A. thaliana* has been demonstrated to accumulate at least 54 flavonoid molecules (breaking down as 35 flavonols, 11 anthocyanins and eight proanthocyanidins). Some structures have been unequivocally identified by isolation and subsequent structural analysis by MS and NMR studies, whilst others were, thus far, only annotated or characterized by UV and/or MS/MS analysis with the comparison of the public data from known flavonoids [14, 15, 17-29].

Although accumulation of anthocyanin, which presents a clear visible phenotypic change in various tissues, has been used as a stress marker metabolite in many publications, details concerning the exact chemical structure of the anthocyanins present are reported in relatively few publications. Major anthocyanin structures in *A. thaliana* have firstly been determined by Bloor and Abrahams [19]. Subsequently, other major intermediates of anthocyanin metabolism were isolated and identified following LC-MS profiling of *PAP1* (*Production of anthocyanin pigment 1*, *AtMYB75*, *At1g56650*) overexpressing Arabidopsis lines [20] and wild-type Arabidopsis grown under a diverse set of severe environmental challenges [30]. Many of these intermediates were subsequently purified (~~from a huge pool of Arabidopsis plants~~) and unequivocally characterized with respect to their structures and relative stereochemistry [26]. The compound named as A11 (cyanidin-3-*O*-[2-*O*-(2-*O*-(sinapoyl)- β -D-xylosyl)-6-*O*-(4-*O*-(β -D-glucosyl)-*p*-coumaroyl)- β -D-glucoside] -5-*O*-[6-*O*-(malonyl)- β -D-glucoside]) is the most highly modified cyanidin type anthocyanin with 3 acylated and 4 glycosylated parts. The compounds, A1 – A10, are presumed to be intermediates or less modified compounds of A11.

Flavonol glycosides in *A. thaliana* consist of the combination of aglycone (kaempferol, quercetin and isorhamnetin), glycosylation (mono, di and tri), and sugar (glucose, rhamnose and arabinose) groups. The abundance of these flavonoid molecules

follows strict spatial regulation [24, 25]. Relatively simple kaempferol and quercetin di-glycosides (di-rhamnosides or glucoside/rhamnoside in -3-*O*- and -7-*O*- positions) and tri-glycosides (di-rhamnosides and mono-glucoside in -3-*O*-, -2"-*O*- and -7-*O*- positions) are commonly distributed in all tissues of wild-type Arabidopsis. In the roots grown on the MS agar plates, four unique flavonol conjugates which are absent in every other tissue, including Arabidopsis roots grown in soil, were detected [24]. On the other hand, flavonols exhibiting unique arabinosylation modifications specifically accumulate in flowers. Furthermore, two flavonol-3-*O*-glucoside-hexosides were specifically found in flowers [25]. Interestingly, a triple knock mutant of Arabidopsis flavonol regulators *PFG1*, *PFG2* and *PFG3*, previously named *AtMYB12* (At2g47460), *AtMYB11* (At3g62610) and *AtMYB111* (At5g49330), displayed accumulation of these flavonol glycosides in pollen [31]. In recent metabolomics study, MS/MS spectral tag-based annotation profile additionally revealed the presence of malonylated flavonols in Arabidopsis leaves (kaempferol/quercetin-hexose-rhamnoside-malonyl) in inflorescences [15]. **In seed of Arabidopsis, quercetin-3-*O*-rhamnoside, biflavonols (dimers of quercetin-rhamnoside), epicatechin and procyanidins accumulated in the seed coat, whereas diglycosylated flavonols were essentially detected in the embryo [22].**

Proanthocyanidins or condensed tannins specifically accumulated **in seed coat along with the maturation of seed conferring** a brown hue [10, 22, 32]. In Arabidopsis these proanthocyanidins are formed using epicatechin as the basic condensing unit at C4-C8 positions. Catechin, which is a stereoisomer of epicatechin, is also known to be one of the building blocks of proanthocyanidins in other species [33-35], but as yet catechin-based proanthocyanidins have not been found in Arabidopsis.

3. Flavonoid diversification in Arabidopsis natural variants

A diverse array of flavonoids in higher plants have evolved according to their wide variety of physiological and ecological functions, which are required to meet adapting and conflicting demands to various environmental pressures under which plant communities grow. Illustrative but by no means comprehensive examples include high-light/UV-stress, cold stress, nutritional deficiencies and pathogen protection wherein the structure: function nexus has been the subject of considerable research [36-41]. Several studies have been made on natural variation of Arabidopsis accessions, in the case of flavonoid biosynthesis, elucidation of qualitative and quantitative differences between accessions have been performed and discussed in combination with stress treatments. Since there are numerous examples to illustrate biological and

ecological importance of anthocyanins in plants, their variations have been much investigated [42-44]. Intriguingly, since the major anthocyanin A11 was isolated in Col-7 [19], it has been found in several other accessions including Col-0 [20, 26] and C24, Can, Co, Cvi, *Ler*, Rsch and Te [45] as a major anthocyanin. However, to date, only limited information is available with respect to quantitative and qualitative differences such as chemical variation of Arabidopsis flavonols and anthocyanins [46]. However, in general the pathways of flavonoid biosynthesis exhibit large variation in their aglycone backbones and tailoring modification which is likely explainable by functional divergence of key genes such as cytochrome P450s (CYPs), 2-oxoglutarate-dependent dioxygenases (2-ODDs) and UDP-glycosyltransferases (UGTs) during the evolution [38, 47-52]. That said natural variance of Arabidopsis anthocyanin levels have been studied particularly with respect to elucidating the functional relationships between the accumulation of anthocyanin and nitrogen limitation [43], cold acclimation [45] or high carbon stress [42].

Despite displaying less variability in chemical diversity than Arabidopsis anthocyanins, accessions-specific flavonol glycosides have been reported in leaves of several accessions, for example, kaempferol 3-*O*- β -glucosyl-(6-*O*- β -glucoside)-7-*O*- α -rhamnoside in Ws-0 [18]; quercetin/kaempferol/isorhamnetin-deoxyhexosyl-dihexoside in *Ler* [53]; kaempferol 3-*O*-rhamnosyl-glucosyl-glucoside-7-*O*-rhamnoside in C24 [23] and kaempferol-hexosyl-hexoside-deoxyhexoside in Ms-0 [54]. Moreover quantitative differences of seed specific biflavonols was observed with higher accumulation in Oy-0, Cvi-0, Bur-0 and St-0 [55]. Whilst accession-specific genes involved in Arabidopsis flavonoid biosynthesis have not yet been found, their elucidation and characterization will likely be critically important for enhancing our understanding plant evolution from the perspective of environmental adaptation.

4. Genes involved in flavonoid biosynthesis

4.1. Shikimate pathway

Flavonoids are synthesized by the combination of the phenylpropanoid and polyketide pathways (Figure 2). The phenylpropanoid pathway provides *p*-coumaroyl-CoA. The polyketide pathway is responsible for C2 chain elongation by utilizing malonyl-CoA as the condensing unit. The phenylpropanoid pathway initiates from the aromatic amino acids phenylalanine and tyrosine, which are synthesized by the

shikimate pathway. The genes involved in the shikimate pathway in *A. thaliana* have been extensively documented in recent literature [56, 57].

4.2. Prior to the branch-point between flavonoids and general phenylpropanoids

Phenylalanine ammonia-lyase (PAL) - Phenylalanine ammonia-lyase (PAL) is the first committed enzyme in the phenylpropanoid pathway leading to the production of lignins, lignans and flavonoids. This enzyme catalyzes the formation of *trans*-cinnamic acid from phenylalanine with the concomitant loss of ammonia. In *A. thaliana*, a small gene family comprising four genes designated *AtPAL1* – *AtPAL4* (*AtPAL1*, At2g37040; *AtPAL2*, At3g53260; *AtPAL3*, At5g04230; *AtPAL4*, At3g10340) encode the isoforms of PAL [58, 59]. They exhibit differential expression patterns and partially complement the function of each other in respective mutation [56, 60-62]. However, *AtPAL1* and *AtPAL2* are presumed to represent the principal isoforms responsible for the formation of phenylpropanoids in *A. thaliana* [56, 61]. In fact, *pal1 pal2* double mutants exhibited a reduced production of anthocyanins and proanthocyanidins [63].

Cinnamic acid 4-hydroxylase (C4H) - Cinnamic acid 4-hydroxylase (C4H) is a cytochrome P450 monooxygenase which catalyzes the hydroxylation of *trans*-cinnamic acid at C-4 position to yield *p*-coumaric acid (4-coumaric acid). In Arabidopsis, there is only one isoform in the subfamily of CYP73 designated CYP73A5 (*AtC4H*, REF3, At2g30490) [64, 65]. Seeds of plants exhibiting a mutation in this gene (*ref3*) exhibited reduced levels of proanthocyanidins in addition to reduced accumulation of wild-type levels of sinapoyl-malate and lignins [66].

4-Coumaric acid:CoA ligase (4CL) - *p*-Coumaric acid needs to be activated to the corresponding CoA thioester to facilitate further conversion. This reaction is catalyzed by 4-coumaric acid:CoA ligase (4CL) with the consumption of ATP as an energy source. Four isoforms of 4CL, *At4CL1* – *At4CL4* (*At4CL1*, At1g51680; *At4CL2*, At3g21240; *At4CL3*, At1g65060; *At4CL5* (*At4CL4*), At3g21230), are present in the *A. thaliana* genome. From the results of expression patterns and phylogenetic similarity, *At4CL3* is suggested to play a role in flavonoid biosynthesis, while *At4CL1* and *At4CL2* are presumed to be involved in lignin production [67]. *At4CL5* (*At4CL4*) exhibits a substrate preference to ferulic acid and sinapic acid over *p*-coumaric acid, suggesting a different function from that of other 4CLs in Arabidopsis [68].

4.3. Formation of malonyl-CoA

Acetyl-CoA carboxylase (ACC) is an Mg^{2+} -ATP dependent biotinylated protein complex that catalyzes the carboxylation of acetyl-CoA into malonyl-CoA [69-71]. The reaction takes place in two steps. First, bicarbonate as the source of carbon is transferred to the biotin molecule covalently bound to ACC (biotin carboxylase). This carbon-carbon bond formation requires energy derived from the hydrolysis of ATP. The second step is the transfer of the carboxyl group to acetyl-CoA to form malonyl-CoA (carboxyltransferase).

Arabidopsis has three ACC isozymes. A heteromeric form of ACC is composed of four distinct subunits: a biotin carboxyl carrier protein (BCCP1, CAC1A, At5g16390), biotin carboxylase (BC, CAC2, At5g35360), carboxyltransferase α subunit (CAC3, At2g38040) and β subunit (accD, AtCg00500) [72]. These subunits form a protein complex in plastids and catalyze the first step reaction in *de novo* fatty acid biosynthesis. A homomeric form of ACC is composed of a large polypeptide with four functional domains. The Arabidopsis genome contains two genes encoding the homomeric type proteins located in a tandem repeat within a 25-kbp region (ACC1, At1g36160; ACC2, At1g36180) [73, 74]. ACC1 is the cytosolic isozyme in Arabidopsis and produces malonyl-CoA for flavonoid biosynthesis. ACC2 has a longer N-terminal sequence, and GFP-fusion protein experiments suggest that this confers a chloroplastic localisation [75]. ACC2 is likely functionally redundant in plastidic malonyl-CoA biosynthesis during seedling development. Biotinylation of ACC is catalyzed by a dual targeting of holocarboxylase synthetase 1 (HCS1, At2g25710) [76]. Cytosolic acetyl-CoA is synthesized by $\alpha_4\beta_4$ heteromeric ATP-citrate lyase [77] and plants deficient in this activity display a complex, bonsai phenotype, with miniaturized organs, smaller cells, aberrant plastid morphology, reduced cuticular wax deposition, and hyperaccumulation of starch, anthocyanin, and stress-related mRNAs in vegetative tissue [77].

The cytosolic pool of malonyl-CoA is required for a variety of reactions including the elongation of very long-chain fatty acids (VLCFAs, $\geq C20$), and the biosynthesis of flavonoids as the substrate of chalcone synthase and anthocyanin malonyltransferase. The plastid envelope is impermeable to malonyl-CoA. ACC1 null mutants are impaired in embryo morphogenesis and produce abnormal seeds which accumulate triacylglycerols lacking VLCFAs [78]. Recently, two ACC1 missense mutants, which were not embryo-lethal, were reported [79, 80]. One mutant line displays a freezing sensitive phenotype and the leaves are deficient in anthocyanin accumulation during cold acclimation. In oil seed rape leaves, decreasing the homomeric ACC activity by antisense RNA technology resulted in decreased

accumulation of flavonoids under UV-B treatment which contrasts to their pattern of increase in the wild type [81]. The highest level of ACC1 mRNA was detected in flowers, where a large variety of flavonols are synthesized (Fig. 1) [82]. ACC activity has similarly been observed to be induced by strong light stress to parsley cell-suspension cultures [83], whilst homomeric ACC activity and transcripts were induced upon addition of yeast or fungal elicitors to alfalfa cell cultures [84]. Moreover, the expression of a set of flavonoid biosynthetic genes including cytosolic *ACC1* were induced upon UV-B stress in Arabidopsis [85]. Also in pea leaves, not the heteromeric ACC, but the homomeric form was induced by UV-B irradiation [86]. Taken together these results suggested cytosolic homomeric ACC is required and coordinately regulated with anthocyanin and isoflavone production under stress conditions.

4.4. Formation of flavonoid scaffolds – the central flavonoid biosynthetic pathway

Flavonoid scaffolds are formed from the building blocks of a phenylpropanoid primer and polyketide condensing units by a series of reactions including condensation, isomerization, oxidation and reduction, which are known to be involved in the central flavonoid biosynthetic pathway. The scaffold structures are further modified by tailoring reactions. In combination this suite of reactions defines the final individual compound structures.

Chalcone synthase (CHS) - Chalcone synthase (CHS) is the first committed enzyme in the biosynthesis of all flavonoids. This enzyme belongs to a family of type III polyketide synthase, which is one of the general polyketide synthases and forms a catalytically-active single polypeptide [87]. CHS catalyzes the so-called Claisen-ester condensation concomitant with CO₂ liberation from malonyl-CoA and acyl-thioester (i.e., *p*-coumaroyl-CoA). Actually CHS catalyzes the formation of a triketide intermediate from *p*-coumaroyl-CoA and three molecules of malonyl-CoA; then spontaneous cyclization of the triketide intermediate results in the formation of naringenin chalcone [87]. There are three CHS-like genes forming a small gene family in *A. thaliana*. Among them, *AtCHS* (*TT4*, At5g13930) has been identified as the gene participating in the synthesis of flavonoids, and thus the mutant of this gene is designated as *transparent testa (tt) 4* since it lacks proanthocyanidin formation in the seed coat (Table 1) [88]. Two other genes, *PKSA/LAP6* (At1g02050) and *LAP5/PSKB* (At4g34850), are known to encode hydroxyalkyl- α -pyrone synthases which are essential for pollen development and the biosynthesis of sporopollenin, the constituent of exine in the outer pollen wall [89, 90].

Chalcone isomerase (CHI) – Chalcone isomerase (CHI) catalyzes the stereospecific cyclization of naringenin chalcone to (2*S*)-naringenin. In *A. thaliana*, there are five CHI-like genes. Among them, *AtCHI* (At3g55120) was identified as the *TT5* gene [7, 10]. The expression of *AtCHI-like1* (*AtCHI-L1*, At5g05270) was induced by the PAP1 transcription factor (AtMYB75, At1g56650) together with *TT5*, suggesting a functional similarity of these two genes [20]. A recent study indicated that non-catalytic CHI-like proteins localized in chloroplasts may function as fatty-acid-binding proteins necessary in proper production of fatty acids in developing seed embryos [91].

Flavanone 3-hydroxylase (F3H) - Flavanone 3-hydroxylase (F3H) is a 2-oxoglutarate-dependent dioxygenase catalyzing the oxygenation at 3-position of flavanone ((2*S*)-naringenin) to form dihydroflavonol (dihydrokaempferol) with the concomitant production of CO₂ and succinate from oxygen and 2-oxoglutarate as co-substrates. At3g51240 is known as the *TT6* gene encoding F3H [92]. The activity of F3H *in vivo* can be partially compensated by two related 2-ODD enzymes in flavonoid biosynthesis, flavonol synthase (FLS) and leucoanthocyanidin dioxygenase /anthocyanidin synthase (LDOX/ANS) by the analysis of “leaky” phenotype of F3H mutant alleles [93]. This promiscuity of 2-ODDs involved in flavonoid biosynthesis has been also supported by the detailed mechanistic study of these reactions [51].

Flavonoid 3'-hydroxylase (F3'H) - Flavonoid 3'-hydroxylase (F3'H) is a cytochrome P450 monooxygenase committed in the hydroxylation of 3'-position of B-ring of flavonoid. This enzyme can accept either dihydrokaempferol or kaempferol as a substrate and convert them to dihydroquercetin and quercetin, respectively. At5g07990 which encodes CYP75B1 has been identified as the *TT7* gene in *A. thaliana* [94].

Flavonol synthase (FLS) - Flavonol synthase (FLS) is the first committed enzyme for the biosynthesis of flavonols branching from the main trunk route to the branch for anthocyanin formation [95]. FLS catalyzing the double bond formation between C-2 and C-3 is one of three 2-ODD enzymes in the flavonoid biosynthesis pathway besides F3H and LDOX/ANS. *FLAVONOL SYNTHASE1* (*AtFLS1*, At5g08640), was first identified as the FLS gene encoding the catalytically active protein [96, 97]. In the Arabidopsis genome, there are five additional genes with high sequence similarity to *FLS*. Although *AtFLS1* predominantly influence flavonoid levels in Arabidopsis [98, 99], the mutant deficient *AtFLS1* still accumulate significant amounts of flavonols. *AtFLS3* (At5g63590) has been identified as a second active FLS [100]. The other FLS-like genes may have alternative noncatalytic functions, however these remain, as yet, unidentified.

Dihydroflavonol reductase (DFR) – The first committed reaction leading to anthocyanin and proanthocyanidin is the reduction of the 4-keto group of dihydroflavonol to the corresponding leucoanthocyanidin. This reaction is catalyzed by dihydroflavonol reductase (DFR) competing the substrate, dihydroflavonol, with FLS. The gene At5g42800 corresponding to *TT3*, is known to encode functional DFR [101].

Leucoanthocyanidin dioxygenase /anthocyanidin synthase (LDOX/ANS) – The first colored compound in the anthocyanin biosynthetic pathway is anthocyanidin which exhibits a conjugated double bond system encompassing A, B and C rings. The enzyme LDOX/ANS catalyzes the formation of anthocyanidin from leucoanthocyanidin with 2-oxoglutarate and oxygen as co-substrates. In Arabidopsis, At4g22880 was assigned as *TT18* it encodes the protein LDOX/ANS. The mutants, *tt11*, *tt17* and *tds4* (*tannin deficient seed 4*), are also ascribed to mutation of this gene [102, 103]. The reaction mechanism of this enzyme has been investigated and was concluded to be a LDOX/ANS-catalyzed 2-oxoglutarate-dependent conversion of leucoanthocyanidin (flavan-3,4-*cis*-diol) to the pseudobase, 3-flaven-2,3-diol, most probably via 3-oxidation, 2,3-desaturation and isomerization, followed by stabilization by glucosylation at the C-3 position [104-106]. Three 2-oxoglutarate- and ferrous iron-dependent oxygenases involved in the flavonoid biosynthetic pathway, F3H, FLS and LDOX/ANS, are closely related by sequence and all catalyze oxidation of the C-ring. In fact, multifunctionality of FLS and LDOX/ANS has been indicated by several lines of evidence [107]. The apparent overlapping substrate and product selectivities of these three enzymes has been explained from the mechanistic view point [51]. The reactions catalyzed by these three enzymes commonly involve the oxidation of C-3 positions of C-ring of flavonoids by the oxygen atom activated by iron of the enzymes [51, 108].

Anthocyanidin reductase (ANR) – For the formation of proanthocyanidins (condensed tannins), anthocyanidin reductase (ANR) is the first committed enzyme in this pathway catalyzing the NADPH-dependent reduction of cyanidin to (-)-epicatechin (*cis*-flavan-3-ol). At1g61720 is designated as the *BANYULS* (*BAN*) gene encoding ANR [109-111]. The similar enzyme leucoanthocyanidin reductase (LAR) responsible for reduction of leucoanthocyanidin to catechin (*trans*-flavan-3-ol) has been reported in legume [33], grapevine [112] and poplar [113]. However, no gene with Arabidopsis homolog of *LAR* has yet been characterized.

Polyphenol oxidase - Polyphenol oxidase (PPO), which is involved in the oxidative polymerization of (-)-epicatechin, has been characterized as a laccase-type enzyme designated TT10 (AtLAC15, At5g48100) [114, 115]. This enzyme is critical for the brown coloring of Arabidopsis seed coat as a function of the fact that it

participates in a polymerization reaction. However, the precise mechanism for regiospecific oxidation of (-)-epicatechin molecule to C4-C8 oxidative linkage has not yet been clarified.

4.5. Tailoring reactions of flavonoid scaffolds

The huge chemical diversity of flavonoids is due to a high order modification by enzymes such as glycosyltransferases, methyltransferases and acyltransferases although structural variations within the flavonoid scaffold structures are relatively limited. These steps are known as tailoring reactions and they are responsible for final determination of physicochemical and biological properties of the flavonoid molecules which accumulated in cells.

Flavonoid glycosyltransferase (FGT) - Glycosylation is essential for stable accumulation of flavonoids in Arabidopsis and occurs at the C-3, C-5 and C-7 positions of flavonoid aglycones. Sugar moieties attached to flavonoid aglycones are also further glycosylated. Flavonoid glycosyltransferase (FGT) catalyzes the glycosylation of flavonoid skeletons. Some of the genes belonging to the family 1 glycosyltransferases (UGTs) which utilize UDP-sugars as sugar donors for glycosylation, have been characterized as FGTs in Arabidopsis. In the Arabidopsis genome there are, depending on the report taken, between 107 and 120 genes encoding family 1 glycosyltransferases [52, 116, 117]. The detailed analysis of flavonoid structures suggest that at least ten FGTs are involved in flavonoid glycosylation. So far, seven genes encoding FGTs were identified (Table 1). Generally, 3-*O*-glycosylation is known to have occurred prior to glycosylation of other position (Figure 3). As 3-*O*-glycosyltransferases which specifically glycosylate at C-3 position of flavonol aglycone but not anthocyanidin, two enzymes, flavonol 3-*O*-rhamnosyltransferase (UGT78D1, At1g30530) [118] and flavonol 3-*O*-arabinosyltransferase (UGT78D3, At5g17030) [25], were identified in Arabidopsis. However, flavonoid 3-*O*-glucosyltransferase (UGT78D2, At5g17050) can transfer glucose to the C-3 position of both flavonol and anthocyanin aglycones [20]. A mutant lacking functional flavonoid 3-*O*-glucosyltransferase was characterized as exhibiting drastically decreased anthocyanin contents, yet still accumulated a small amount of the anthocyanin A11. These data indicate that 3-*O*-glucosylation by UGT78D2 plays a major role in the stable accumulation of anthocyanins and it furthermore seems likely that Arabidopsis has additional gene(s) encoding anthocyanin 3-*O*-glucosyltransferase(s) [20]. Anthocyanin 5-*O*-glucosyltransferase (UGT75C1, At4g14090), flavonol 7-*O*-glucosyltransferase (UGT73C6, At2g36790) and flavonol

7-*O*-rhamnosyltransferase (UGT89C1, At1g06000) have also been identified [20, 118, 119]. The mutation of anthocyanin 5-*O*-glucosyltransferase resulted in the accumulation of anthocyanins lacking a glucose moiety at the C-5 position [20]. In the *ugt73c6* knockout mutant, flavonol 7-*O*-glucosides were missing. On the other hand, *ugt89c1* mutants accumulated unknown flavonols. UGT79B1 (At5g54060) is an anthocyanin 3-*O*-glucoside: 2''-*O*-xylosyltransferase [120]. Total anthocyanin is drastically decreased in *ugt79b1* knockout mutants, suggesting that additional 2''-*O*-xylosylation of 3-*O*-glucoside of cyanidin is required for stable anthocyanin accumulation in *Arabidopsis* (Figure 4). Recently, it was additionally found that glycoside hydrolase (GH)-type FGTs transfer the sugar moiety from an acyl-glucose to anthocyanins in *Dianthus caryophyllus* (carnation) and *Delphinium grandiflorum* (delphinium) [121, 122]. The *Arabidopsis* ortholog, AtBGLU10 (At4g27830) is presumably involved in glucosylation of the coumaroyl moiety on anthocyanin A11 [123]. This GH-type FGT reaction occurs inside vacuoles resulting in the storage form of anthocyanins.

Phylogenetic analyses of FGTs indicated that FGTs form distinct clusters on the basis of their regioselectivity for glycosylation [124]. When considered from an evolutionary perspective, *Arabidopsis* UGTs can be divided into 12 groups based on phylogenetic analysis [116]. More extensive analysis on UGTs from six plant species (*Arabidopsis thaliana*, *A. lyrata*, *Populus trichocarpa*, *Oryza sativa*, *Selaginella moellendorffii*, *Physcomitrella patens*) indicated that these UGTs can be classified into 24 orthologous groups [52]. An orthologous group is defined as a group containing genes that have diverged in each species from those of their common ancestor. In both instances, flavonoid 3-*O*-glycosyltransferases form a distinct group, suggesting that the function of flavonoid 3-*O*-glycosyltransferases was established before the divergence from *P. patens* and thus flavonoid 3-*O*-glycosyltransferases acquired substrate specificity for sugar donor following plant specification. The UGT families can be classified into 24 orthologous groups, which are defined as groups containing genes that had diverged in each species from those of their common ancestor [52]. FGTs except for flavonoid 3-*O*-glycosyltransferases belong to groups including multiple UGT subfamilies. For example, anthocyanin 5-*O*-glucosyltransferase (UGT75C1) belongs to a group consisting of UGT74, UGT75 and UGT84 subfamilies. UGT74s and UGT84s are involved in glucosinolate biosynthesis and phenylpropanoid modification/ auxin deactivation, respectively. During the branching processes to UGT74s, UGT75s and UGT84s from a common ancestor, UGT75C1 may have acquired the substrate specificity for anthocyanin. Thus, FGTs except for flavonoid 3-*O*-glycosyltransferases may have gained the substrate specificity for flavonoids independently.

Flavonoid methyltransferase (FMT) – In Arabidopsis, there are 24 SABATH-type methyltransferase genes [125]. AtOMT1 (At5g54160) has been characterized as being involved in the methylation of flavonols forming isorhamnetin a fact supported by the evidence from *in vivo* [24] and *in vitro* experimental evidence [126]. AtOMT1 also participates in the production of lignins and sinapoyl esters [127].

Flavonoid acyltransferase (FAT) - Acyltransferases can also be classified within the BAHD and serine carboxypeptidase-like (SCPL) families. The BAHD family was named after the first four biochemically characterized enzymes in this family (BEAT, AHCT, HCBT, and DAT) and all use acyl coenzyme A thioesters as acyl donors [128]. By contrast, the SCPL family use acyl-activated sugars such as 1-*O*- β -glucose esters [129]. BAHD-type acyltransferases are predicted to be localized in cytosol (or cytoplasmic surface of the ER) whereas SCPL-type acyltransferases are vacuolar enzymes [124].

The Arabidopsis genome contains 64 and 53 genes encoding BAHD- and SCPL-type acyltransferases, respectively [117] and of these two BAHD-type and one SCPL-type acyltransferases are involved in flavonoid acylation. Anthocyanin 5-*O*-glucoside malonyltransferase (At3g29590) and anthocyanin 3-*O*-glucoside coumaroyltransferases (At1g03940 and At1g03495) have been identified as BAHD type acyltransferases and these acylations contribute to the stability of anthocyanins at neutral pH [130]. The gene At2g23000 was identified as an SCPL-type acyltransferase specifically functioning as an anthocyanin 3-*O*-glucoside-2''-*O*-xyloside: sinapoyltransferase [131].

5. The anthocyanin modification pathway in Arabidopsis

The pathway from cyanidin to the most highly modified anthocyanin A11 requires seven modification steps by four glycosyltransferases and three acyltransferases (Figure 5). Enzymatic characterization has been carried out for all modifying enzymes with the exception of cyanidin 3-(6''-*p*-coumaroylglucoside): glucosyltransferase, facilitating a fairly complete overview of the major routes of anthocyanin modification.

Flavonoid 3-*O*-glucosyltransferase is the first committed modification enzyme [20, 132]. Anthocyanin 3-*O*-glucoside: 2''-*O*-xylosyltransferase recognizes cyanidin 3-*O*-glucoside, but not cyanidin 3,5-*O*-diglucoside as a substrate. Anthocyanin 3-*O*-glucoside: 6''-*O*-coumaroyltransferase prefers cyanidin 3-*O*-glucoside, but shows very minor activity for cyanidin 3,5-*O*-diglucoside. These data strongly suggest that

2''-O-xylosylation and 6''-coumaroylation occur prior to 5-O-glucosylation. This is also supported by the fact that the recombinant UGT75C1 failed to 5-O-glucosylate cyanidin 3-O-glucoside (Y. Nishiyama, T. Tohge, M. Yamazaki and K. Saito, unpublished data), similarly as the homologous *Petunia hybrid* enzyme was unable [133]. These data suggest that UGT75C1 prefers 2''-O-xylosylated anthocyanins as substrates. In sharp contrast, the properties of anthocyanin 5-O-glucosyltransferases from *Perilla frutescens* and *Verbena hybrida* revealed that both exhibited a broad substrate specificity accepting cyanidin 3-O-glucoside besides bis-glycosylated anthocyanins [134]. Anthocyanin malonyltransferase and sinapoyltransferase are a BAHD-type and an SCPL-type acyltransferase, respectively. The sub-cellular localization of these acyltransferases suggests that cytosolic malonyltransferase acts prior to the vacuolar sinapoyltransferase. Thus, cyanidin is thought to be modified following the order 3-O-glucosylation, xylosylation or coumaroylation, 5-O-glucosylation and finally malonylation.

Recently, in addition to FGTs and FATs, it was reported that several enzymes affect flavonoid modification pathway although they do not interact directly with flavonoid molecules. For example sinapate glucosyltransferase, AtSGT1 (bright trichomes 1, AtBRT1, At3g21560, UGT84A2), catalyzes the conversion of sinapate to 1-O-sinapoyl glucose, an acyl donor for acyltransferases [135]. In *ugt84a2* knockout mutants, a major sinapoylated anthocyanin A11 is drastically decreased and instead, a non-sinapoylated anthocyanin A5 (cyanidin 3-O-[2''-O-(xylosyl)-6''-O-(p-coumaroyl) glucoside] 5-O-malonylglucoside, Figure 5A) is detected as the major anthocyanin [120]. This suggests that UGT84A2 is a major supplier of the sinapoyl moiety for anthocyanins and that limited supply of 1-O-sinapoyl glucose as a consequence of the mutation in *ugt84a2* suppresses production of sinapoylated anthocyanins. Intriguingly, in the *sng1-5* mutant (deletion in genes encoding *sinapoylglucose:malate sinapoyltransferase 1*, *AtSNG1*, At2g22990 and *anthocyanin 3-O-[2''-O-(xylosyl)-glucoside sinapoyltransferase*, *SAT*, At2g23000), anthocyanin A8 (cyanidin 3-O-[2''-O-(xylosyl) 6''-O-(p-O-(glucosyl) p-coumaroyl) glucoside] 5-O-[6''-O-(malonyl) glucoside] , Figure 5A) accumulated as the major anthocyanin [136]. The difference in the major anthocyanin species in *ugt84a2* and *sng1-5* mutants suggests that: (1) sinapoyltransferase may have a higher affinity to A5 than anthocyanin 3-O-6''-O-coumaroylglucoside: glucosyltransferase and thus inhibits further glucosylation of A5 as a consequence of tight binding of A5 in *ugt84a2* mutants or (2) anthocyanin 3-O-6''-O-coumaroylglucoside: glucosyltransferase utilizes 1-O-sinapoyl glucose as sugar donor and the glucosyltransferase activity may be also suppressed by the limited supply of 1-O-sinapoyl glucose in *ugt84a2* mutants.

6. Flavonol modification pathway in Arabidopsis

Based on the flavonoid structures identified to date it would appear that the flavonol modification pathway needs three steps at the most (Figure 3). 3-*O*-Glycosylation occurs first. In the case of flavonols, the C-3 position is glycosylated with glucose, rhamnose or arabinose. Therefore, mutation of one of 3-*O*-glycosyltransferase genes would result in the increase of flavonols glycosylated by other flavonol 3-*O*-glycosyltransferases. The order of flavonol 7-*O*-glycosyltransferase and glycosyltransferase which glycosylate the sugar moiety attached to flavonol aglycones (GGTs) is unknown. However, anthocyanin GGTs prefer anthocyanin 3-*O*-glucoside over anthocyanidin 3,5-*O*-diglucosides [120, 137, 138]. It suggests that flavonol GGTs may also prefer flavonol 3-*O*-glucoside over flavonol 3-*O*-glucoside-7-*O*-glycosides.

UDP-rhamnose synthase 1 (RHM1, AtROL1, At1g78570) was identified to be an enzyme which affects flavonol modification pathway even though it does not interact directly with flavonol molecules [25]. *RHM1* was isolated as a gene whose expression manner is highly correlated with those of flavonoid biosynthetic genes. In *rhml* knockout mutants, flavonol 3-*O*-rhamnoside derivatives are significantly decreased and flavonol 3-*O*-glucoside derivatives are increased. Arabidopsis has three UDP-rhamnose synthase genes (*RHM1*; *RHM2*, *AtMUM4*, At1g53500; *RHM3*, At3g14790) [139-141]. Comparison of the flavonol profiles in RHM knockout mutants indicated that RHM1 plays a major role in supply of the UDP-rhamnose precursors for the flavonol modification pathway. Interestingly the manner of flavonol 3-*O*-glycosylation is more affected by the limitation of UDP-rhamnose supply than 7-*O*-glycosylation is.

7. Subcellular translocation of flavonoid in plants

In addition to the structural enzymes discussed above transport proteins are also essential for flavonoid biosynthesis. Functional characterization of the screened mutant series named either *tt* or *tds* has led to a better understanding of flavonoid transport proteins and their transport mechanism [88, 103, 142]. Proanthocyanidin is thought to be stored in the vacuoles of seed coat endothelial cells following oligomerization and polymerization of PA intermediates which are transported from the cytosolic face of the endoplasmic reticulum (ER) [143, 144]. Characterization of the

tt12 mutant encoding a membrane protein of the multi-drug and toxic efflux (MATE) transporter family revealed that the TT12 protein (At3g59030) is related to vacuolar transport of proanthocyanidin in Arabidopsis seed coats [145]. However, whilst TT12 protein when expressed in yeast could transport cyanidin-3-*O*-glucoside it could not transport catechin-3-*O*-glucoside, flavonol glycosides or proanthocyanidin dimers [146]. Furthermore, although there is evidence that catechin-3-*O*-glucoside inhibits TT12-mediated transport of cyanidin-3-*O*-glucoside, flavan-3-ol and flavonol glycosides had no effect on anthocyanin transport. This fact suggests that transport catalyzed by the TT12 protein is confined to flavan-3-ol-glycosides [146]. The flower flavonoid transporter (FFT, AtDTX35), a member of the MATE family, was isolated by the identification of the mutation resulting in floral and growth phenotypes and reduction of floral kaempferol di-glucoside. The expression of this gene is highly localized to epidermal guard cells in flower tissues [147].

Over 15 years ago a glutathione *S*-transferase-like (GST) was identified as an essential protein for anthocyanin and proanthocyanidin transport to the vacuole. This protein, also known as *Bronze2*, was isolated from an anthocyanin deficient mutant (*bz2*) in maize [148]. Interestingly, *TT19* (*TT14*, *GSTF12*, At5g17220) also encodes an Arabidopsis GST-like protein [143]. In Arabidopsis, there are three GST-like genes, which are up-regulated with *PAP1* overexpression and their gene products exhibited a weak GST activity [149]. However, TT19 is almost exclusively involved in anthocyanin accumulation. To date several flavonoids related GST-like genes have been found in diverse plant species. Plant flavonoid related GST-like genes being found across the different types of GST family which are sub-divided on the basis of sequence identify, for example, maize *Bz2* [148], soybean *GmGST26A* [150] and parsley *PcGST1* [151] belong to the Tau type GSTs whereas petunia *AN9* [150, 152], cyclamen [153] and Arabidopsis *TT19* [143] belong to the Phi type GSTs [154]. Furthermore, petunia *AN9* could complement the anthocyanin accumulation of the Arabidopsis *tt19* mutant but not its deficiency in proanthocyanidin production. Despite the relatively common identification of changes in flavonoids in GST-like mutants the function of GST-like in the transport processes is still very much ambiguous [152, 155] since whilst the GST-likes are themselves directly involved as components of the flavonoid transport system despite their name the glutathione molecule itself may not be required for flavonoid transport. This fact notwithstanding it is clear that GST-like function in the cytosol in order to maintain regular accumulation of proanthocyanidin precursors such as epicatechin and epicatechin-glycosides in the vacuole [144, 156]. Recent study with a TT19–GFP fusion protein indicated that TT19 is localized not only in the cytoplasm and

nuclei, but also on the tonoplast [157]. Flavonol specific GST-like proteins have not yet been identified, however one candidate GST-like, At1g10370, has been discussed in the context of belonging to the co-expression network of flavonol biosynthetic genes [25]. Further genetic and biochemical work is however required in order to confirm the hypothesized function of this gene.

Arabidopsis AHA10 (TT13, At1g17260), a putative P-type H⁺-ATPase been isolated from the *aha10* mutant which shows vacuolar morphological defects and reduction of proanthocyanidin accumulation levels in seeds [10, 158]. This enzyme is considered to act in tandem with the activity of the MATE transporter TT12 to maintain a H⁺/flavonoid-antiport function in the Arabidopsis. Interesting although the *tt12* knockout mutant accumulates epicatechin-glycosides in seeds a considerably higher level of epicatechin was observed in *aha10* mutant seeds [32]. AHA10 is thought to be localized on the cytosolic face of the ER or on vesicles involved in PA transport from the cytosol to the vacuole and it is hence hypothesized that vesicle trafficking-mediated PA transport may require AHA10 to supply energy [144].

8. Future prospects

The majority of chemical diversity of flavonoid molecules in Arabidopsis has been characterized by the efforts of the decade. The genes involved in the synthesis of these diverse molecules have been also intensively identified by use of cutting-edge functional genomics approaches [159], although several missing genes/enzymes need to be identified and subsequently characterized further. However, the biological significance of this enormous diversification of flavonoids during developmental changes and **respective** ecotype specificity should be addressed by further investigation. The functionality of diversity could be related to the adaptation of Arabidopsis plants to environments during the evolution. These findings could be the basis for further engineering of flavonoids [160] and optimization of the metabolic pathway [161]. The study of flavonoid metabolism in Arabidopsis should additionally be extended to non-model medicinal or exotic plant species producing flavonoids or even other pharmacologically-active compounds [162-164]. Biotechnological application by transgenic technology for the promotion of human health by engineering of flavonoid is based on an in-depth understanding of fundamental mechanism of the biosynthetic mechanisms is also a promising area for future research [165, 166]. Relatively inexpensive high-throughput sequencing technology in combination with emerging metabolomics technologies [167, 168] will likely facilitate the mining of yet more genes

involved in this specialized metabolic pathway of plants [169].

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Table 1. Genes for the flavonoid biosynthesis

Protein function	Gene	<i>transparent testa</i>	Locus	Ref.
	designation	mutant		
phenylalanine ammonia-lyase	PAL1		At2g37040	[58]
	PAL2		At3g53260	[59]
cinnamic acid 4-hydroxylase	C4H		At2g30490	[64]
4-coumaric acid:CoA ligase	4CL3		At1g65060	[67]
acetyl-CoA carboxylase	ACC1		At1g36160	[73, 74]
chalcone synthase	CHS	<i>tt4</i>	At5g13930	[88]
chalcone isomerase	CHI	<i>tt5</i>	At3g55120	[88]
	CHI-L1		At5g05270	[20]
flavanone 3-hydroxylase	F3H	<i>tt6</i>	At3g51240	[92]
flavonoid 3'-hydroxylase	F3'H	<i>tt7</i>	At5g07990	[94]
flavonol synthase	FLS1		At5g08640	[95]
	FLS3		At5g63590	[98, 100]
dihydroflavonol reductase	DFR	<i>tt3</i>	At5g42800	[101]
leucoanthocyanidin dioxygenase/anthocyanidin	LDOX/ANS	<i>tt18, tt11, tt17,</i>	At4g22880	[109]
anthocyanin reductase	ANR	<i>ban (banyuls)</i>	At1g61720	[111]
laccase	LAC15	<i>tt10</i>	At5g48100	[114]
glutathione S-transferase	GSTF12	<i>tt19, tt14</i>	At5g17220	[10, 143]
multi-drug and toxic efflux (MATE) transporter	TT12	<i>tt12</i>	At3g59030	[145]
P-type H ⁺ -ATPase	AHA10	<i>tt13, aha10</i>	At1g17260	[10, 158]
flavonoid 3-O-glucosyltransferase	F3GlcT		At5g17050	[20, 132]
flavonol 3-O-rhamnosyltransferase	F3RhaT		At1g30530	[118]
flavonol 3-O-arabinosyltransferase	F3AraT		At5g17030	[25]
flavonol 7-O-glucosyltransferase	F7GlcT		At2g36790	[118]
flavonol 7-O-rhamnosyltransferase	F7RhaT		At1g06000	[119]
anthocyanin 5-O-glucosyltransferase	A5GlcT		At4g14090	[20]
anthocyanin 3-O-glucoside: 2"-O-xylosyltransferase	A3G2"XylT		At5g54060	[20, 120]
anthocyanin 3-O-6"-O-coumaroylglucoside:	BGLU10		At4g27830	[123]
glucosyltransferase				
methyltransferase	OMT1		At5g54160	[24, 126]
anthocyanin malonyltransferase (BAHD)	A5GlcMalT		At3g29590	[125]
anthocyanin coumaroyltransferase (BAHD)	A3GlcCouT		At1g03495	[20, 130]
	A3GlcCouT		At1g03940	[20, 130]
anthocyanin sinapoyltransferase (SCPL)	A3Glc2"XylSinT		At2g23000	[131]

Legends to figures

Figure 1. Flavonoid diversity in Arabidopsis.

(A) Structures of anthocyanins, flavonols and proanthocyanidins detected in *A. thaliana*.
(B) Accumulation sites of flavonoids in Arabidopsis tissues. Grey color indicates the sites where the flavonoid molecules were detected. *Key*: C, cyanidin; K, kaempferol; Q, quercetin; I, isorhamnetin; E, epicatechin; mal, malonyl; *p*-cou, *p*-coumaroyl; sina, sinapoyl; glc, glucose; rha, rhamnose; ara, arabinose; hex, hexose; deoxyhex, deoxyhexose; pent, pentose. Flavonoid derivatives were found in Arabidopsis leaves [18, 20, 23, 25, 28, 30, 119], root [20, 24, 25, 28, 29], flowers [15, 25, 28] and seeds [10, 21, 22, 28, 32] by LC-MS or LC-UV/VIS-MS studies, and identified or characterized by 1D- and 2D-NMR, IR, UV and MS studies [17, 19, 25, 26].

Figure 2. General biosynthetic pathway of phenylpropanoid and flavonoid skeletons.

PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid: CoA ligase; ACC, acetyl-CoA carboxylase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; OMT1, *O*-methyltransferase 1; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase.

Figure 3. Proposed flavonol modification pathway in Arabidopsis

The compounds detected in Arabidopsis are shown. White arrows indicate proposed but unidentified pathways. OMT1, *O*-methyltransferase 1; F3AraT, flavonol 3-*O*-arabinosyltransferase; F3GlcT, flavonoid 3-*O*-glucosyltransferase; F3RhaT, flavonol 3-*O*-rhamnosyltransferase; F7GlcT, flavonol 7-*O*-glucosyltransferase; F7RhaT, flavonol 7-*O*-rhamnosyltransferase.

Figure 4. Ds transposon insertion mutants of UGT79B1

(A) Phenotype of the wild-type (F-Nossen), Ds53, Ds54 and Arabidopsis transposon tagged lines, *ugt79b1-1* (*Ds53-4592-1*) and *ugt79b1-2* (*Ds-54-1263-1*). Plants were grown on one-half-strength Ms-agar medium containing 5% sucrose in a growth chamber at 22 °C with 16h/8h light and dark cycles for 7 days.

(B) HPLC/PDA/MS analyses of the *ugt79b1* mutant lines.* a compound which has the

m/z value corresponding to cyanidin 3-*O*-(6"-*O*-*p*-coumaroylglucoside)-5-*O*-(6"-*O*-malonylglucoside); ** a compound which has the *m/z* value corresponding to cyanidin 3-*O*-6"-*O*-*p*-coumaroylglucoside.

Figure 5. Proposed anthocyanin modification pathway

The metabolic grid for the biosynthesis of A11. Black and pale grey arrows indicate potential glucosylation (Glc), xylosylation (Xyl), coumaroylation (Cou), sinapoylation (Sin), and malonylation (Mal). Dark grey arrows on the metabolic grid indicate putative major anthocyanin modification route. C3G, cyanidin 3-*O*-glucoside; C3G5G, cyanidin 3,5-*O*-diglucoside; C3G2"X, cyanidin 3-*O*-xylosyl(1→2)glucoside.

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A

Anthocyanins (A1 – A11)

Flavonols (f1 – f35)

Proanthocyanidins (p1 – p8)

B

	immature seed	mature seed	leaf	flower	root	stem	aglycone	R1	R2	R3
A1							C	H	H	H
A2							C	H	H	mal
A3							C	H	p-cou	H
A4							C	sina	H	H
A5							C	H	p-cou	mal
A6							C	H	p-cou-glc	H
A7							C	sina	p-cou	H
A8							C	H	p-cou-glc	mal
A9							C	sina	p-cou	mal
A10							C	sina	p-cou-glc	H
A11							C	sina	p-cou-glc	mal
f1							K	H	rha	rha
f2							K	H	glc	rha
f3							K	H	rha (1→2) glc	rha
f4							K	H	glc (1→6) glc	rha
f5							Q	OH	rha	rha
f6							Q	OH	glc	rha
f7							Q	OH	rha	glc
f8							Q	OH	rha (1→2) glc	rha
f9							K	H	conjugate	
f10							K	H	hex+deoxyhex	—
f11							K	H	conjugate	
f12							K	H	rha+conjugate	—
f13							Q	OH	hex+deoxyhex	—
f14							I	OCH ₃	glc	rha
f15							I	OCH ₃	rha	glc
f16							Q	OH	glc	H
f17							K	H	rha	H
f18							K	H	glc	H
f19							K	H	ara	rha
f20							K	H	rha	glc
f21							K	H	glc+hex	—
f22							K	H	pent+deoxyhex	rha
f23							Q	OH	ara	H
f24							Q	OH	rha	H
f25							Q	OH	ara	rha
f26							Q	OH	glc+hex	—
f27							I	OCH ₃	rha	H
f28							I	OCH ₃	glc	H
f29							I	OCH ₃	ara	rha
f30							I	OCH ₃	rha	rha
f31							I	OCH ₃	glu+hex	—
f32							I	OCH ₃	rha (1→2) glc	rha
f33							K	H	rha + hex + mal	
f34							Q	OH	rha + hex + mal	
f35							I	OCH ₃	rha + hex + mal	
p1							E		epicatechin	
p2							E		procyanidin B2 (n = 0)	
p3							E		epicatechin trimer (n = 1)	
p4							E		epicatechin tetramer (n = 2)	
p5							E		epicatechin pentamer (n = 3)	
p6							E		epicatechin hexamer (n = 4)	
p7							E		epicatechin heptamer (n = 5)	
p8							E		epicatechin hexoside

Fig. 3

A

B