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REVIEW PAPER

Current understanding of the pathways of flavonoid biosynthesis in model and crop plants

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Abstract

Flavonoids are a signature class of secondary metabolites formed from a relatively simple collection of scaffolds. They are extensively decorated by chemical reactions including glycosylation, methylation, and acylation. They are present in a wide variety of fruits and vegetables and as such in Western populations it is estimated that 20–50 mg of flavonoids are consumed daily per person. *In planta* they have demonstrated to contribute to both flower color and UV protection. Their consumption has been suggested to presenta wide range of health benefits. Recent technical advances allowing affordable whole genome sequencing, as well as a better inventory of species-by-species chemical diversity, have greatly advanced our understanding as to how flavonoid biosynthesis pathways vary across species. In parallel, reverse genetics combined with detailed molecular phenotyping is currently allowing us to elucidate the functional importance of individual genes and metabolites and by this means to provide further mechanistic insight into their biological roles. Here we provide an inventory of current knowledge of pathways of flavonoid biosynthesis in both the model plant *Arabidopsis thaliana* and a range of crop species, including tomato, maize, rice, and bean.

Key words: Anti-oxidant, Arabidopsis, crop species, flavonoids, human health, tomato.

Introduction

Polyphenolic compounds represent one of the most numerous and widely distributed groups of substances in the plant kingdom (Tohge *et al.*, 2013*a*). Flavonoids are by far the largest class of polyphenols, estimated to comprise over 8000 metabolites. They bear a common diphenylpropane (C_6-C_3-C_6) backbone in which two aromatic rings are linked via a three-carbon chain (see Tohge *et al.*, 2013*a*). The A ring is normally formed from a molecule of resorcinol or phloroglucinol, synthesized via the less well characterized acetate pathway, and has a characteristic hydroxylation pattern at the 5′ and 7′ positions (Croft, 1998). The B ring comes from the comprehensively characterized shikimate pathway (Tohge *et al.*, 2013*b*; Chen *et al.*, 2016) and is commonly 4′-, 3′4′-, or 3′4′5′-hydroxylated. Flavonoids can be further subdivided into six major subclasses - and in excess of 5000 total subclasses on the basis of variation in the heterocyclic C-ring (Harborne, 1993) - namely flavones, flavonols, flavanones, flavanols, anthocyanidins, and isoflavones (Fig. 1). Flavonoids are classified as secondary metabolites in plants, since they have been believed not to play a role in plant growth (Saito *et al.*, 2013). However, as mentioned below, recent evidence has implicated flavonols as playing a role in the phototropic response (Silva-Navas *et al.*, 2016; Tohge and Fernie, 2016). They are, in addition, ubiquitously distributed in plants. The total carbon flux through the flavonoid pathway represents approximately 20% of the

Abbreviations: AVI, anthocyanin vacuolar inclusion; CHS, chalcone synthase; FLS, flavonol synthase; ILs, introgression lines; tt, *transparent testa*; UV-B, ultraviolet-B; QTL, quantitative trait loci.

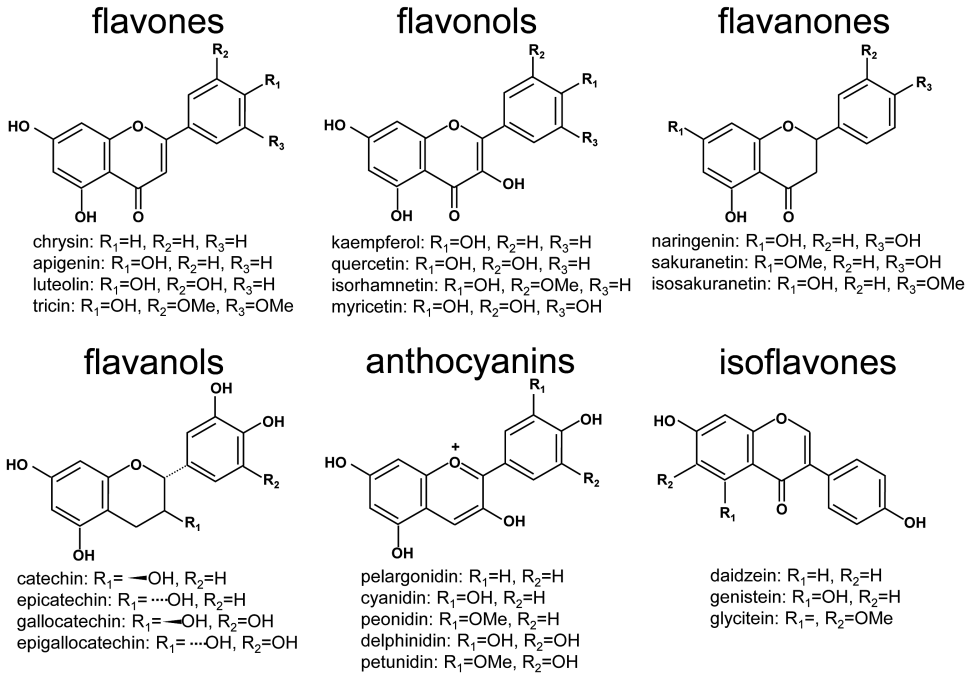

Fig. 1. Structure of major flavonoid aglycones.

total carbon flux through a typical plant cell and so is by no means minor (Haslam, 1993). Flavonoids exhibit a wide range of biological activities, including protection against ultraviolet-B (UV-B), high carbon, low nitrogen and cold stress, as well as defense against herbivores and pathogens *in planta* (Peters and Constabel, 2002; Torregrosa *et al.*, 2004; Foster-Hartnett *et al.*, 2007; Samanta *et al.*, 2011; Schulz *et al.*, 2015). In root research, flavonoids have also been implicated as important constituents of root exudates (Monchgesang *et al.*, 2016), being important in interacting with hormones as part of the phototropic response (Kuhn *et al.*, 2016; Silva-Navas *et al.*, 2016), and playing an apparently important role in pollen fertility (Mo *et al.*, 1992; Pollak *et al.*, 1993; Taylor and Hepler, 1997; Ferreyra *et al.*, 2013, 2015). They are also implicated as having beneficial health properties against a number of chronic diseases when taken up in the diet by animals (Halliwell *et al.*, 2005; Wang *et al.*, 2009). These features have been comprehensively reviewed in the last five years (Martin *et al.*, 2011; Martin *et al.*, 2013; Saito *et al.*, 2013; Tohge and Fernie, 2017). We will therefore not dwell on their discussion here. Suffice it to say, enhancing the flavonoid content of food and feed via metabolic engineering (see for example Butelli *et al.*, 2008; Zhang *et al.*, 2014; Zhang *et al.*, 2015) or by modern genome assisted breeding programs (for details see Fernie and Klee, 2011; Alseekh *et al.*, 2015; Scossa *et al.*, 2016) remains an important and achievable goal, both for improving crop yield security and human nutrition. Given the vast number of studies concerning the transcriptional regulation of flavonoid biosynthesis, we will not touch upon this aspect of flavonoid regulation in any detail here but rather refer the interested reader to previous reviews on this subject (Stracke *et al.*, 2001; Dubos *et al.*, 2010; Stracke *et al.*, 2010; Tohge *et al.*, 2013*a*; Tohge *et al.*, 2015*a*).

The biological activities of flavonoids depend, to a large extent, on their structural diversity. The advent of metabolomics and next generation sequencing has rapidly accelerated our ability to collect species-specific inventories of the metabolites, as illustrated by the KNApSAcK family databases (Afendi *et al.*, 2012; http://kanaya.naist.jp/KNApSAcK). These advances have also enabled us to perform cross species cataloguing of the structural and regulatory genes involved in metabolite synthesis and catabolism, using software such as PLAZA 3.0 (Proost *et al.*, 2009; http://bioinformatics.psb.ugent.be/plaza), PlaNet (Mutwil *et al.*, 2011; http://aranet.mpimp-golm.mpg.de) and FamNet (Ruprecht *et al.*, 2016; http://aranet.mpimp-golm.mpg.de/famnet.html). In this review, we will summarize recent research aimed at understanding the flavonoid metabolic network at both the chemical and molecular levels. To this end, we will summarize current understanding of the underlying pathways in the model plant Arabidopsis, as well as the crop species tomato, maize, rice, and beans. We will conclude by discussing how the torrent of data emerging from projects such as the 1001 Arabidopsis genomes project (1001 Genomes Consortium, 2016; http://1001genomes.org/) and the 1000 plants project (https://sites.google.com/a/ualberta.ca/onekp), will allow us to expand both structure function relationships and translational research into flavonoids beyond these major species. Such research will potentially allow the isolation and characterization of yet further flavonoids with as yet undiscovered bioactive properties.

Flavonoid research in Arabidopsis

As stated in the comprehensive recent review of Saito and colleagues, linking the metabolome to the genome is challenging,

even in Arabidopsis for which the available genomic resources far outstrip those of other species (Saito *et al.*, 2013). The coverage of this earlier review, regarding the biochemical reactions that constitute the main trunk pathway of flavonoid biosynthesis, is extensive and indeed far beyond the scope that we can manage here. That said, there have been a number of important features uncovered regarding the so-called modifications or decorative reactions of flavonoids in the short time since the publication of this last major review. In addition to addressing these features, we will summarize novel functional roles for the metabolites themselves, which have been postulated or even proven within the last three years. Before coming to these advances, it is however prudent for us to briefly describe the main trunk pathway of flavonoid biosynthesis, since this is by and large conserved across plant species. Flavonoid synthesis occurs at the convergence of the shikimate and acetate pathways, with the former providing *p*-coumaroyl-CoA and the latter being responsible for C2 chain elongation. Phenylalanine synthesized in the shikimate pathway (Fraser and Chapple, 2011; Maeda and Dudareva, 2012), is cleaved by phenylammonia-lyase (PAL) to yield ammonia and *trans*-cinnamic acid, which is then used in the production of lignins, lignans and flavonoids. Subsequently, cinnamic acid 4-hydrolase (C4H), a cytochrome 450 monooxygenase, hydroxylates the C4 position of cinnamic acid yielding *p*-coumaric acid. Seeds of plants exhibiting a mutation in *C4H* are compromised in their ability to produce proanthocyanidins, sinapoyl malate and lignins (Schilmiller *et al.*, 2009). For further metabolism, *p*-coumaric acid needs to be activated by an ATP-consuming condensation reaction catalyzed by *p*-coumaric acid:CoA ligase (4CL). There are four isoforms (At4CL1-At4CL4) of this enzyme in the Arabidopsis genome but only 4CL2 displays the expression and kinetic characteristics consistent with a role in flavonoid biosynthesis (Hamberger and Hahlbrock, 2004).

Malonyl-CoA formation is catalyzed by a series of reactions, which are shared between flavonoid production, and the elongation of very long chain fatty acids (Baud *et al.*, 2003). However, considerably less is known regarding the role of this pathway with respect to flavonoid formation and future research is required in order that a more complete picture can be obtained (Fig. 2). That said, plants deficient in ATP-citrate lyase have been noted to hyperaccumulate anthocyanins (Fatland *et al.*, 2005). In contrast, antisense inhibition of Acetyl-CoA carboxylase activity in oil seed rape resulted in a decreased accumulation of flavonoids under UV-B treatment (White *et al.*, 1998). Once malonyl-CoA and *p*-coumaroyl-CoA have been formed, they are converted into flavonoid scaffolds by a complex series of reactions including condensations, isomerizations, oxidations and reductions (Saito *et al.*, 2013). The elucidation of this pathway was greatly reliant on the *transparent testa* (*tt*) seed color mutants (Koornneef, 2004). The process begins with the action of chalcone synthase, the enzyme mutated in the colorless seed coat, *tt4* mutant (TT4, AtCHS; Winkel-Shirley *et al.*, 1995; Austin and Noel, 2003) (Fig. 2 and Table 1). Chalcone isomerase was identified as the gene mutated in *tt5* (TT5, AtCHI; Winkel-Shirley, 2001; Lepiniec *et al.*, 2006). Flavanone 3-hydroxylase catalyzes oxygenation at the 3′-position of flavone ((2*S*)-naringenin) to yield dihydro-kaempferol, but is also able to substitute for flavonol synthase and dihydroflavonol reductase for anthocyanin biosynthesis (Turnbull *et al.*, 2004; Araujo *et al.*, 2014). Flavanone 3-hydroxylase is encoded by the gene corresponding to *tt6* (TT6, AtF3H; Pelletier and Shirley, 1996). The enzyme flavone 3′-hydroxlase (TT7, AtF3′H; Schoenbohm *et al.*, 2000) catalyzes hydroxylation at the 3′-position of either dihydrokaempferol or kaempferol and converts them to dihydroquercetin or quercetin, respectively. Flavonol synthase (AtFLS1) catalyzes the first step branches of the trunk pathway towards anthocyanin formation and it appears that there is second active isoform of AtFLS1 in Arabidopsis (AtFLS3; Owens *et al.*, 2008; Preuss *et al.*, 2009; Stracke *et al.*, 2009). Dihydroflavonol reductase (TT3, AtDFR; Shirley *et al.*, 1992) competes with FLS for dihydroflavonol, yielding the corresponding leucoanthocyanin. Interestingly, the ortholog of maize flavone synthase I (ZmFNSI-1) found in Arabidopsis (DMR6/AtFNSI) has been characterized as having flavone synthase activity, as an analogy to the function identified in maize ZmFNSI-1 (Falcone-Ferreyra *et al.*, 2015). The enzyme anthocyanin synthase (TT18, AtANS, LDOX) next utilizes leucoanthocyanidin, which is the first colored compound of the pathway. The mutants, *tt4*, *tt11* and *tt17* and *tannin deficient seed 4*, are all ascribed to mutations in the anthocyanin synthase gene (Abrahams *et al.*, 2003; Bowerman *et al.*, 2012).

While the core pathway described above is the high flux bearing backbone of flavonoid biosynthesis, the chemical diversity in the family is due to the high number of tailoring modifications carried out by a variety of glycosyltransferases, methyltransferases, and acyltransferases. Glycosylation is essential for the stable accumulation of flavonoids (Mazza and Brouillard, 1987; Luo *et al.*, 2007; Lee *et al.*, 2017) in Arabidopsis and occurs at -OH moieties of the C3, C5 and C7 positions of flavonoid aglycones, with sugar moieties attached to flavonoid aglycones also being glycosylated themselves (Saito *et al.*, 2013; Tohge *et al.*, 2015*a*). A total of nine genes encoding flavonoid glycosyltransferases have been identified with the most recent being reported by Ishihara and colleagues in 2016. However, on the basis of flavonoid structures it has been predicted that at least ten flavonoid glycosyltransferases are present in Arabidopsis. We do not discuss these here but rather refer the reader to the review of Saito and colleagues (2013), for details. Recently flavonol 3-*O*-glucoside:2″-*O*-glucosyltransferase (F3Glc:2″XGlcT), which is involved in glycosylation of pollen specific flavonol-glycosides (flavonol 3-*O*(-2″-*O*-glucosyl)glucoside), was characterized in Arabidopsis (Yonekura-Sakakibara *et al.*, 2014). In addition, as alluded to above, the most recently identified flavonol glucosyl transferase, AtBGLU6 (flavonol 3-*O*-glucoside:6″-*O*-glucosyltransferase, F3Glc:6″GlcT), was identified by a screen of 81 Arabidopsis ecotypes, alongside a quantitative trait loci (QTL) analysis for variation in flavonol 3-*O*-gentiobioside 7-*O*-rhamnoside content (Ishihara *et al.*, 2016). These analyses defined the causal single nucleotide polymorphism that discriminated between ecotypes which could produce flavonol 3-*O*-gentiobioside 7-*O*-rhamnoside

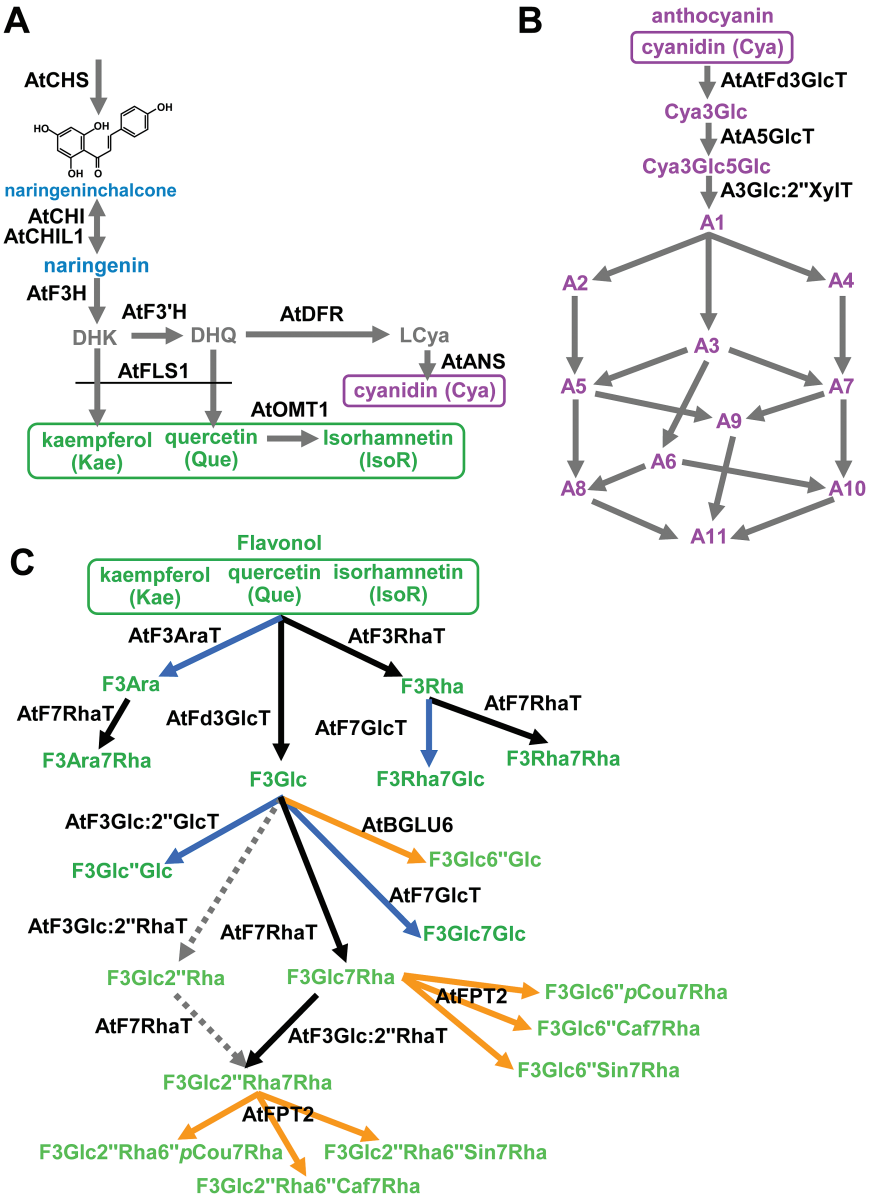

Fig. 2. Arabidopsis flavonoids biosynthetic pathway. Descriptions of genes are presented in Table 1. Different colors of metabolites correspond to: blue, flavanones; green, flavonol; purple, anthocyanin. Different colors of enzymatic steps correspond to: blue, flower specific steps; orange, accessions specific step; dot line, not in the wild-type.

from those which could not. Interestingly, the genes in question encode both AtBGLU10 and AtBGLU6, neither of which belongs to the canonical family of flavonol glycosyltransferases (UDP-sugar dependent glycosyltransferase 1 family, UGT1), which use UDP-conjugates as their activated sugar donor substrate. In addition to glycosyltransferases, methyltransferases and acyltransferases confer important modifications to flavonoids in Arabidopsis. The methyltransferases and acyltransferases of the BEATAHCT/HCBT/DAT (BAHD) and serine carboxypeptidase-like (SCPL) families have been comprehensively reviewed elsewhere (D'Auria and Gershenzon, 2005; Saito *et al.*, 2013) and very few recent advances on these reactions have been made. In contrast, a novel class of phenylacylated flavonols, comprising a total of 18 different metabolites, was recently characterized by a battery of chemical analytical techniques. Furthermore, the gene responsible for their synthesis was cloned and demonstrated to be a flavonol-phenylacyltransferase (AtFPT2, AtF3Glc:6″PheAT; Tohge *et al.*, 2016). While such modifying enzymes have been documented to occur in scots pine (Bakowska-Barczak, 2005; Kaffarnik *et al.*, 2005), tomato (Tohge *et al.*, 2015*b*) and other Brassica vegetables (Cartea *et al.*, 2011), the compounds identified in Arabidopsis and subsequently named saiginols differ from others reported with respect to the position at which the phenylacylation reaction occurs. Intriguingly, this modification provides enhanced UV-B absorbent properties to the saiginols, which confers a fitness advantage to the plants that produced them following exposure to prolonged UV-B (Tohge *et al.*, 2016).

Most of the constituent enzymes of flavonoid metabolism in Arabidopsis, alongside the chemical structure of many of the metabolic intermediates (Nakabayashi *et al.*, 2009;

Table 1. *Flavonoid biosynthetic genes characterized in Arabidopsis thaliana*

Name	Synonyms	Arabidopsis Gene Identifier	Function	Reference
AtCHS	TT4	At5g13930	Chalcone synthase	Austin and Noel, 2003
AtCHI	TT5	At3g55120	Putative chalcone isomerase	Winkel-Shirley, 2001
AtCHIL		At5g05270	Chalcone isomerase-like	Jiang *et al.*, 2015
AtF3H	TT6	At3g51240	Flavanone 3-hydroxylase	Turnbull *et al.*, 2004
AtF3′H	TT7	At5g07990	Flavone 3′-hydroxylase	Schoenbohm *et al.*, 2000
AtFLS1		At5g08640	Flavonol synthase	Owens *et al.*, 2008
AtFLS3		At5g63590	Flavonol synthase	Preuss *et al.*, 2009
AtDFR	TT3	At5g42800	Dihydroflavonol reductase	Shirley *et al.*, 1992
AtANS	TT18	At4g22880	Anthocyanin synthase	Abrahams *et al.*, 2003
AtBAN	BANYULUS	At1g61720	Anthocyanin reductase	Devic *et al.*, 1999
AtLAC15	TT10	At5g48100	Polyphenol oxidase	Pourcel *et al.*, 2005
AtFNSI	DMR6	At5g24530	Flavone synthase I activity enzyme	Falcone Ferreyra *et al.*, 2015
AtF3RhaT	UGT78D1	At1g30530	Flavonol 3-*O*-rhamnosyltransferase	Jones *et al.*, 2003
AtF3AraT	UGT78D3	At5g17030	Flavonol 3-*O*-arabinosyltransferase	Yonekura-Sakakibara *et al.*, 2008
AtFd3GlcT	UGT78D2	At5g17050	Flavonol 3-*O*-glucosyltransferase	Tohge *et al.*, 2005
AtA5GlcT	UGT75C1	At4g14090	Anthocyanin 5-O-glucosyltransferase	Tohge *et al.*, 2005
AtF7GlcT	UGT73C6	At2g36790	Flavonol 7-O-glucosyltransferase	Jones *et al.*, 2003
AtF7RhaT	UGT89C1	At1g06000	Flavonol 7-O-rhamnosyltransferase	Yonekura-Sakakibara *et al.*, 2008
AtA3Glc:2″XylT	UGT79B1	At5g54060	Anthocyanin 3-*O*-glucoside:2″-*O*-xylosyltransferase	Tohge *et al.*, 2005
AtA3Glc6″Cou:GlcT	AtBGLU10	At4g27830	Anthocyanin 3-*O*-(p-coumaroyl) glucoside:glucosyltransferase	Miyahara *et al.*, 2013
AtF3Glc:6″GlcT	AtBGLU6	At1g60270	Flavonol 3-*O*-glucoside:6″-*O*-glucosyltransferase	Ishihara *et al.*, 2016
AtOMT1		At5g54160	Flavonol 3′-*O*-methyltransferase	Muzac *et al.*, 2000
AtFOMT-like	CCOAOMT7	At4g26220	Flavonoid *O*-methyltransferase	Wils *et al.*, 2013
AtA5Glc:6″MalT	At5MAT	At3g29590	Anthocyanin 5-*O*-glucoside:malonyltransferase	D'Auria *et al.*, 2007
AtA3Glc:6″CouT1		At1g03940	Anthocyanin 3-*O*-glucoside: p-coumaroyltransferase	Luo *et al.*, 2007
AtA3Glc:6″CouT2		At1g03495	Anthocyanin 3-*O*-glucoside: p-coumaroyltransferase	Luo *et al.*, 2007
AtA3Glc2″Xyl:2″SinT	SAT/ AtSCPL10	At2g23000	Anthocyanin 3-*O*-glucoside-2″-*O*-xyloside:sinapoyltransferase	Fraser *et al.*, 2007
AtF3Glc:6″PheAT	AtFPT2	At2g22960	Flavonol-phenylacyltransferase	Tohge *et al.*, 2016
AtTT12	TT12	At3g59030	MATE transporter	Debeaujon *et al.*, 2001
AtAHA10	TT13	At1g17260	putative P-type H+-ATPase	Baxter *et al.*, 2005
AtDTX35	FFT	At4g25640	MATE transporter	Thompson *et al.*, 2010
AtGSTF12	TT19	At5g17220	Glutathione S-transferase like	Kitamura *et al.*, 2004

Tohge *et al.*, 2015*a*) have been identified. These studies have collectively facilitated the reconstitution of the major metabolic pathways of anthocyanin and flavonol biosynthesis (Saito *et al.*, 2013). This metabolic framework thus serves as an important blueprint from which those of crop species can be deduced and modified, as we will discuss in the sections below. However, before setting out to do so, the issue of flavonoid compartmentation should be discussed since a large proportion of our understanding of this important process comes from work performed in Arabidopsis. Transport proteins were first recognized to be important in the transparent testa and tannin deficient seed screens detailed above (Winkel-Shirley *et al.*, 1995; Abrahams *et al.*, 2003). Proanthocyanidin is believed to be stored in the vacuoles of seed coat endothelial cells following oligomerization and polymerization of proanthocyanidin intermediates, which are transported from the cytosolic facing side of the endoplasmic reticulum (Kitamura *et al.*, 2004; Zhao *et al.*, 2010). While *TRANSPARENT TESTA 12* (*TT12*) encodes a multidrug and toxic efflux (MATE) transporter related to vacuolar proanthocyanidin transport in the same tissue (Debeaujon *et al.*, 2001). Further studies, however, indicated that transport via TT12 is confined to flavan-3-ol glycosides (Marinova *et al.*, 2007). A more recent study has postulated that AtAHA10, a putative P-type H^+-ATPase, acts in concert with TT12, to maintain an H^+/flavonoid antiport function in Arabidopsis. Consistent with such a role is the fact that *aha10* mutants show vacuolar defects and reduced proanthocyanidin accumulation (Baxter *et al.*, 2005; Lepiniec *et al.*, 2006). However, a considerably lower epicatechin-glucoside level is seen in the *tt12* mutant than in the *aha10* mutant (Kitamura *et al.*, 2010). That said, further support for the concerted action of these two transporters came from the recent identification that *TRANSPARENT TESTA 13* (*TT13*) encodes AtAHA10, as well as a series of elegant complementation experiments that confirm its function in tandem with TT12 (Appelhagen *et al.*, 2015). Another MATE transporter has been demonstrated to be expressed in floral guard cells and when mutated the flowers exhibit decreased levels of floral kaempferol di-glucosides (Thompson *et al.*, 2010). *TRANSPARENT TESTA*

19 (*TT19*) meanwhile encodes a glutathione *S*-transferase like protein that is almost exclusively involved in both proanthocyanidin and anthocyanin accumulation (Kitamura *et al.*, 2004). Although a reduction in flavonoid content is a fairly common feature of *tt19* mutants (Mueller *et al.*, 2000; Smith *et al.*, 2003), the function of TT19 in anthocyanin transport remains unclear.

The above section has dealt with the membrane transporter-mediated pathway, however, considerable recent advances have also been made in studying the vesicle trafficking pathway. Ichino and colleagues screened a library of Arabidopsis mutants with defects in vesicle trafficking, and isolated the *gfs9* mutant, which was characterized by abnormal pale tan-colored seeds caused by low level flavonoid accumulation (Ichino *et al.*, 2014). They demonstrated that *gfs9* is allelic to the unidentified *tt9* mutant. GFS9 is a peripheral membrane protein localized to the Golgi apparatus and its deficiency causes several membrane trafficking defects, including the missorting of vacuolar proteins, vacuole fragmentation, the aggregation of enlarged vesicles and the proliferation of autophagosome-like structures. A recent paper described the study of anthocyanin vacuolar inclusion (AVI) formation in cotyledons of different Arabidopsis genotypes grown under anthocyanin inductive conditions (Chanoca *et al.*, 2015). This study demonstrated that cytoplasmic anthocyanin aggregates in close contact with the vacuolar surface are directly engulfed by the vacuolar membrane in a process reminiscent of microautophagy, yet neither endosomal or prevacuolar trafficking nor the autophagy ATG5 protein is involved in the formation of AVIs. However, the formation of AVIs is promoted by both an increase in cyanidin 3-*O*-glucoside derivatives and by depletion of the glutathione *S*-transferase TT19. The authors additionally postulated that this novel microautophagy-like mechanism also mediates the transport of other flavonoid aggregates into the vacuole. Very recently, a study demonstrated that AVIs form when the concentration of aromatically acylated anthocyanins reaches a level that aggregates when the pH of the compartment is between 4.5 and 6.5 (Kallam *et al.*, 2017). The authors of this study posit, in contrast to what Chanoca and colleagues speculate, that the formation of AVIs is an inevitable consequence of their chemistry but that there is a possibility that some glycosylations have evolved or been retained to reduce aggregation. Alternatively, they suggest that in extreme cases, such as in the black regions of lisianthus flowers, the formation of AVIs may have been harnessed. It will be interesting to study the conditional hierarchies involved in flavonoid transport to the vacuole by comparing and contrasting mutants of the various routes. Further studies generating a number of crosses between mutants of the biosynthetic and transport functions will be useful as tools for combining metabolomics and co-expression analysis, ultimately providing clues to vacuolar transport functions as demonstrated in a proof of concept study in barley (Tohge *et al.*, 2011). Coupling such molecular studies with emerging tools such as fluorescence lifetime imaging microscopy (FLIM), as recently described by (Chanoca *et al.*, 2016), in order to provide information concerning flavonoid trafficking should greatly facilitate advances in our understanding of the regulation of transport between the cytosol and vacuole.

Tomato species

Tomato (*Solanum lycopersicum*) is one of the most important fleshy fruit crops and has served as a model fruit-bearing organism for many decades (Tomato Genome Consortium, 2012). In terms of nutrition, due to their rich polyphenolic content, tomato fruits represent an important constituent of the Western diet (Tieman *et al.*, 2012; Martin, 2013). The history of tomato flavonoids began with the characterization of quercetin 3-*O*-glucoside in the outer epidermis of tomato peels (Wu and Burrell, 1958) and quercetin 3-*O*-rutinoside (rutin) in tomato paste (Rivas and Luh, 1968). The first step of flavonoid biosynthesis, catalyzed by CHS, was initially characterized in tomato in the early 1990s (SlCHS1 and SlCHS2; O'Neill *et al.*, 1990) (Fig. 3 and Table 2). Furthermore, given that CHS RNAi transgenic tomato fruits displayed impaired pollen tube growth, further study of this enzyme led to novel insights in the mechanisms underlying parthenocarpic fruit development (Schijlen *et al.*, 2007).

In tomato, anthocyanins are readily observed as red pigmentation in a variety of tissues including hypocotyls, the lower epidermis of cotyledons, the first true leaves particularly near vascular tissues, and cortical cells at the base of stem and leaf hairs (von Wettstein-Knowles, 1967; Butelli *et al.*, 2008; Zhang *et al.*, 2014; Tohge *et al.*, 2015*b*). Overexpression of the SlDFR gene complemented the *anthocyanin without* (*aw*) mutant thus establishing its identity (Bongue-Bartelsman *et al.*, 1994). Subsequently, a flavonoid 3′5′-hydroxylase (F3′5′H) enzyme, which accepts flavones, flavanones, dihydroflavonols and flavonols as substrates, was cloned and characterized (SlCYP75A31, SlF3′5′H; Olsen *et al.*, 2010). Interestingly, the preferred substrate of SlDFR is dihydromyricetin, which is converted from dihydrokaempferol and dihydroquercetin by the SlF3′5′H enzyme. In addition, tomato SlFLS prefers dihydroquercetin and dihydrokaempferol to dihydromyricetin; therefore SlDFR and SlFLS do not compete for a common substrate (Bovy *et al.*, 2002). In an early study, overexpression of petunia *phCHI* was demonstrated to result in a higher production of flavonoids in tomato (Muir *et al.*, 2001). More recently, tomato SlCHI1 was functionally characterized as being able to complement the phenotype of glandular trichomes of the *anthocyanin free* (*af*) mutant of *S. lycopersicum*, which produces neither flavonoids nor terpenoids (Kang *et al.*, 2014).

Anthocyanin over-accumulating tomato has been used for the annotation of genes involved in anthocyanin biosynthesis in tomato via the integration of transcriptomic and metabolomic approaches. The first example of this was use of the *anthocyanin 1* (*ant1*) mutant (Mathews *et al.*, 2003) isolated from an activation-tagging screen. Analysis of gene expression in this mutant revealed several genes encoding proteins involved in anthocyanidin biosynthesis, such as anthocyanin glycosyltransferase and transporters. Later transgenic lines overexpressing *Del* and *ROS1* snapdragon

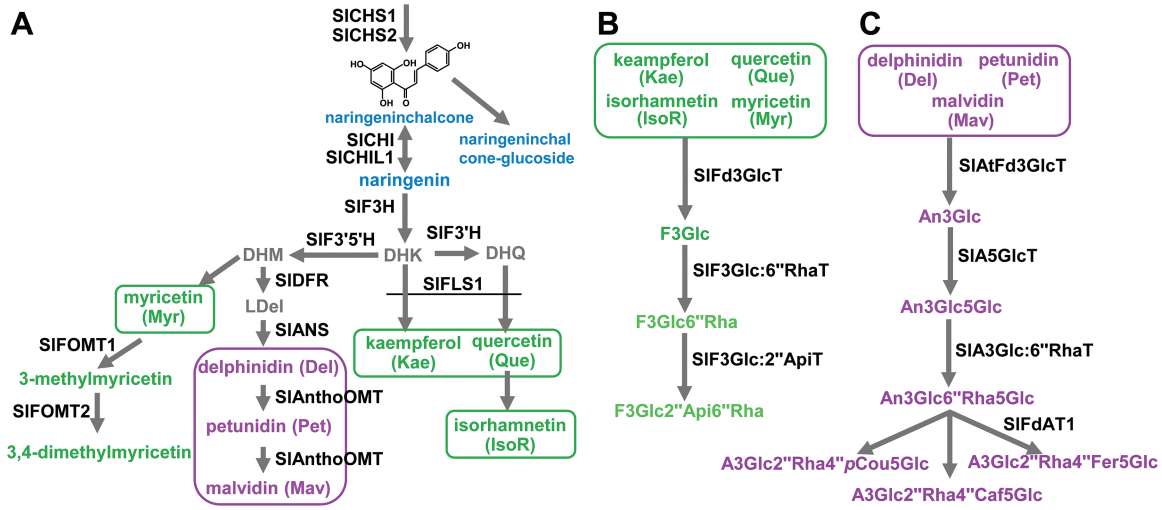

Fig. 3. Tomato flavonoids biosynthetic pathway. Descriptions of genes are presented in Table 2. Different colors of metabolites correspond to: blue, flavanones; green, flavonol; purple, anthocyanin.

Table 2. *Flavonoid biosynthetic genes characterized in tomato species*

Name	Synonyms	*Solanum lycopersicum* Gene Identifier	Function	Reference
SlCHS1		Solyc05g053550	Chalcone synthase	O'Neill, *et al.*, 1990
SlCHS2		Solyc09g091510	Chalcone synthase	O'Neill, *et al.*, 1990
SlCHI1		Solyc05g010320	Chalcone isomerase	Kang *et al.*, 2014
SlCHIL		Solyc05g052240	Chalcone isomerase-like	Tohge *et al.*, 2015*b*
SlF3H		Solyc02g083860	Flavanone 3-hydroxylase	Zhang *et al.*, 2015
SlF3′H		Solyc03g115220	Flavone 3′-hydroxylase	Tohge *et al.*, 2015*b*
SlF3′5′H	SlCYP75A31	Solyc11g066580	Flavonoid 3′5′-hydroxylase	Olsen *et al.*, 2010
SlFLS		Solyc11g013110	Flavonol synthase	Bovy *et al.*, 2002
SlDFR		Solyc02g085020	Dihydroflavonol reductase	Bongue-Bartelsman *et al.*, 1994
SlANS		Solyc08g080040	Anthocyanin synthase	Tohge *et al.*, 2015*b*
SlAnthOMT		Solyc06g06450	Anthocyanin *O*-methyltransferase	Gomez Roldan *et al.*, 2014
ShMOMT1	MOMT1	Solyc06g083450	Myricetin 3′/5′-O-Methyltransferases	Schmidt *et al.*, 2011
ShMOMT4			Myricetin 3′-*O*-methyltransferase	Kim *et al.*, 2014
SlFdAT1	SlFd3Glc6″Rha4‴PAT	Solyc12g088170	Flavonoid-3-*O*-rutinoside-4‴-*O*-phenylacyltransferase	Tohge *et al.*, 2015*b*
SlFd3GT		Solyc10g083440	Flavonoid 3-*O*-glucosyltransferase	Tohge *et al.*, 2015*b*
SlGST		Solyc02g081340	Glutathione *S*-transferase	Tohge *et al.*, 2015*b*

transcription factors were documented to harbor anthocyanin hyperaccumulating fruit (Butelli *et al.*, 2008). Integration of data from gene expression profiling of *Del/ROS1* transgenic fruits and tomato seedlings, revealed 57 candidate tomato anthocyanin biosynthetic genes including an anthocyanin *O*-methyltransferase (SlAnthOMT; Gomez Roldan *et al.*, 2014). Recently, in order to assess tomato anthocyanin biosynthetic structures more fully, the major anthocyanins [TA1: delphinidin-3-*O*-(4‴-*p*Cou)-6″-*O*-Glc)Glc-5-*O*-Glc (nasunin) and TA2; Delphinidin-3-*O*-(4‴-*p*Cou)-6″-*O*-Glc) Glc-5-*O*-Glc (petanin)] were purified and characterized from *Del/ROS1* transgenic tomato fruits (Tohge *et al.*, 2015*b*). Both TA1 and TA2 have been characterized in several Solanaceae species, such as eggplant and petunia. They were found in young leaves of *S. lycopersicum* as well as the related wild species *S. pennellii*, which is characterized by its extreme stress tolerance (Bolger *et al.*, 2014). Integration of chemical structure and transcriptomic data with phylogenetic analysis, suggested functions for the anthocyanin decorating enzymes anthocyanin-3-*O*-glucosyltransferase (SlA3GlcT), anthocyanin-5-*O*-glucosyltransferase(SlA5GlcT),anthocyanin-3-*O*-glucoside-6″-*O*-rhamnosyltransferase (SlA3Glc6″RhaT), andanthocyanin-3-*O*-rutinoside-4‴-*O*-phenylacyltransferase (SlFdAT1) in tomato. Further investigation of the function of Solanaceae species-specific candidate genes using recombinant enzyme assays and metabolite profiling of transgenic tobacco confirmed that *SlFdAT1* encodes a flavonoid-3-*O*-rutinoside-4‴-*O*-phenylacyltransferase(SlFdAT1,SlFd3Glc6″Rha4‴ PAT; Tohge *et al.*, 2015*b*). Interestingly, a significant reduction of phenylalanine was also observed in the *Del/ROS1* transgenic tomato. It was previously demonstrated that flavonoid and volatile biosynthesis compete with one another

(Dal Cin *et al.*, 2011). Further dissection of the regulation of Phe biosynthesis and turnover will likely be crucial in understanding this important metabolic crossroad. Alongside such studies the identification of the major biosynthetic genes (SlCHI, SlCHIL, SlF3H, SlF3′H, SlFLS, SlANS, SlFd3GT, SlGST), which are well conserved among plant species have been annotated on the basis either of gene homology (Tohge *et al.*, 2015*b*; Zhang *et al.*, 2015), or, at least for a subset of species, by looking at co-expression data (Mutwil *et al.*, 2011; Ruprecht *et al.*, 2016).

Beyond the genetic variation found in mutagenized tomato populations, naturally existing variation is particularly useful since wild species could be used as sources for genetic improvement of crop quality. A major goal of modern tomato breeding is to screen crossable wild Solanum species for valuable traits such as resistance against various biotic and abiotic stresses (Legnani *et al.*, 1996; Frankel *et al.*, 2003) and quality traits conferred by the content of primary metabolites (Schauer *et al.*, 2005) and secondary metabolites (Alseekh *et al.*, 2015). Wild tomato species, such as *S. pennellii*, *S. pimpinellifolium* and *S. chmielewskii*, have been used as a source to develop their introgression lines (ILs) in *S. lycopersicum*. These populations can be used to identify QTLs that improve crop quality (Zamir, 2001). In some studies, these populations have been used to identify QTLs for flavonoid biosynthesis. Liquid chromatography-mass spectrometry (LC-MS) profiling of fruit pericarp of the set of *S. pennellii*-derived *S. lycopersicum* ILs, resulted in the identification of a total of 69 flavonoid metabolic QTLs (Alseekh *et al.*, 2015). Furthermore, LC-MS profiling of fruits of ILs derived from a cross between *S. lycopersicum* and the wild species *S. chmielewskii* revealed a robust flavonoid metabolic QTL region on chromosome 5 (Ballester *et al.*, 2016).

The presence of flavonoids in cuticles of tomato fruits has been previously reported (Luque *et al.*, 1995; Baker *et al.*, 2006; Mintz-Oron *et al.*, 2008). Non-glycosylated aglycones, such as myricetin, methylated at the 3-hydroxyl (-OH) position accumulate in glandular trichomes of tomato leaves (Schmidt *et al.*, 2011; Schmidt *et al.*, 2012). Research focusing on flavonoid biosynthesis using species comparison between domesticated and wild tomatoes revealed a divergence in several genes. The first two genes identified were 3′/5′ *O*-methyltransferases (ShMOMT1) and 4′/7 O-methyltransferases (ShMOMT2) (Schmidt *et al.*, 2011; Schmidt *et al.*, 2012), which confers *O*-methylation to flavonol aglycone. Eran Pichersky and colleagues found that glandular trichomes of the wild tomato, *Solanum habrochaites*, produce myricetin derivatives that are all methylated at the 3-hydroxyl position and some are additionally methylated at one or more of the 3′, 4′, 5′, and 7 hydroxyl positions (Schmidt *et al.*, 2011; Schmidt *et al.*, 2012). Furthermore in the domesticated tomato, *S. lycopersicum*, SlMOMT enzymes encoded by the apparent orthologs of ShMOMT2 and ShMOMT3 were partially characterized biochemically and shown to have activity similar to that of the corresponding *S. habrochaites* enzymes (Schmidt *et al.*, 2011; Schmidt *et al.*, 2012). Interestingly, *SlMOMT1* in wild species has a natural deletion in its first exon, but this does not affect its ability for *O*-methylation (Schmidt *et al.*, 2012). SlMOMT4 was identified as an *S. habrochaites*-specific 3′ O-methyltransferase, which is absent in the reference genome of *S. lycopersicum* (Kim *et al.*, 2014). These combined studies mean that tomato flavonoid biosynthesis is partially well characterized but by no means as comprehensively as that of Arabidopsis.

Maize and rice

Monocots are the most economically important group of plants with regard to food and feed and hence for human and animal nutrition. However flavonoid biosynthesis in monocot species, such as maize and rice, is relatively poorly understood in comparison to Arabidopsis and tomato. This is likely because grains generally contain considerably lower amounts of flavonoids. Despite this fact, maize was the initial model species for gene discovery in flavonoid biosynthesis. In very early studies, analysis of maize seed color mutants, such as *pericarp color* (*p*), *anthocyanin* (*a*), and *bronze* (*bz*), were related to several major anthocyanin biosynthetic genes: *zmCHS* (*colorless2*, *C2*; Cone *et al.*, 1986), *zmF3′H* (*purple aleurone1*, *Pr*; Larson *et al.*, 1987), *zmDFR* (*A1*; Schwarz-Sommer *et al.*,1987), *zmANS* (*A2*; Styles and Coe, 1986), *zmFd3GT* (*Bz*1; Ralston *et al.*, 1988), and *zmGST* (*Bz2*; Marrs *et al.*, 1995). Since the 1950 discovery of transposable elements, which jumped in and out of flavonoid biosynthesis genes of the maize kernel (Fedoroff, 2012; McClintock, 1950), seminal genetic experiments reliant on visible phenotyping were carried out to unravel the molecular mechanisms underlying flavonoid biosynthesis. These early studies of discovery of major flavonoid biosynthetic genes using maize mutants, as well as flower pale-colored mutants, established the strategy for the functional characterization of flavonoid biosynthetic genes in plant science.

Unlike dicots, which accumulate *O*-glycosylated flavonols as the major type of flavonoids, monocot species predominantly produce flavone *C*-glycosides, namely glycoflavones (Brazier-Hicks *et al.*, 2009; Mutwil *et al.*, 2011; Tohge *et al.*, 2013*a*). Glycoflavones are formed in many monocots and a limited range of dicot plant species, producing large chemical variation and a diverse range of biological functions including roles as siderophores, antioxidants, and antibiotics (Hultin, 2005). A number of glycoflavones with different aglycones, chrysin, apigenin, luteolin, and tricin (Fig. 1), were detected and characterized in wheat (Cavaliere *et al.*, 2005; Wojakowska *et al.*, 2013), rice (Chen *et al.*, 2014; Yang *et al.*, 2014; Matsuda *et al.*, 2015), and maize (Wen *et al.*, 2014). The chemical structure and biosynthetic pathways of both flavone *C*-glycosides and flavone *O*-glycosides in leaf blades and germinating seeds of rice have also been recently identified (Gong *et al.*, 2013; Yang *et al.*, 2014). Moreover, comprehensive profiling utilizing genome-wide association studies (GWAS) of maize kernels (Wen *et al.*, 2014) has been carried out. Additionally, transgenic maize overexpressing P1 (MYB) resulted in a higher resistance to the major maize pest earworm due to an over-accumulation of maysin (*C*-glycosyl flavone) in the corn silk (Johnson *et al.*, 2007).

The biosynthesis of flavones starts from flavanones through two different types of flavone synthase (FNS) enzymes, FNS-I (2-ODD) and FNS-II (P450)(Lee *et al.*, 2008). The gene encoding FNS-I was previously identified in parsley and is classified as belonging to the 2-ODD gene family (Martens *et al.*, 2001). Later on, rice FNS-I was characterized (OsFNS-I; Lee *et al.*, 2008). Among 2-ODD genes, FNS-I, FLS, F3H and ANS are all involved in flavonoid biosynthesis (Lee *et al.*, 2008; Bredebach *et al.*, 2011; Araújo *et al.*, 2012; Tohge *et al.*, 2013*a*; Table 3). On the other hand, flavone formation is catalyzed by FNS-II, a member of the cytochrome P450 protein family. OsFNS-II (OsCYP93G2) was characterized as a key branch point enzyme channeling flavanones to the biosynthesis of tricin O-linked conjugates in rice (Lam *et al.*, 2014). Tricin, which was recently established as a true monomer in grass lignins, is particularly interesting due to the importance of acting as a monomer in the lignification of monocots (Lan *et al.*, 2016*a*,*b*). The function of species-conserved structural genes of rice encoding CHS, CHI, F3H, F3′H, DFR, and ANS were biologically confirmed by functional complementation in the appropriate Arabidopsis *tt* mutants (Shih *et al.*, 2008). Additionally, recent transcriptome analysis of P1 maize mutants provided global gene annotation involved in maize flavonoid biosynthesis, such as ZmF2H1, as well as sugar-nucleotide conversion (Morohashi *et al.*, 2012). Interestingly maize FLS genes, *zmFLS1* and *zmFLS2*, were found in the syntenic monocot FLS region as duplicated genes (Ferreyra *et al.*, 2012). Several flavonoid-*O*-glycosyltransferases, such as zmUGT706C1, zmUGT707A3, and zmUGT706D1, have been reported in maize (Ko *et al.*, 2008). Indeed, the enzyme responsible for *C*-glycosylation to 6- and 8-flavones was firstly identified as a member of the flavonoid *C*-glucosyltransferase (FCGT) gene family from rice (OsFCGT; Brazier-Hicks *et al.*, 2009) and maize (Szalma *et al.*, 2005). Maize zmUGT708A6 is found as a bifunctional glycosyltransferase that can produce both *O*- and *C*-glycosylated flavonoids (Falcone Ferreyra *et al.*, 2012). Importantly, from studies of flavones accumulating *salmon silk* mutants (*sm1* and *sm2*; McMullen *et al.*, 2004), orientin-rhamnosyltransferase (SM2, zmUGT91L1) for the glycosylation of maysin was recently characterized (Casas *et al.*, 2016). Orientin-rhamnosyltransferase confers resistance to maize earworm as described above.

Similar to the presence of cuticle flavonoids found in tomato species, a non-glycosylated-flavanone, sakuranetin, has been identified as a phytoalexin from UV irradiated rice leaves (Kodama *et al.*, 1992). The recent transcriptomic analysis of UV-treated rice leaves annotated three OsCHSs (OsCHS1–3) and two OsCHIs (OsCHI1 and OsCHI2) that are highly expressed during the accumulation of sakuranetin. Furthermore, the gene invoved in the methylation step of sakuranetin was characterized as a naringenin *O*-methyltransferase (OsNOMT, Os12g13800; Shimizu *et al.*, 2012). Interestingly, expression of OsNOMT was induced by jasmonate and UV treatment in rice leaves prior to sakuranetin accumulation. This pathway alongside that of maysin is a clear research priority regarding cereal flavonoids, however, despite the early use of maize as a model considerable additional research on the core pathway is also needed.

Beans

Flavonoid metabolism in legumes is particularly interesting due to the presence of isoflavonoids, a highly specialized subclass of flavonoids that play important roles as phytoalexins and as signals for nodulation. Interestingly, they are almost entirely restricted to the subfamily Papilionoideae (Veitch, 2009). We focus here in describing flavonoid metabolism in common beans (*Phaseolus vulgaris*), the most relevant

Table 3. *Flavonoid biosynthetic genes in monocot species presented in this review*

Name	Synonyms	Function	Reference
OsFNS-I		Flavone synthase I	Lee *et al.*, 2008
OsFNS-II	OsCYP93G2	Flavone synthase II	Lam *et al.*, 2014
OsFCGT		Flavone-C-glycosyltransferase	Brazier-Hicks *et al.*, 2009
OsNOMT		naringenin 7-O-methyltransferase	Shimizu *et al.*, 2012
ZmCHS	C1	Chalcone synthase	Cone *et al.*, 1986
ZmF3′H	Pr	Flavone 3′-hydroxylase	Larson *et al.*, 1987
ZmDFR	A1	Dihydroflavonol reductase	Schwarz-Sommer *et al.*, 1987
ZmANS	A2	Anthocyanin synthase	Styles and Coe, 1986
ZmFd3GT	Bz1	Flavonoid 3-*O*-glucosyltransferase	Ralston *et al.*, 1988
ZmGST	Bz2	Glutathione *S*-transferase	Marrs *et al.*, 1995
ZmF2H1		Flavanone 2-hydroxylase	Morohashi *et al.*, 2012
ZmFLS1		Flavonol synthase 1	Ferreyra *et al.*, 2012
ZmFLS2		Flavonol synthase 2	Ferreyra *et al.*, 2012
ZmUGT706C1		Flavonoid-O-glycosyltransferase	Ko *et al.*, 2008
ZmUGT707A3		Flavonoid-O-glycosyltransferase	Ko *et al.*, 2008
ZmUGT706D1		Flavonoid-O-glycosyltransferase	Ko *et al.*, 2008
ZmFCGT		Flavone-C-glycosyltransferase	Szalma *et al.*, 2005
ZmUGT708A6		Flavonoid-O- and -C-glycosyltransferase	Ferreyra *et al.*, 2013
ZmUGT91L1		Orinetin-rhamnosyltransferase	Casas *et al.*, 2016

legume for direct human consumption. We draw on the parallel knowledge from soybean (*Glycine max*), given that their genomes display considerable synteny (McClean *et al.*, 2010; Reinprecht *et al.*, 2013).

Extensive reviews of the biosynthesis of isoflavonoids were published in the last few years (Du *et al.*, 2010; Wang, 2011; Veitch, 2013). In short this pathway shares the core pathway with other flavonoids up to CHS, which can than produce either naringenin-chalcone or isoliquiritigenin via a coupled reaction with the legume-specific chalcone reductase (CHR; Bomati *et al.*, 2005). The following step is performed by subtypes of CHI, namely type I CHIs, which convert only naringenin-chalcone to naringenin, while legume specific type II CHIs convert both naringenin-chalcone and isoliquiritigenin to naringenin and liquiritigenin, respectively (Jez *et al.*, 2000; Ralston *et al.*, 2005). The isoflavonoid backbone is finally produced via hydroxylation of flavanone at the C2 position and subsequent migration of the aryl moiety from C2 to C3 in a step catalyzed by the CYP93C isoflavone synthase (IFS; Jung *et al.*, 2000). Dehydration of 2-hydroxyisoflavanones occurs either spontaneously or in a reaction catalyzed by 2-hydroxyisoflavanone dehydratase (HID; Akashi *et al.*, 2005), producing genistein from naringenin and daidzein from liquiritigenin. Isoflavone backbones are further modified, usually by glycosyltransferases and methyltransferases, and their products are transported to the vacuole where they accumulate. In soybean, five genes encoding UGT1 type glycosyltranferases, namely Fd3GlcT (Kovinich *et al.*, 2010), F3Glc6ppRhaT (Rojas Rodas *et al.*, 2014), F3Glc/Gal2ppGlcT (Di *et al.*, 2015) and GmIf7GlcT (Noguchi *et al.*, 2007), were enzymatically characterized to date. Alternatively, isoflavones can be used as substrates for the production of antimicrobial pterocarpans, starting with 2′ or 3′ hydroxylation by P450s isoflavone 2′-hydroxylase (I2′H) and isoflavone 3′-hydroxylase, respectively (Barz and Welle, 1992), followed by stereospecific NADPH dependent reduction to isoflavanone by isoflavone reductase (IFR; Wang *et al.*, 2006), and formation of the dihydrofuran ring by pterocarpan synthase (Barz and Welle, 1992), producing the basic pterocarpan backbone that can again be further modified.

Common beans accumulate isoflavonoids at much lower levels than soybeans. Nevertheless genestein and daidzein (Díaz-Batalla *et al.*, 2006; de Lima *et al.*, 2014), as well as its petrocarpan derivatives (Woodward, 1980), were identified in *P. vulgaris* beans and sprouts, and are mainly associated with biotic interactions in the roots. Common beans contain a number of CHSs that show different expression patterns upon infection or wounding, suggesting a role in phytoalexin response (Ryder *et al.*, 1984; Ryder *et al.*, 1987). Common beans also contain a CHI knwon to show affinity to both naringenin-chalcone and isoliquiritigenin (Blyden *et al.*, 1991; Dixon *et al.*, 1982). More recently PvIFR1 was identified by screening for genes involved in the symbiotic interaction between *P. vulgaris* and *Rhizobium etli* (Meschini *et al.*, 2008; Table 4). PvIFR1 is expressed in roots and induced by N deficiency. Its silencing altered expression of auxin regulated genes affecting shoot and root growth, as well as nodule formation (Rípodas *et al.*, 2013).

Table 4. *Flavonoid biosynthetic genes in beans presented in this review*

Name	Function	Reference
GmFd3GlcT	Flavonoid-O-glucosyltransferase	Kovinich *et al.*, 2010
GmF3Glc6ppRhaT	Flavonoid-3-Glc-6″-O-rhamosyltransferase	Rojas Rodas *et al.*, 2014
GmF3Glc/Gal2ppGlcT	Flavonoid-3-Glc/Gal-2″-O-glucosyltransferase	Di *et al.*, 2015
GmIf7GlcT	Isoflavone-7-O-glucosyltransferase	Noguchi *et al.*, 2007
PvIFR1	Isoflavone reductase	Meschini *et al.*, 2008

The composition of flavonoids other than isoflavonoids is also an interesting trait in common beans since they are associated with seed coat and pod skin color, an important feature from a commercial perspective. Extensive genetic investigations identified different Mendelian genes controlling color, namely P, C, J[L], D[Z], G, B, V, and Rk, and color pattern, namely T, D[Z], J[L], Gy, Bip, and Ana, in seed coats, and associated them with RAPD markers (Prakken, 1970; Prakken, 1972; Bassett *et al.*, 2002; McClean *et al.*, 2002; Bassett, 2007). Later on, attempts to elucidate the relationship between genes and flavonoid biosynthesis established that V influences the hydroxylation pattern, producing a trihydroxylated B ring (Feenstra, 1960). This finding was reinforced by the phytochemical characterization of different genotypes showing the presence of the anthocyanins delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, cyanidin 3,5-diglucoside, petunidin 3-*O*-glucoside, pelargonidin 3-*O*-glucoside, and malvidin 3-*O*-glucoside (Takeoka *et al.*, 1997; Choung *et al.*, 2003) associated with the presence of a dominant *V* allele (Beninger *et al.*, 1999; Reinprecht *et al.*, 2013). While in recessive *v* genotypes, only flavonols kaempferol 3-O-glucoside and kaempferol 3-O-glucoside-O-xyloside were detected and no anthocyanins (Beninger, 1998). Finally, the *V* gene was recently mapped to chromosome Pv6 in a region syntenic to the soybean seed coat color gene *W1* and associated with F3′5′H (Yang *et al.*, 2010; Reinprecht *et al.*, 2013; Zabala and Vodkin, 2007). The *C* gene promotes the production of flavonols and anthocyanins interacting with different alleles of *V* to produce only: mono- and dihydroxylated flavonols (Cvlae), mono- to trihydroxylated flavonols and trihydroxylated anthocyanins (CV), mono- and dihydroxylated flavonols and anthocyanins (Crvlae), or only trihydroxylated anthocyanins (CrV; Feenstra, 1960). The presence of the *j* allele results in a similar pattern as described for *C* but with absence of dihydroxyflavonoids in the presence of vlae, while *J* produces leucoanthocyanidins and increases the production of flavonols and anthocyanins by 5-fold (Feenstra, 1960). Proanthocyanidins in common beans are composed mainly of catechin monomers with minor amounts of gallocatechin and afzelechin (Díaz *et al.*, 2010). These were absent in genotypes recessive for *j* (Beninger *et al.*, 1998), which was hypothesized to encode dihydroflavonol reductase (Hosfield, 2001; Konzen and Tsai, 2014). That said, *J* mapped to a region containing MYB123, which is syntenic

to soybean TT2 (Reinprecht *et al.*, 2013). Comparison of different alleles for *B* revealed it to regulate the amount of anthocyanins, with lines recessive for *b* having only 19% of the anthocyanin content of those expressing the dominant *B* (Beninger *et al.*, 2000). However, in anthocyanin-less genotypes there was no difference in astragalin levels between *GB* and *gb*, which was significantly lower in *Gb* (Beninger *et al.*, 1999). Beninger and colleagues propose that gene *B* regulates the precursor of any compound before dihydrokaempferol, probably at the level of CHS or CHI, and its strong linkage with the pathogen resistance gene *I* may be due the production of a shared precursor for isoflavonoid biosynthesis (Beninger *et al.*, 2000). More recent work done by Hu and colleagues demonstrated that the presence of a unique anthocyanin, malvidin 3,5-diglucoside, accounts for the difference in color, namely purple versus green, between two different varieties of bean pods and showed the differential expression of 11 anthocyanin structural genes and five regulatory genes in different developmental stages and light conditions. They interpreted these collective results to suggest that PvMYB1, PvMYB2 and PvTT8-1 play critical roles in regulating anthocyanin biosynthesis in purple kidney bean pods (Hu *et al.*, 2015). The above examples reveal that while great progress has been made in our understanding of legume flavonoid metabolism, considerable further work is needed before a fuller understanding of the metabolic and regulatory pathways underpinning the accumulation of these metabolites is fully understood.

Summary

Flavonoids are a large class of secondary metabolites formed from a diversity of aglycones that are extensively decorated by chemical reactions, including glycosylation and acylation. Here we summarize the current understanding of flavonoid biosynthesis in the model plant Arabidopsis, as well as the crop species tomato, maize, rice, and beans. As described in the above sections, knowledge of structural genes and chemical structures relating to flavonoid biosynthesis has been updated via a combination of several approaches, such as analysis of natural mutants, transgenic plants and ILs, for genomic, metabolomic, and transcriptomic analyses. Maize and Arabidopsis were often used for the discovery of genes involved in flavonoid biosynthesis because of the natural transposon mutants of maize, Arabidopsis T-DNA insertion mutants and genome sequence data, which are very useful for this purpose. In recent studies focusing on natural diversity of flavonoid biosynthesis, several key genes involved in the production of accessions- or species-specific flavonoids were characterized in plant species. Studying of their diversity and convergence in the flavonoid pathway provides a scaffold for understanding of species-by-species chemical diversity, which allows us to expand to cross species translational research of flavonoid biosynthesis. Given that even within a single tissue a wide diversity of individual flavonoid species are present, an important research priority is to disentangle which of these are functionally important in conferring tolerance to various stresses. Before we do this, however, we need to ask the more fundamental question as to whether they are all important or if some of them coincidentally arose during evolution. We believe that they are all important given that their synthesis costs are relatively high. Teasing out the *in vivo* functions and relative importance of each and every metabolite of this class will be a particularly arduous task. However, since flavonoids offer a legion of protective functions, both *in planta* and following dietary intake by animals, we argue that it is increasingly important to understand the functional roles of those diverse flavonoids.

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