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Flavonols modulate plant development, signaling, and stress responses

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Abstract

Flavonols are plant specialized metabolites with important functions in plant growth and development. Isolation and characterization of mutants with reduced flavonol levels, especially the *transparent testa* mutants in *Arabidopsis thaliana*, have contributed to our understanding of the flavonol biosynthetic pathway. These mutants have also uncovered the roles of flavonols in controlling development in above- and below-ground tissues, notably in the regulation of root architecture, guard cell signaling, and pollen development. In this review, we present recent progress made towards a mechanistic understanding of flavonol function in plant growth and development. Specifically, we highlight findings that flavonols act as reactive oxygen species (ROS) scavengers and inhibitors of auxin transport in diverse tissues and cell types to modulate plant growth and development and responses to abiotic stresses.

Keywords

Flavonols; Flavonoids; Reactive oxygen species; Root development; Stomatal closure; Pollen development

Biosynthesis of flavonols

Flavonoids are a group of plant specialized metabolites that have multiple important functions including conferring flower color to attract pollinators and acting as antioxidants that regulate levels of reactive oxygen species (ROS) to control plant growth, development, and fertility [1–3]. The general structure of flavonoids consists of two benzene rings linked

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by a heterocyclic ring. Flavonoids are divided into subgroups based on modifications of the heterocyclic ring, with the major groups being flavonones, flavanols, flavonols, anthocyanins, and proanthocyanins [1]. The biosynthetic pathway of flavonoids has become a topic of broad interest due to their importance in modulating plant form and function, as well as their pharmacological and nutritional benefits [4–6].

This review focuses on flavonols due to recent evidence suggesting they have unique functions in controlling plant signaling and development. Flavonols are distinguished from other groups of flavonoids by the hydroxylation of one of the benzene rings. Each flavonol has a distinct pattern of hydroxylation of the benzene ring [7]. Three core flavonols are the most prevalent across the plant kingdom, which are the monohydroxylated kaempferol, the dihydroxylated quercetin, and the trihydroxylated myricetin, shown in Figure 1. Flavonols are further modified by the addition of diverse moieties to the hydroxyl groups, with carbohydrate modifications being the most frequent [8]. These decorations contribute to the immense chemodiversity found in the flavonol subgroup. Another well-characterized modification is the methylation of quercetin that yields isorhamnetin [9].

The enzymes of the flavonoid biosynthetic pathway in Arabidopsis were identified through isolation and characterization of mutants that lacked brown proanthocyanin pigmentation in their seed coat, or testa [10]. The resulting *transparent testa (tt)* mutants were used to elucidate the biochemical pathway in Arabidopsis by mapping each mutation to genes encoding pathway enzymes. This genetic approach was feasible in Arabidopsis as most pathway enzymes are encoded by single genes [11]. Studies in other species have revealed similar enzymatic sequences, with multiple genes encoding isoenzymes that catalyze most steps of this biosynthetic pathway [12,13].

Flavonoids are synthesized from 4-coumaroyl-CoA, a phenylalanine derivative, and three molecules of malonyl-CoA, a fatty acid derivative, to produce naringenin chalcone through a reaction catalyzed by chalcone synthase (CHS), as shown in Figure 1. The cysteine residue in the active site of CHS has evolved to enhance its nucleophilicity and this increase in catalytic activity is suggested to have contributed to the diversification of the flavonoid biosynthetic pathway in terrestrial plants [14]. Naringenin chalcone is isomerized to naringenin by the enzyme chalcone isomerase. Naringenin is the precursor for flavonol biosynthesis and is converted to dihydrokaempferol by flavanone 3-hydroxylase (F3H). The flavonol precursor dihydrokaempferol can be converted to three different products by three different enzymes. Dihydrokaempferol can be converted to the other flavonol biosynthesis intermediates dihydroquercetin and dihydromyricetin by flavonoid 3-hydroxylase (F3’H) and flavonoid 3’5-hydroxylase (F3’5’H), respectively. FLS then oxidizes dihydrokaempferol, dihydroquercetin, and dihydromyricetin to the flavonols kaempferol, quercetin, and myricetin, respectively.

Pathway enzymes have multiple subcellular localizations to control where specific pathway products accumulate. For example, CHS and CHI enzymes were localized in the nucleus of cortical and epidermal cells of the root tip in Arabidopsis using immunofluorescence [15], and flavonol accumulation was detected in the nucleus [16]. In grape berry, CHS has also been found associated with the plastid and rough endoplasmic reticulum [17].

Given the many functional roles of flavonoids, their biosynthesis may occur in different tissues to allow distinct functions within these tissues. Most striking is the synthesis of anthocyanins, which is turned off in roots, pollen, and other locations, allowing higher accumulation of flavonols to modulate development, since dihydroflavonols are not converted to these downstream anthocyanin metabolites [16,18]. Genes encoding flavonoid biosynthetic pathway enzymes are highly regulated to allow both tissue specific expression and to allow pathway activity to be regulated by changes in environmental and hormonal signals.

Flavonoid biosynthesis is regulated by intrinsic and extrinsic factors

The flavonoid biosynthetic pathway is tightly regulated by intrinsic factors such as hormones and diverse external biotic and abiotic factors, which control the transcription of pathway enzymes. Expression of flavonoid biosynthetic genes depends on complexes of transcription factors. Members of the R2R3-MYB and bHLH families are required for all pathway enzymes to be expressed and a WD40 protein is a third component of complexes that regulate the downstream pathway enzymes that produce anthocyanins [19]. In Arabidopsis, three R2R3-MYB family transcription factors, MYB11, MYB12, and MYB111, activate the expression of the biosynthetic genes encoding CHS, CHI, F3H, and FLS in a tissue-specific manner [20]. Mutations *mMYB12* block flavonol synthesis in roots, while *MYB111* is required for pathway activity in cotyledon and primary leaves [21]. Other cell types show different transcription factor requirements. In Arabidopsis pollen, the triple mutant *myb11 myb12 myb111* [22] accumulates flavonols, suggesting flavonol synthesis is independent of these three transcription factors in this cell type. However, leaves of this triplHe mutant or a single mutant in *myb111* were incapable of synthesizing flavonols while maintaining anthocyanin production in leaves. This suggests that dihydroflavonols were still synthesized but were directly channeled into anthocyanins due to the absence of FLS activity [21].

Flavonol biosynthesis can be regulated by multiple plant hormones. Ethylene increases flavonol accumulation in guard cells to regulate stomatal aperture [23] and in roots to regulate gravitropism [16,24]. In roots, ethylene and auxin increase flavonol synthesis by increasing the abundance of transcripts encoding multiple flavonol biosynthetic enzymes [16]. These responses are dependent on *MYB12* as neither hormone alters flavonol accumulation in a *myb12* mutant [16]. Auxin and ethylene increase transcripts encoding CHS, CHI, and FLS, but F3H is only induced by auxin [16]. This results in different flavonol accumulation patterns with kaempferol increasing in response to both hormones, while auxin, but not ethylene, increases quercetin, the product of F3’H. The WRKY23 transcription factor is also required for flavonol induction by auxin, as a *wrky23* mutant has reduced *MYB12* transcripts and no induction of flavonol biosynthesis in roots [25].

One notable function of flavonols is protecting plants from environmental fluctuations that may be stressful. The flavonol biosynthetic pathway is thought to have evolved in plants during their colonization of land, when plants were first faced with new environmental stresses including ultraviolet (UV) light [14]. Many studies have reported increased flavonol levels in plants exposed to UV-B [26]. While the exact mechanism of flavonols in UV-B protection remains to be established, studies have demonstrated that excess flavonols

provide protection against UV-B, such as the Arabidopsis transgenic line over-expressing flavonol synthase [27] and the UV-B tolerant Arabidopsis *uvt1* mutant, which does not sustain leaf damage under UV-B stress [28]. Studies have also reported increased expression of the genes encoding flavonol biosynthetic enzymes CHS, F3H and FLS in different plants exposed to UV-B [28–30]. Flavonol biosynthesis under visible light in the absence of UV-B wavelengths is regulated through COP1, an E3 ubiquitin ligase. COP1 ubiquitinates HY5, a TF that activates expression of R2R3-MYB proteins [31]. Additionally, red and blue light induce flavonol synthesis through actions of HY5 and cryptochromes, respectively [32,33].

In addition to UV-B radiation, flavonols offer protection from a host of other abiotic stresses, which are increasing with climate change [34]. For example, drought conditions have been shown to induce accumulation of quercetin and kaempferol in Arabidopsis [35] and several tomato varieties [36]. External stimuli that result in increased flavonol production also include heat, high salinity [37]. and ozone; the latter of which increases flavonols by increasing nitrate reductase activity to elevate nitric oxide, which acts as a signaling molecule [38], These stresses are often tied to increased flavonol production to prevent stress-induced inhibition of plant growth and development [39].

Flavonols modulate reactive oxygen species (ROS) homeostasis and auxin transport

Flavonols have been shown to regulate plant growth, development, and physiology through two distinct mechanisms: maintenance of ROS homeostasis and inhibition of auxin transport. The tightly regulated production of ROS is necessary for molecular signaling involved in different plant processes [40]. However, excess ROS accumulation, e.g. during abiotic stresses, generates oxidative stress that damages cellular macromolecules [41]. As a result, plants need intricate mechanisms to maintain ROS homeostasis. ROS levels are a function of production by biosynthetic enzymes, transport, and scavenging [42]. Plants have evolved different enzymatic and non-enzymatic systems for ROS scavenging and flavonols are important players in mediating this homeostasis [43]. Indeed, their chemical structure, with its many hydroxyl groups, can act as a powerful electron donor, making flavonols potent antioxidants/reductants [44]. Compared to other plant antioxidants such as phenolic acids, flavonols have a more potent antioxidant capacity, based on their ability to neutralize singlet oxygen and hydrogen peroxide [45,46]. The chemistry by which flavonols are predicted to donate electrons to convert hydrogen peroxide to water is shown in Figure 1. as modified from [46]. The resulting semiquinone intermediate is predicted to dismutate back to quercetin or to a quinone that may be reduced back to quercetin via a NAD(P)H dependent quinone reductase. The following sections review the linkages between flavonol regulation of development through modulation of ROS homeostasis.

A second activity of flavonols is to modulate polar auxin transport, which moves auxin long distance in plant tissues [47]. Polar transport is mediated by auxin influx carriers, including AUX1 and LAX (like AUX1) proteins, and efflux carrier proteins, which include both PIN (Pin formed) and ABCB (ATP Binding Cassette B) proteins [48–50]. The polarity of auxin transport is largely driven by asymmetric location of the PIN proteins [51], the activity of

which is highly regulated through transcriptional controls, covalent protein modification, and elaborate cell biological mechanisms that control the amount, activity, and localization of these proteins [52].

The ability of flavonols to reduce polar auxin transport has been demonstrated through genetic approaches using Arabidopsis *tt* mutants [53]. Auxin transport is elevated in inflorescences, hypocotyls, and roots of Arabidopsis plants with *tt4* mutations [16,54–56]. In contrast. *tt7* and *tt3* mutants, which have elevated kaempferol or elevated kaempferol and quercetin, respectively, show reduced polar auxin transport [16,55,57]. The increased polar auxin transport in the *tt4* mutant can be reversed by chemical complementation with naringenin, which is a downstream pathway intermediate [55]. Flavonols also regulate auxin distribution by redirecting PIN-mediated auxin efflux during root gravitropic response [58,59], to facilitate asymmetries in auxin at the root tip needed for root gravitropism. A recent report suggests that the flavonol quercetin competes with the key auxin efflux inhibitor *N*-1-naphthylphthalamic (NPA) acid by directly interacting with PIN protein [60].

Additional evidence suggests that flavonols modulate auxin transport mediated by ABCB transporters [61]. When ABCB proteins were expressed in a heterologous system, transport of auxin across the membrane was reduced by quercetin [62–64]. Additionally, the effect of *tt4* on root gravitropism is lost in an *abcb4* double mutant, consistent with flavonoids targeting ABCB proteins *in vivo* [65]. Quercetin, and to a lesser extent kaempferol, disrupts the protein complex between ABCB1 and TWISTED DWARF1 (TWD1), an immunophilin that regulates ABCB1 transport activity [66], suggesting that flavonols may inhibit auxin transport by altering protein complexes needed for maximal auxin transport. The resulting differences in auxin transport in these flavonol-deficient mutants have been tied to changes in growth and development, as described below.

Flavonols regulate root development

The role of flavonols in controlling root development has been demonstrated using genetic approaches. Arabidopsis has been utilized in a majority of these studies due to the genetic simplicity of its flavonol biosynthetic pathway, where single genes encoding pathway enzymes has allowed identification of mutant phenotypes in single mutant lines [67]. Developmental roles of flavonols are best characterized using *tt4* mutants with defects in the gene encoding the first enzyme in the flavonol pathway, chalcone synthase (CHS) [68]. Multiple *tt4* alleles have been examined with reported root phenotypes that include increased numbers of root hairs [69] and lateral roots [40,55,70], as well as impaired root gravitropism [16,24,56]. However, not all of these mutant alleles are equivalently well characterized, with the genetic and biochemical activities of the point mutant alleles in the Ler background being less well characterized than the Col-0 alleles [68], so this review will focus on insight from the Col-0 alleles. The root developmental phenotypes in *tt4* are similar in *fls* mutants, which catalyze the final step in flavonol synthesis, consistent with reduced flavonol levels controlling mutant phenotypes [40,69]. Additional studies have examined the developmental function of specific flavonols by using mutants that are impaired in branch point enzymes, revealing distinct functions of different flavonols in aspects of root development [16,40,69].

In roots, flavonols modulate development via antioxidant activity [71] or as modulators of auxin transport [53].

In Arabidopsis, flavonols are negative regulators of root hair formation [69]. In both *tt4* and *fls* mutants, there are significant increases in the number of root hairs that form from trichoblast (root hair forming) cells near the root tip [69]. The effect of the *tt4* mutation can be reversed by genetic complementation with a *CHS-GFP* transgene and chemical complementation with naringenin, a metabolite downstream of CHS in the flavonol pathway, as shown in Figure 2. An examination of the root hair phenotype of mutants in genes encoding branchpoint enzymes revealed that quercetin is the active flavonol controlling this process, as the *tt7* mutant, defective in *F3'H*, the enzyme that produces dihydroquercetin, has an identical phenotype to *tt4* (Figure 2). LC-MS was used to demonstrate that this mutant has elevated kaempferol and no detectable quercetin [69].

In root hairs, flavonols function by controlling the levels of reactive oxygen species. Two *tt4* alleles have elevated ROS in root hair forming cells, as revealed with a generic ROS sensor and with a hydrogen peroxide selective reporter [69]. ROS levels using these reporters are returned to wild-type levels upon chemical complementation with naringenin [69]. Flavonol synthesis and accumulation is highest in root cortical cells, which are adjacent to the epidermal tissues from which root hairs form, as demonstrated by staining with the flavonol-specific dye, diphenylboric acid 2-aminoethyl ester (DPBA), and localization of *FLS-GFP* and *CHS-GFP* reporters. Cortical cells have low levels of ROS, while root hair forming epidermal cells have high levels of ROS and lower levels of flavonols. Together these results suggest a model in which flavonols modulate root hair formation by controlling ROS in Arabidopsis root hairs [69]. Similarly, in the *are* mutant of tomato, which has a defect in the gene encoding F3H and reduced flavonol levels, there is an increased number of root hairs with elevated ROS in these root hairs, suggesting that this flavonol function may be conserved across species [72].

Lateral root formation is another process that is negatively regulated by flavonols, however studies have demonstrated that kaempferol controls this process, which differs from the active flavonol controlling root hair formation. Flavonols accumulate in lateral root primordia, as judged by DPBA staining and by visualization of GFP fusions to either CHS or FLS [40]. Mutants in either *tt4* or *fls* have increased numbers of lateral roots [40,55,70] when plants are grown under sucrose conditions that enhance flux through the flavonoid pathway [70]. This phenotype is also reversible by genetic and chemical complementation [40]. In contrast to increased lateral root formation in *tt4*, the *tt7* mutant has a reduced number of emerged lateral roots. Since the combined number of lateral root primordia and emerged lateral roots are similar in *tt7*, this phenotype is due to a defect in lateral root emergence [67]. Using LC-MS, the levels of kaempferol are elevated by ~2-fold in roots of *tt7*, with kaempferol accumulation increased within the lateral root primordia, as detected with DPBA [40]. This suggests that kaempferol, rather than quercetin, is the active flavonol regulating the emergence of lateral roots. Examination of ROS accumulation in these mutants, revealed reduced superoxide accumulation in the lateral root primordia of *tt7* consistent with a role of flavonols in regulating ROS to modulate lateral root formation [40]. These findings, combined with those described above on root hair initiation,

provide evidence for the functional specificity of flavonol intermediates controlling different developmental responses.

Earlier experiments examined whether these lateral root phenotypes in flavonol deficient mutants were tied to altered auxin transport. Polar auxin transport from the shoot toward the root tip (rootward transport, which was formerly called acropetal transport) is required for lateral root formation as mutations or auxin transport inhibitor treatments that block this polarity of auxin transport impaired lateral root formation [73]. Rootward auxin transport in *tt4* is elevated consistent with the absence of a negative regulator of transport [56].

Several studies have implicated flavonols in controlling root gravitropism via modulation of auxin transport, which is required for gravitropism. Consistent with flavonols negatively regulating auxin transport, the *tt4* mutant has increased auxin transport in roots using radioactive auxin transport assays, which measured shootward transport (formerly called basipetal transport) [16,56], which is the polarity of auxin transport that controls root gravitropism [74]. The *tt4* mutant fails to establish an asymmetric auxin gradient at the root tip which is needed for root gravitropic curvature [16,56]. Like root hair formation, the *tt4* and *tt7* mutants have similarly impaired gravitropic responses, consistent with quercetin regulating both responses [16]. As this flavonol-regulated auxin movement occurs through epidermal cells [16], which also give rise to root hairs, these findings suggest two quercetin functions in this cell type. It is important to note the possibility that these functions are linked, as multiple studies have shown that auxin transport is regulated by reactive oxygen and reactive nitrogen species [75,76] suggesting that auxin transport regulation by flavonols may be tied to their antioxidant activity.

Flavonol are involved in above-ground vegetative development and in gas exchange

In addition to their role in root architecture, flavonols have profound effects on leaf development and leaf physiology, including gas exchange through stomata. The absence of flavonols in the *tt4-1* (*CHS*) mutant contributes to a smaller leaf area and slower inflorescence growth rate [77]. On the leaf surface, several striking phenotypes have been tied to altered flavonol biosynthesis, including changes in the morphology of pavement cells and trichomes and the aperture of stomata. Rhamnosylated flavonols were shown to regulate the shape of pavement cells in Arabidopsis, with the flavonol rhamnosyl-deficient *rol1* mutant having brick-shaped instead of jigsaw-shaped pavement cells [78]. This mutant also displayed deformed trichomes [78]. While the flavonol biosynthetic mutant *af* in tomato, which is deficient in *CHI1*, had normally formed trichomes, it showed a reduced density of glandular trichomes [79]. These trichomes failed to produce the high levels of flavonols found in the wildtype trichomes and had elevated levels of ROS [80]. Additionally, trichomes of the *af* mutant accumulated reduced levels of terpenoids, which are plant specialized metabolites that mediate the defense against herbivores in leaves. The reduction in terpenoid synthesis was attributed to elevated ROS levels [80]. Due to the lower terpenoid pools, the *af* mutant was more susceptible to diverse herbivores [79].

The stomatal pores, which are found on the leaf surface, regulate entry of CO_2 for photosynthesis and exit of water during transpiration. Flavonols accumulate within the two guard cells, which flank the stomata, as demonstrated by localization with the flavonol dye DPBA in Arabidopsis, tomato, and apple [23,81,82]. Flavonols accumulate in both the cytosol and nuclei of guard cells [23,82], at substantially higher levels than the surrounding pavement cells, as shown in Figure 3. Flavonol accumulation appears to be the result of flavonol synthesis in guard cells as *CHS-GUS* and *FLS-GFP* reporters are expressed within this cell type [23,83].

Stomatal closure is highly regulated by light levels and the hormone ABA, which signals reduced water availability [84]. In response to elevated ABA, localized ROS accumulation in guard cells drives stomatal closure [85,86]. Consistent with the role of flavonols as antioxidants, these molecules function in guard cells to regulate stomatal aperture through the scavenging of ROS [43]. The Arabidopsis *CHS* mutant allele, *tt4-2*, and the tomato *F3H* mutant *are* (*anthocyanin reduced*), both have reduced flavonol levels and accumulate higher levels of ROS in guard cells and display an enhanced rate of stomatal closure [23,82]. In contrast, a flavonol overproducing tomato mutant, *aw* (with a defect in the gene encoding DFR), has a dampened response to ABA-dependent stomatal closure. Elevated flavonol levels appear to confer drought tolerance in Arabidopsis, as shown by a transgenic line overexpressing the transcription factors MYB12 and PAP1 that drive flavonol synthesis [35]. This improved drought response was also seen in a maize mutant containing elevated levels of flavonols [87].

Flavonol synthesis is upregulated in guard cells in several species in response to hormones such as ethylene [23], the growth promoting molecule 5-Aminolevulinic acid (ALA) [88], and the drought hormone abscisic acid (ABA) [89]. Both ethylene and ALA were found to increase flavonol levels, as judged by DPBA staining, and led to more open stomata consistent with flavonols reducing ROS levels. In contrast, an untargeted metabolomic study in guard cell protoplasts from *Brassica napus* found that ABA increased flavonol metabolites under conditions where ABA induced stomatal closure [89]. Since ABA-induced stomatal closure requires an initial ROS burst [90,91], it would be interesting to evaluate the kinetics of flavonol upregulation in the same species in response to these hormones as ABA might trigger a delayed synthesis of flavonols following the elevated ROS burst after ABA treatment as a protective mechanism to prevent ROS from reaching damaging levels.

Flavonols enhance reproduction

Flavonols are known to play an important role in plant sexual reproduction in multiple species [92]. Successful sexual reproduction requires the development of viable pollen, the male gametophyte, within the locules of anthers. These locules are lined by the tapetum, a cell layer that contributes nutrients and cell wall materials to the developing pollen. During pollination, mature pollen is released from anthers and lands on a stigma where it germinates a pollen tube that navigates the stigma and style to fertilize ovules [93]. Evidence for the role of flavonols throughout the developmental sequence of plant sexual reproduction has come from studies with mutants in multiple species that have defects in pollen development or tube growth. These species include tomato, petunia, tobacco, maize,

rice and apple [18,94]. Mutations in *CHS* block flavonol synthesis, which has enabled researchers to uncover the role of flavonols in reproduction. For example, the white pollen in a flavonol deficient maize mutant is unable to pollinate [95]. In petunia, the white anther (*wa*) *CHS*-mutant produces pollen that is unable to germinate. The sterility in this mutant is rescued by exogenous addition of kaempferol and genetic complementation with a *CHS* transgene [96]. *In vitro* germination assays showed that addition of kaempferol to flavonol deficient petunia pollen rescues tube growth and germination [97]. The essential role of flavonols in pollen tube formation is further highlighted in rice, as evidenced in the *CHS* mutant line that shows lower pollen germination rate and an inability to produce functional pollen tube, while maintaining normal pollen morphology [98]. RNAi silencing of the *CHS* gene in tomato results in the production of fruits with complete absence of seeds [99]. Similarly, RNAi silencing of *FLS* in tobacco results in reduced pollen germination and pollen tube elongation [100]. However, the role of flavonols in reproduction are not limited to pollen-specific accumulation, as flavonols localized to the pistil have been shown to aid in pollen tube growth and seed set in apples [94]. Intriguingly, there is one species in which flavonols have not been found to control reproduction; Arabidopsis *tt* mutants do not exhibit reproductive defects [101]. However, a putative flavonol transport Arabidopsis mutant showed reduced pollen viability and seed set [102].

Insight into how flavonols aid in pollen development was revealed through examination of the flavonol-deficient tomato mutant *are*, which produces fewer viable pollen and has reduced pollen tube germination, elongation, and higher rates of pollen tube rupture [18], as shown in Figure 4. The reduced pollen tube growth and the pollen tube integrity defects of this mutant were rescued with genetic complementation with an *F3H* transgene [18]. The levels of reactive oxygen species (ROS) in this mutant were elevated, consistent with flavonols acting as antioxidants in pollen [18]. Addition of ascorbic acid and the ROS synthesis inhibitor diphenylene iodinium both reduced ROS and increased pollen tube growth [18], consistent with a role of flavonols in promoting pollen viability by scavenging excess ROS.

ROS can increase to damaging levels during diverse abiotic stresses, including heat stress [41], and flavonols may function to prevent this damage [18]. The pronounced reduction in pollen viability and pollen tube growth was linked to overaccumulation of ROS during heat stress [18]. Metabolomic analysis of tomato pollen at different developmental stages revealed that microspores accumulate high levels of flavonols after acute heat stress of 38°C [103], suggesting that flavonol increases may reduce the negative effects of heat stress on pollen viability. Additionally, transcriptomic analysis of a thermotolerant and thermosensitive rice variety during meiosis showed higher expression of flavonol biosynthetic genes in the thermotolerant variety in response to heat stress [104].

Despite the importance of flavonols for pollen development and tube growth, it is not clear yet whether flavonols are produced within pollen or whether these molecules move into pollen from surrounding tissues. Indeed, during pollen development of Brassica, flavonols first accumulate in the tapetum and are then deposited on the pollen coat after tapetal programmed cell death [105,106]. In tomato, DPBA staining has shown that the flavonols accumulate both within pollen grains and on the surface of grains [18]. Transcriptomic

studies of developing pollen have shown that the abundance of transcripts encoding some flavonol biosynthetic genes is relatively low [107] which aligns with the observation that at least part of the flavonols accumulating in mature pollen are likely derived from the tapetum [105,106].

Conclusions

Recent evidence has revealed critical functions for flavonols in the regulation of plant growth and development in a variety of tissues across many species. Genetic and chemical approaches have been combined to yield insight into the localization of the synthesis and accumulation of products of the flavonol biosynthetic pathway, providing insight into controls of their synthesis and their functions in specific tissues. Mechanistically, flavonols can influence growth and development through inhibition of auxin transport, regulating distribution of this hormone to modulate development. Additionally, flavonols can act as scavengers of reactive oxygen species to maintain homeostasis to ensure productive signaling while preventing excess levels from accumulating, resulting in cellular damage. The antioxidant capacity of flavonols provides a protective role in plant responses to environmental stresses, including elevated temperature and drought, which are increasing in prevalence as our climate changes. Altogether this evidence provides important insight into flavonol-dependent regulation of plant growth and development, as well as a potential avenue by which to enhance plant tolerance to detrimental environmental stress.

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Abbreviations

CHS	chalcone synthase
CHI	chalcone isomerase
FNS	flavone synthase
F3H	flavanone 3-hydroxylase
IFS	isoflavone synthase
DFR	dihydroflavonol 4-reductase
LAR	leucoanthocyanidin reductase
ANS/LDOX	anthocyanidin synthase/leucoanthocyanidin dioxygenase
OMT	O-methyltransferase
RT	rhamnosyl transferase

UFGT	UDP-glucose flavonoid 3-O-glucotransferase
F3′5′H	flavonoid 3′5′-hydroxylase
F3′H	flavonoid 3′-hydroxylase
FLS	flavonol synthase
OMT-1	O-methyltransferase-1
tt	*transparent testa*
ROS	reactive oxygen species
LAX	like AUX1
PIN	Pin formed
ABCB	ATP Binding Cassette B
NPA	*N*-1-naphthylphthalamic acid
TWD1	TWISTED DWARF1
DPBA	diphenylboric acid 2-aminoethyl ester
are	*anthocyanin reduced*
rol1	*repressor of Irx1*
af	*anthocyanin free*
aw	*anthocyanin without*
ALA	5-Aminolevulinic acid
ABA	abscisic acid
wa	white anther
DCF	dichlorofluorescein
H_2O_2	hydrogen peroxide
PO1	peroxy orange 1
FDA	fluorescein diacetate
PI	propidium iodide
AC	Ailsa Craig
nr	*neverripe*

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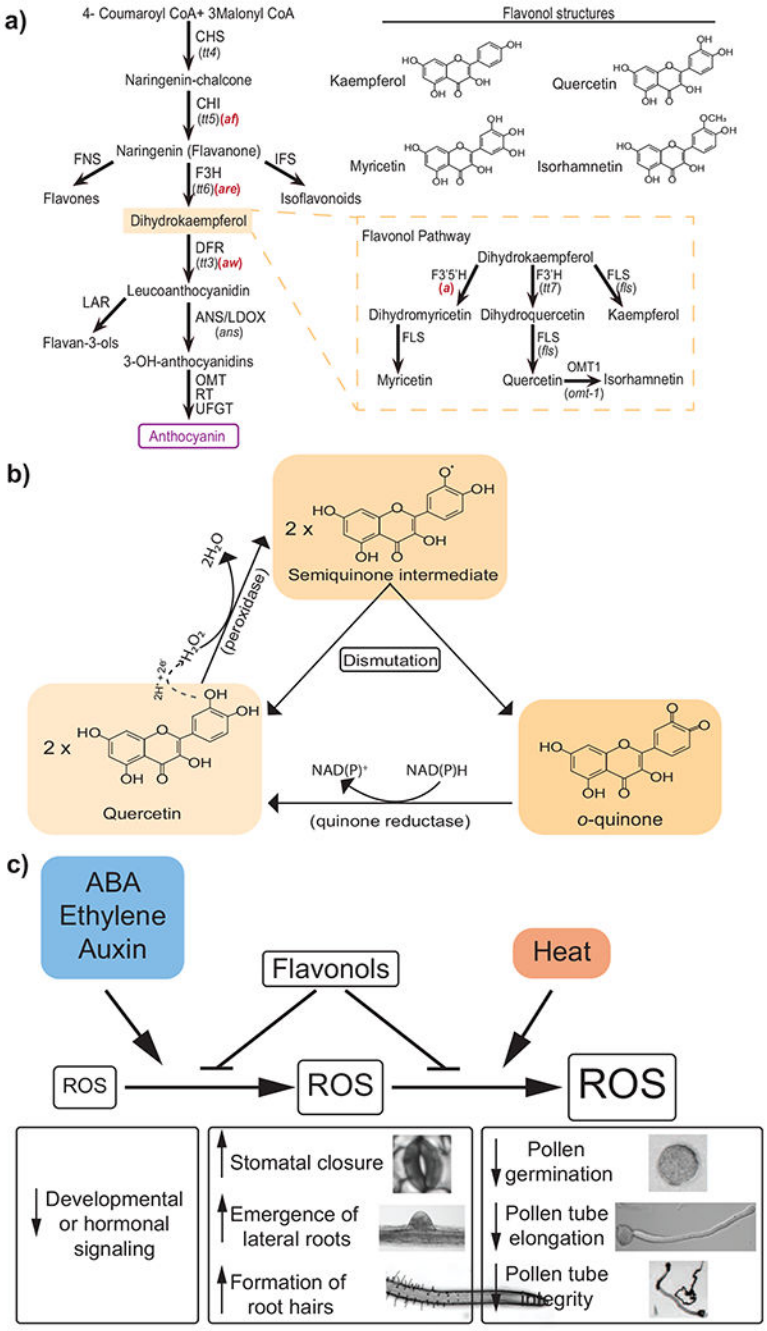

Figure 1. The flavonoid biosynthetic pathway.

Enzymes are indicated next to arrows with mutant names in parentheses under the enzyme abbreviation. Arabidopsis mutants are in black and tomato mutants are in red. The flavonol biosynthesis branch is illustrated in the yellow dashed box. Chemical structure of specific flavonols are presented on the upper right. Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; IFS, isoflavone synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; ANS/LDOX, anthocyanidin synthase/leucoanthocyanidin dioxygenase; RT, rhamnosyl transferase;

UFGT, UDP-glucose flavonoid 3-O-glucotransferase; F3’5’H, flavonoid 3’5’-hydroxylase; F3’H, flavonoid 3’-hydroxylase; FLS, flavonol synthase; OMT-1, O-methyltransferase-1. Figure adapted from [64 & 66], B. The predicted mechanism by which the flavonol quercetin donates electrons to convert hydrogen peroxide to water is shown, along with a potential mechanism by which reduced flavonols are regenerated. The transfer of electrons and protons to hydrogen peroxide are indicated above the dashed arrow. Types of enzymes that may catalyze these chemical reactions are presented in parentheses. It is also possible that the quinone reductase may convert the quinone back to a semiquinone. C. This diagram summarizes environmental or hormonal factors that increase levels of reactive oxygen species (ROS) and the role of flavonols as modulators of ROS to control developmental and physiological responses. Factors affecting ROS levels are indicated in colored boxes and the resulting changes in ROS concentration are depicted by increased size of boxes. Hormonal and environment increases in ROS levels are indicated by arrows and the ability of flavonols to reduce ROS accumulation is indicated by a blunt-end arrow. Observed developmental or physiological changes linked to the different ROS levels are indicated below the ROS changes. Abbreviation: ABA, abscisic acid.

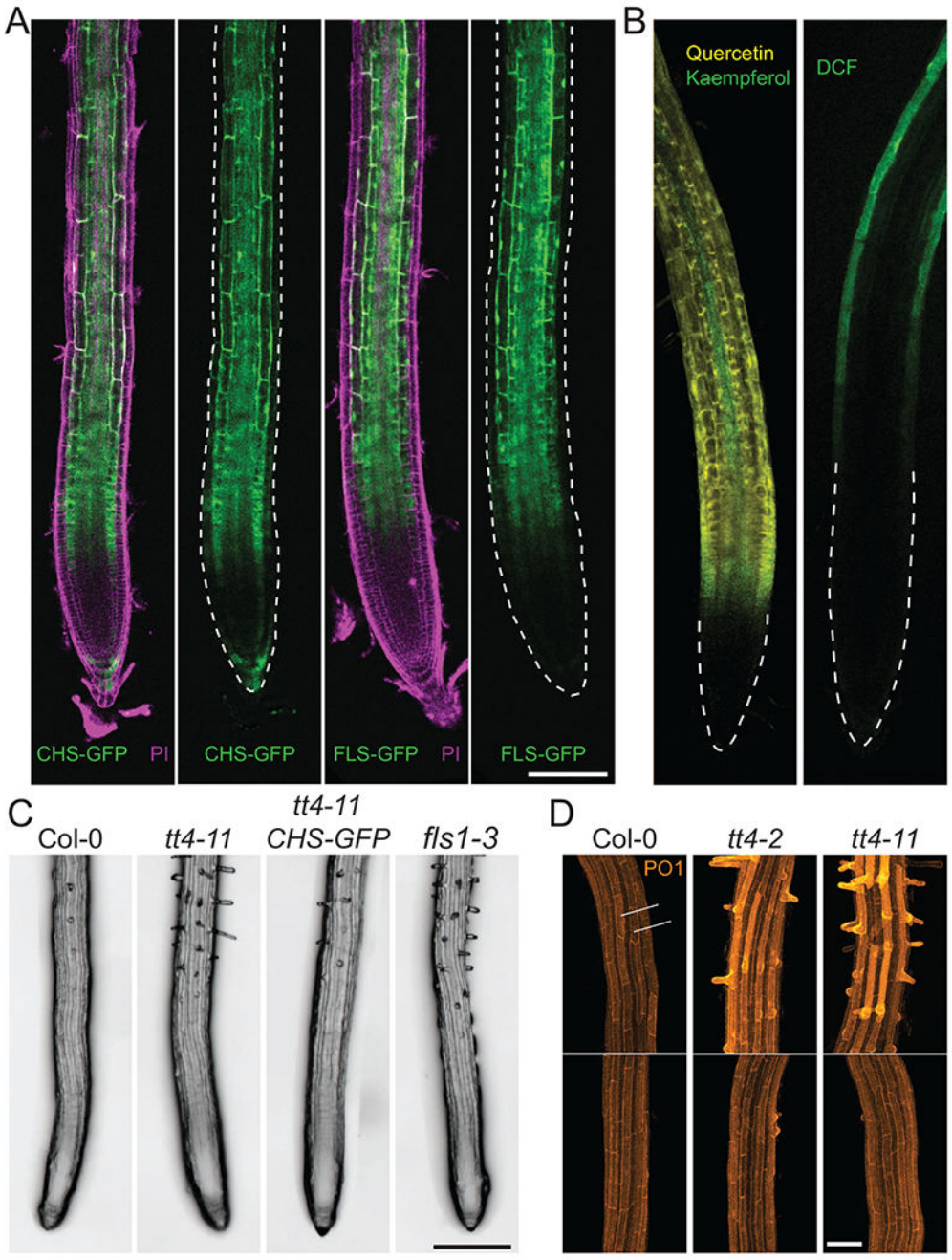

Figure 2. The flavonol biosynthetic machinery is localized to the root epidermis to regulate root hair initiation.

(a) Fluorescence of transgenic Arabidopsis lines containing CHS-GFP and FLS1-GFP reporters (green) stained with the cell wall probe propidium iodide (PI) (magenta) show that flavonol biosynthetic enzymes localize to the root epidermis. Images without PI channel have epidermal tissues outlined by dashed lines. Scale bar =100 μm (b) LSCM images of WT Arabidopsis roots treated with either the flavonol-selective probe, DPBA, with yellow fluorescence of quercetin and green fluorescence of kaempferol (left image), or the fluorescence of the general ROS sensor, dichlorofluorescein (DCF) (green, right image),

reveals ROS accumulate in higher levels in root epidermal tissues where flavonols are less abundant. (c) Representative images of root hair number in WT Arabidopsis and mutants with impaired flavonol accumulation, *tt4-ll and fls1-3*, and *tt4-11* complemented with a *CHS-GFP transgene* (*ttd-11 CHS-GFP*) show increased root hair numbers in mutants with defects in flavonol synthesis. Scale bar = 200 μm (d) Confocal images of WT, *tt4-2,* and *tt4-11* Arabidopsis lines stained with the H_2O_2 probe, Peroxy orange 1, display elevated H_2O_2 accumulation in root hair forming cells (denoted as 1, 3, and 5) relative to nonhair cells (2 and 4). H_2O_2 accumulation is increased in *tt4-2* and *tt4-11,* though this phenotype can be restored to WT levels through treatment with the flavonol precursor, naringenin. Scale bar = 50 μm. Adapted from Ref. [64].

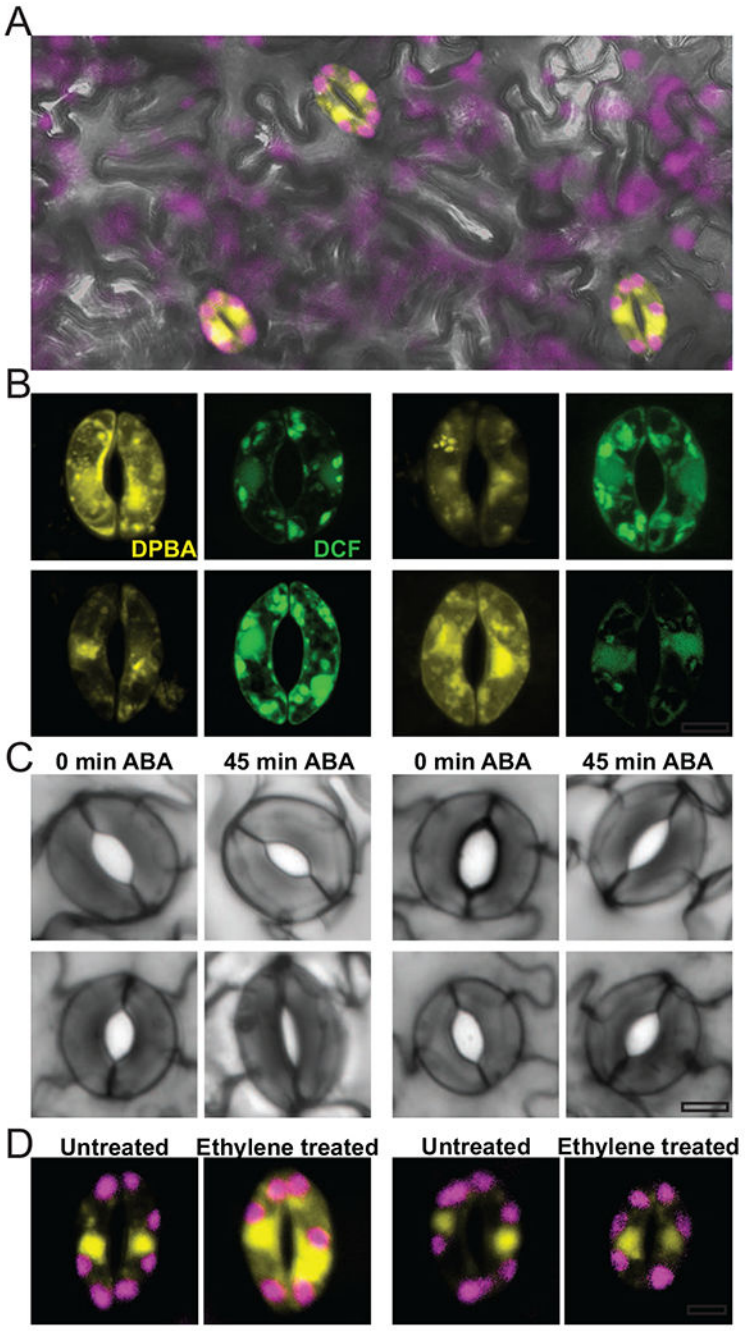

Figure 3. Flavonols accumulate within guard cells to influence stomatal aperture.

(a) Confocal micrograph of a VF36 tomato leaf stained with the flavonol-selective probe DPBA (yellow) reveals flavonol accumulation in guard cells. Chlorophyll autofluorescence is visualized in magenta, (b) Fluorescence of DPBA (yellow) or the general ROS sensor dichlorofluorescein (DCF) (green) in guard cells of the *are* and *aw* tomato mutants and their respective wild-type parental lines, VF36 and AC, reveals an inverse relationship between flavonol levels and general ROS accumulation, (c) Representative images of stomatal aperture of guard cells treated with ABA show that the *are* (*anthocyanin reduced*) mutant

with decreased flavonols and elevated ROS levels has enhanced rates of stomatal closure, while the anthocyanin without (aw) mutant with increased flavonols and decreased ROS accumulation has reduced rates of stomatal closure, (d) DPBA fluorescence is increased in guard cells following treatment with ethylene gas, while they remain unchanged in the ethylene insensitive *Neverripe* mutant. Scale bars = 5 μm. (Adapted from Ref. [78]. Copyright 2017 American Society of Plant Biologists.

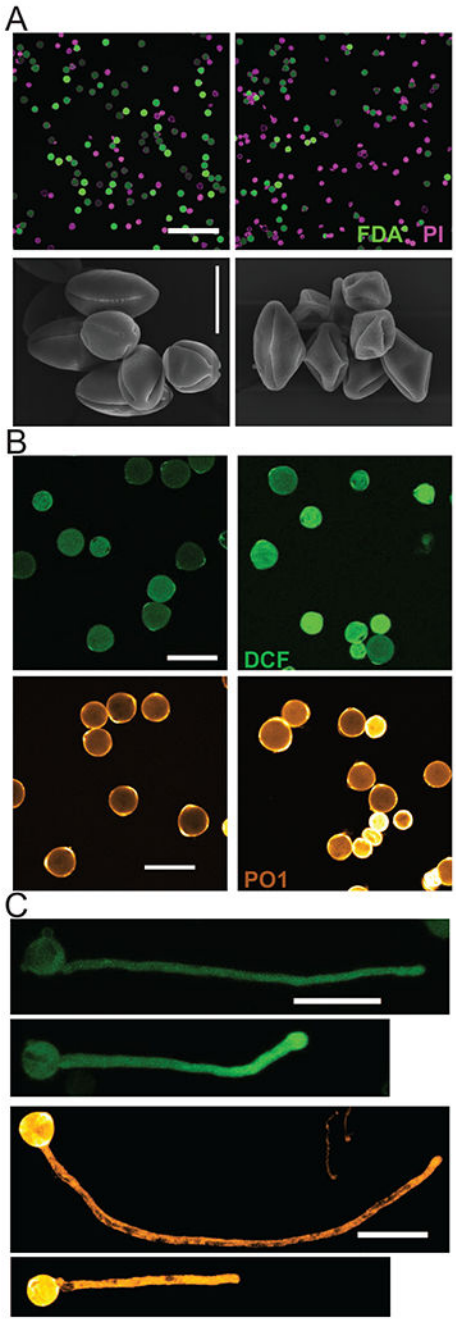

Figure 4. Flavonols promote pollen development and tube growth in tomato.

(a) Confocal images of VF36 and flavonol-deficient *are* mutant pollen grains stained with fluorescein diacetate (FDA, green; live grains) and propidium iodide (PI, magenta; dead grains) and scanning electron micrographs show that the *are* mutant produces less viable pollen. Scale bars = 200 μm for confocal images and 20 μm for electron micrographs, (b) The general ROS sensor, DCF, and the H_2O_2-selective chemical probe, Peroxy orange 1 (PO1) reveal increased ROS accumulation in pollen grains of the *are* mutant. Scale bar = 50 μm (c) Confocal micrographs display significantly impaired pollen tube growth as well as

increased levels of ROS in the *are* mutant. Scale bar = 50 μm (top) and 100 μm (bottom). Adapted from Ref. [18]. Copyright 2018 Proceedings of the National Academy of Sciences.