

Flavonoids in plant rhizospheres: secretion, fate and their effects on biological communication

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Abstract Flavonoids, one of the most-described group of plant “specialized metabolites”, consist of more than 10,000 structurally diverse compounds. Most flavonoids accumulate in plant vacuoles as glycosides, with some released by the roots into rhizospheres. These flavonoids are involved in biological communications with rhizobia, arbuscular mycorrhizal fungi, plant growth promoting rhizobacteria, pathogens, nematodes, and other plant species. Both aglycones and glycosides of flavonoids are found in root exudates and in soils. This review describes researches on the mechanisms of flavonoid secretion and the fate of flavonoids released into rhizospheres. This review also discusses the direction of future research that may elucidate the specific roles of flavonoids in biological communications in rhizospheres, enabling the utilization of flavonoid activities and functions in agricultural practice.

Key words: Biological communication, flavonoid, rhizosphere, root exudates.

Introduction

Plants synthesize a wide range of low molecular weight compounds, which are active in plant defenses against biotic and abiotic stresses and also act as attractants or repellents of other organisms. These compounds have recently been described as “specialized” rather than “secondary” metabolites, and their number has been estimated at more than 200,000 (Dixon and Strack 2003). In contrast to the primary metabolites, which are synthesized by almost all organisms, each specialized metabolite is usually synthesized by a few plant families, or even by a few species, suggesting that, during evolution, these plants acquired the ability to synthesize new specialized metabolites, which conferred adaptive advantages to these plants (Pichersky and Lewinsohn 2011).

Flavonoids are a well-described group of plant specialized metabolites, consisting of more than 10,000 different compounds (Ferrer et al. 2008). Flavonoids are a group of phenylpropanoid compounds synthesized from 4-coumaroyl-CoA and malonyl-CoA, with the flavonoid skeleton often undergoing various types of chemical modification, such as glycosylation, hydroxylation, acylation, methylation, malonylation, and prenylation, which result in diverse structures and functions. Flavonoids are classified into several groups

based on their structures and the functional groups on the C-ring, including the chalcones, flavanones, flavones, flavonols, anthocyanidins and catechins (Aoki et al. 2000) (Figure 1). Among them are the isoflavonoids, which occur mostly in legume plants and are biosynthesized from flavanones such as liquiritigenin and naringenin via 2-hydroxylation catalyzed by the cytochrome P450 enzyme isoflavone synthase (IFS).

Flavonoid functions in plants include the regulation of auxin transport, the modulation of reactive oxygen species (ROS), the coloring of flowers, and in protection against UV light (Falcone Ferreyra et al. 2012). Flavonoids are also important compounds for human life, functioning as phytoestrogens, anti-oxidants, anti-inflammatory and anti-carcinogenic compounds and in cancer prevention (Garcia-Lafuente et al. 2009; Miadokova 2009).

Due to their importance in biological processes in planta as well as in enhancing human quality of life, flavonoids have been intensively studied in various fields. This review focuses on flavonoids in rhizospheres, which are small regions around the roots of plants (Hartmann et al. 2008), where flavonoids play important roles in biological communications. Because there have been several recent reviews on flavonoids in rhizospheres (Cesco et al. 2012; Cheynier et al. 2013;

Abbreviations: ABC, ATP-binding cassette; AMF, arbuscular mycorrhiza fungi; CHS, chalcone synthase; IFR, isoflavone reductase; IFS, isoflavone synthase; MATE, multidrug and toxic compound extrusion; PAL, phenylalanine ammonia-lyase; PGPR, plant growth promoting rhizobacteria.

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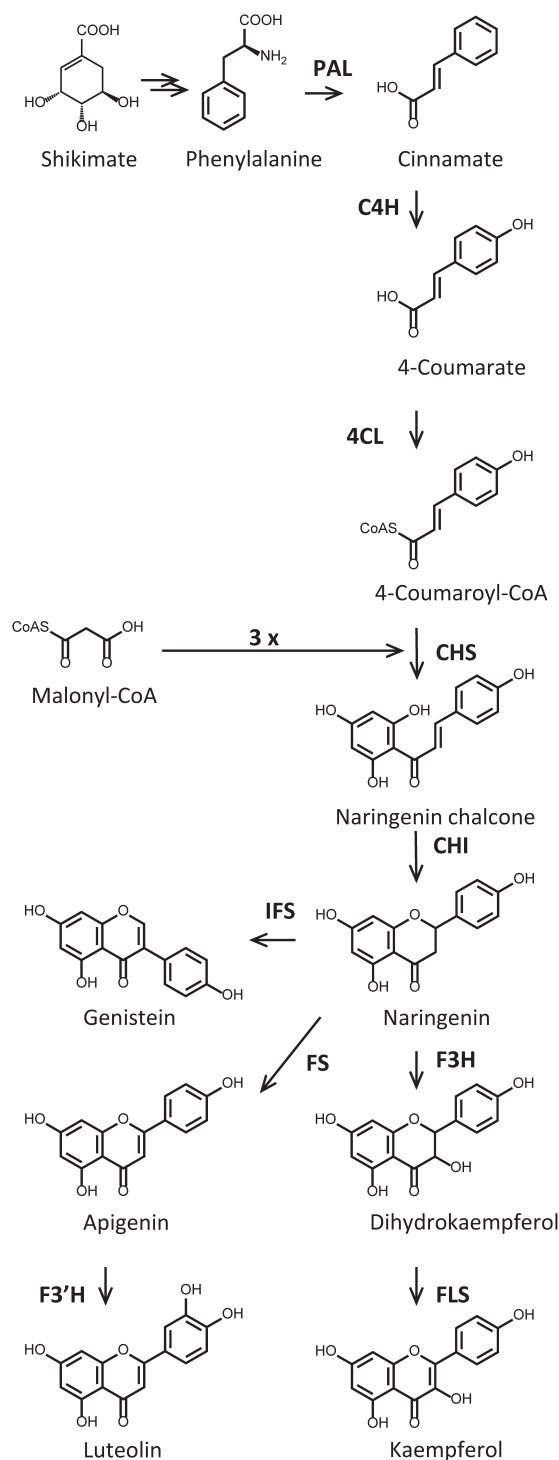


Figure 1. Biosynthetic pathway for flavonoids. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; IFS, isoflavone synthase; FS, flavone synthase; F3'H, flavonoid 3'-hydroxylase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase.

Hassan and Mathesius 2012; Weston and Mathesius 2013), this review does not include all relevant studies, but concentrates on the potential of flavonoids to act as plant-mediated signaling compounds for sustainable agriculture.

Secretion and fate of flavonoids in rhizospheres

Secretion of flavonoids from plant roots

Flavonoids are synthesized by coupling of the phenylpropanoid and polyketide pathways, with 4-coumaroyl-CoA and malonyl-CoA, respectively, being the direct precursors (Figure 1). In the phenylpropanoid pathway, phenylalanine and tyrosine are metabolized to form flavonoid skeletons as well as lignins and lignans. Genes involved in this pathway leading to the core structures of flavonoids have been characterized in detail in various plants (Saito et al. 2013). These genes have been used for metabolic engineering in various plant species as well as in microorganisms (for review see Suzuki et al. in press). Flavonoid diversity is largely due to the modification of the core structure by enzymes such as glycosyltransferases, prenyltransferases, methyltransferases and acyltransferases. These modifications confer biological activities on the resultant compounds, as well as being important for their transport and accumulation in plant cells. In general, flavonoids exist as glycosides in vacuoles, with various transporters responsible for the vacuolar accumulation of flavonoid glycosides; these include, for example, members of the ATP-binding cassette (ABC) and multidrug and toxic compound extrusion (MATE) transporter families (Shitan and Yazaki 2013; Zhao and Dixon 2010), while such transporters have not been identified from soybean so far. The most common isoflavone in soybeans (*Glycine max*) is daidzein, an aglycone commonly glucosylated by GmIF7GT, UDP-glucose:isoflavone 7-O-glucosyltransferase (Noguchi et al. 2007), followed by malonylation by GmIF7MaT, malonyl-CoA:isoflavone 7-O-glucoside-6''-O-malonyltransferase (Suzuki et al. 2007). These glucosides and malonylglucosides show high accumulation in vacuoles (Barz and Welle 1992).

Flavonoid aglycones and glycosides can be found in root exudates. The concentrations of flavonoids vary widely in plants grown axenically in mineral nutrient solution, depending on plant species, plant growth conditions and sampling techniques ((Cesco et al. 2010), our unpublished results). In *Arabidopsis*, root exudate contents and concentrations were shown to correlate with the metabolic activities of bacterial communities in rhizospheres (Chaparro et al. 2013, 2014).

There are two major mechanisms for flavonoid secretion from roots: 1) passive processes, such as root turnover, root injury and root decomposition from root cap and border cells, and 2) active processes; i.e., root exudation (Shaw et al. 2006)(Figure 2). Using plasma membrane vesicles prepared from soybean roots, these roots were shown to possess an ATP-dependent transporter that functions in the secretion

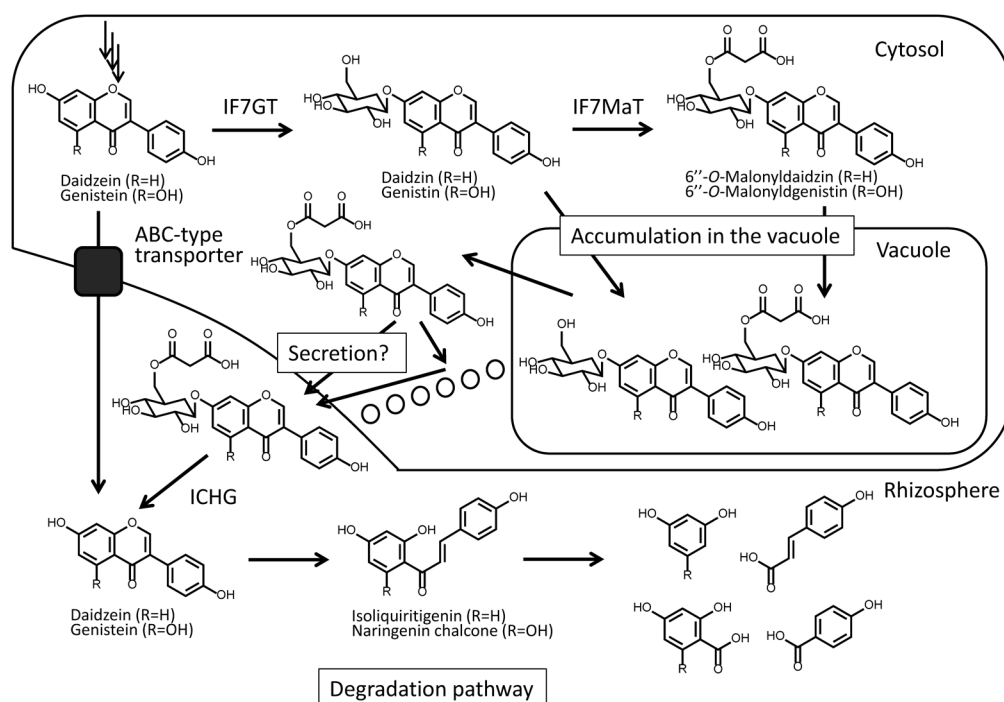


Figure 2. Biosynthesis, accumulation and secretion of isoflavones in soybean roots. Soybeans biosynthesize isoflavones, primarily daidzein and genistein, both of which are glucosylated and subsequently malonylated in the cytosol. The resulting glucosides and malonylglucosides accumulate in the vacuoles. The possible degradation products in the rhizosphere are also shown.

of genistein into the rhizosphere (Sugiyama et al. 2007). Biochemical transport assays using various inhibitors have suggested that an ABC transporter is the primary candidate for this process. This ABC-type transporter was shown to be specific for isoflavonoid aglycones, whereas similar biochemical transport assays using isoflavonoid glucosides such as daidzin and genistin as substrates did not show ATP-dependent transport into plasma membrane vesicles (our unpublished results). These results suggest that soybean roots have different mechanisms for the secretion of aglycones and glucosides (Figure 2). For example, although daidzein and genistein are synthesized in the cytosol, their glucosides, such as daidzin, genistin, malonyldaidzin and malonylgenistin, accumulate in vacuoles. The mechanisms by which metabolites that accumulate in the vacuoles are secreted into apoplasts remain to be determined; one possible mechanism could be vesicle-mediated secretion as illustrated in Figure 2. Several specialized metabolites of plants, such as nicotine, accumulate in vacuoles and are secreted into apoplasts. Characterization of these secretion mechanisms is of particular importance in future studies.

Apoplastic β-glucosidase has been identified in soybean roots, and an isoflavone conjugate-hydrolyzing β-glucosidase, GmICHG, indicated in Figure 2, has been purified from roots of soybean seedlings (Suzuki et al. 2006). Immunocytochemical experiments using antibodies against this protein showed that GmICHG

localized in the apoplasts is present in the epidermis, endodermis, and stele tissues of the root, as well as in root hairs. Isoflavonoid aglycones are a more active form, mediating legume-rhizobial interactions as well as in defenses against pathogens in rhizospheres. Apoplastic β-glucosidase may help plants rapidly secrete large quantities of the active forms of flavonoids, stored in vacuoles, into rhizospheres.

Flavonoids are also secreted by non-leguminous plants into rhizospheres. For example, at least 54 flavonoid compounds have been identified in *Arabidopsis* (Saito et al. 2013), with flavanone detected among the secreted phenolic compounds, such as coumarin, syringic acid and vanillic acid, in root exudates (Toussaint et al. 2012). An analysis of the effects of various mutations in ABC transporter genes of *Arabidopsis* on the composition of root exudates and soil microbial communities showed that root exudates from the *abcg30/pdr2* mutant contained more phenolic compounds and fewer sugars than the exudates of wild type plants (Badri et al. 2009). Because this mutation altered the expression of many genes, the differences in root exudate composition may not have been due to the direct effects of mutation in this ABC transporter gene but to the pleiotropic effects of the gene mutation. Although candidate genes involved in the secretion of flavonoids and phenolics have been identified, transporters responsible for flavonoid exudation into the rhizosphere have not yet been determined.

Fate of flavonoids in the rhizosphere

In natural soils, flavonoids are released from plant roots, with manure application potentially increasing flavonoid contents in agricultural soils. The flavonoid composition of soil was quantitatively investigated in grassland fields in the presence and absence of applied manure (Hoerger et al. 2011b). The amounts of formononetin, daidzein, biochanin A and genistein were higher in topsoil (0–10 cm) than in subsoil (10–20 cm), but flavonoid contents were not significantly increased by the application of manure. Moreover, manure application under conditions of good agricultural practice did not result in additional excretion of flavonoids into drainage water.

Legume plants are widely used as green manure. Formononetin, medicarpin and kaempferol have been detected in soils during the growth of white clover (*Trifolium repens*), presumably due to root exudation (Carlsen et al. 2012). High concentrations of the glycosides of kaempferol and quercetin were observed within one day of application of white clover to soil as green manure. However, after 16 days, most of the flavonoids were aglycones, with substantial amounts of kaempferol persisting in the soil while other flavonoids were degraded.

Because flavonoids can be aerobically metabolized by bacteria such as rhizobia and *Pseudomonas*, the measurement of the actual concentration of flavonoids in soil is technically challenging. Chemical properties of soil also affect the fate of flavonoids in soil, e.g., the concentration of catechin was about 4-fold higher in alluvial soil at pH 7 than in volcanic ash at pH 4 (Furubayashi et al. 2007). Minerals in soil such as Fe and Cu were shown to promote the degradation of catechin, while Ca was shown to protect flavonoids from degradation (Pollock et al. 2009), suggesting that mineral composition and contents in soil are important in determining the fate of flavonoids. Soil microbes, as a predominant factor, strongly affect the fate of flavonoids in soil. A study of the half-lives of various flavonoids in sterile and non-sterile soils showed that, in the latter, naringenin and formononetin were degraded within 4 and 24 h, respectively, with formononetin having a longer lag time in non-sterile soils (Shaw and Hooker 2008). Two major isoflavonoids from soybean, daidzein and genistein, had short half-lives in soil, of 37.2 and 6.0 min, respectively (Guo et al. 2011). These extremely short half-lives may have been due to the experimental design, in which isoflavonoids were added to soils collected from fields in which soybeans were continuously grown, with these soils likely containing active isoflavone-catabolizing bacteria.

In general, flavonoid glucosides are degraded more rapidly in non-sterile than in sterile soils. For example, the half-lives of flavonoid glycosides produced by *Alliaria petiolata* were shorter under non-sterile (3 to 12 h) than

sterile (12 to 46 h) conditions. Moreover, these flavonoid glycosides were not detected in bulk soils, confirming their instability in soil (Barto and Cipollini 2009). The half-lives of flavonoid glucosides may be short due to their rapid hydrolysis by glycosidase enzymes from both plants and microbes (Hartwig and Phillips 1991). One exception was formononetin-7-O-glycoside, which was detected in the rhizosphere soil of alfalfa (*Medicago sativa*), suggesting that this flavonoid glycoside was secreted from roots in large quantities or was unexpectedly stable in rhizosphere soil (Leon-Barrios et al. 1993).

Adding to the difference between sterile and non-sterile conditions, rhizosphere microbial communities whose composition depends on the growth condition of plants (Chaparro et al. 2014; Mougél et al. 2006; Sugiyama et al. 2014) are also important in affecting the fate of flavonoids in the rhizosphere. The rate of flavonoid degradation by rhizosphere microbial communities may increase during plant growth because these rhizosphere soils are exposed to the continuous release of flavonoids from roots (Shaw et al. 2006). When microbial communities are exposed to high concentrations of flavonoids, flavonoid-degrading microbes will likely prevail around the roots. The addition to soils of *Arabidopsis* root exudates collected from liquid medium was found to modify the microbial composition of these soils, depending on the fractions of root exudates; i.e. phenolic compounds exhibited positive correlation with more operational taxonomic units than other fractions (Badri et al. 2013). Because the microbial community is one of the most important determinants of the fate of flavonoids in soils, the half-life of a particular flavonoid in soil may largely differ depending on the soil microbes.

Extraction methods are another key factor for the measurement of flavonoid concentrations in soils. Organic solvents such as methanol, ethanol, acetone and ethyl acetate have been widely used to extract flavonoids from soil. A systematic comparison of various solvents for the extraction of formononetin from soil, with quantification relative to ¹³C-labeled internal standards, found that a mixture of methanol and acetonitrile had the highest extraction efficiency, followed by methanol (Hoerger et al. 2011a). Moreover, extraction was increased at higher temperature but not at higher pressure. Due to differences in soils and extraction methods it is not feasible to compare reported flavonoid concentrations and fate; as summarized in a review, contradictory results have been obtained (Cesco et al. 2012).

Effect on biological communication

Interaction with rhizobia

Interactions between legume plants and rhizobia are of particular importance in agriculture and ecology. The molecular mechanisms of bacterial and plant

perception, signaling, organogenesis and nitrogen fixation have been intensively studied, with many components essential for these symbiotic interactions identified (Popp and Ott 2011; Wang et al. 2012; Werner 2007; Yokota and Hayashi 2011). Due to the chemotaxis of rhizobia to root exudates, these rhizobia can find their host legume plants in soils. The bacterial proteins responsible for the recognition of plant signal molecules were identified as being NodD, a LysR-type regulator acting as a transcriptional activator for other nod genes, and NodA, NodB, and NodC, which together synthesize the backbone structure of a Nod factor, lipochitooligosaccharide, responsible for inducing symbiotic signals in the roots of host plants (Sugiyama and Yazaki 2012). Using a reporter system consisting of a nod promoter fused to LacZ, signaling molecules secreted by legume plants were identified as flavonoids, such as luteolin in alfalfa, 7,4'-dihydroxyflavone and geraldone in white clover, and daidzein and genistein in soybean, (Djordjevic et al. 1987; Kossak et al. 1987; Peters et al. 1986; Redmond et al. 1986). The induction of *nod* gene expression by flavonoids was studied using alfalfa-*S. meliloti* interaction as a model (Peck et al. 2006), showing that only host specific flavonoid, luteolin, up-regulates the expression of *nod* genes, while noninducing flavonoids such as naringenin compete with luteolin for binding to NodD1.

Upon the perception of Nod factors, legume plants rapidly respond to signals, leading to nodule formation on roots. Genes induced by these bacterial factors have been identified, but less is known about which metabolites, especially specialized metabolites, are increased, decreased, or accumulate during the nodulation process. Flavonoid biosynthesis genes have been shown to be induced during the early stage of nodulation; for example, *B. japonicum* induces the expression of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) in soybeans (Estabrook and Sengupta-Gopalan 1991). Nod factor also up-regulates the expression of isoflavone reductase (IFR) on suspension cells of alfalfa (Savoure et al. 1994), as well as inducing the expression of peroxidases, chitinases, WRKY transcription factors and IFS in the roots of *L. japonicus* (Nakagawa et al. 2011). The presence of flavonoids in the roots was shown to be essential in the stimulation of Nod factor synthesis in infection threads (Zhang et al. 2009). During infection, the contents of kaempferol are increased, inhibiting auxin transport and causing auxin accumulation at nodule initiation sites, thus stimulating cell division and nodule organogenesis (Mathesius et al. 1998; Wasson et al. 2006). In *Medicago truncatula*, the silencing of genes in different branches of the flavonoid pathway, such as chalcone synthase, the key entry-point enzyme for flavonoid biosynthesis, IFS and flavone synthase, showed that flavonols like kaempferol

act as auxin transport inhibitors during nodulation, while flavones such as 7,4'-dihydroxyflavone act as nod-gene inducers (Zhang et al. 2009).

Differences in flavonoid composition have been observed in legume roots infected with symbiotic rhizobia. In alfalfa, for example, the inoculation of rhizobia was shown to strongly alter the flavonoid composition in roots, with medicarpin and its glucoside as well as formononetin-7-O-(6''-O-malonylglycoside) found only in infected roots (Dakora et al. 1993). Co-inoculation of the common bean (*Phaseolus vulgaris*) with *Azospirillum* and *Rhizobium* was found to increase the exudation of nod-gene-inducing flavonoids from roots, as well as relieving symbiotic interactions under conditions of salt stress (Dardanelli et al. 2008). These results suggest that plants respond to bacteria in rhizospheres by altering the flavonoid composition of both roots and root exudates, possibly to facilitate and fine-tune symbiotic interactions with bacteria.

In addition to legume plants, more than 200 species from eight families of non-legume plants, collectively called actinorhizal plants, can interact symbiotically with nitrogen-fixing actinomycetes, particularly Frankia species. Flavonoids also play important roles in these interactions. Flavonoids were shown to accumulate in actinorhizal nodules (Laplaze et al. 1999). Among the flavonoid-like compounds extracted from red alder (*Alnus rubra*), one possible flavanone enhanced nodulation, whereas two other compounds were shown to act as inhibitors (Benoit and Berry 1997). To assess the roles of flavonoids in Frankia-actinorhizal symbiosis, eight flavonoid compounds were extracted from the fruits of *Myrica gale*, with two dihydrochalcone compounds found to enhance the growth and nitrogen fixation of compatible, but not incompatible, Frankia strains (Popovici et al. 2010). Transcriptome analysis revealed that genes involved in flavonoid synthesis and auxin transport were activated in the roots of *C. glauca*, suggesting the important roles of flavonoids and auxin during the Frankia infection process and nodule organogenesis (Hoche et al. 2011). The signaling pathway involved in Frankia-actinorhizal symbiosis has not been fully characterized, but transcriptome analysis showed that similar expression patterns of symbiotic genes in legume and actinorhizal plants, suggesting the involvement of a common or similar pathway for actinorhizal symbiosis.

Interaction with arbuscular mycorrhizal fungi

Mycorrhizal fungi are major components of the soil microbial community, contributing to the transfer of nutrients, especially phosphorus, from soils to plants. Among the mycorrhizal fungi, arbuscular mycorrhizal fungi (AMF) symbiotically interact with around 80% of plant species widely distributed in the terrestrial

ecosystem (Parniske 2008), whereas families such as the Brassicaceae and Chenopodiaceae are not hosts of these fungi (Smith and Read 1997). Fossil records have suggested that arbuscular mycorrhizal symbiosis originated at least 420 to 460 million years ago, coinciding with the origin of the first terrestrial plants, suggesting that ancestral AMF was involved in land plant survival (Redecker et al. 2000; Remy et al. 1994; Simon et al. 1993). Compounds in root exudates have been shown to facilitate interactions with AMF. Using the root exudates of *L. japonicus* grown in nutrient solution, the chemical structure of the signaling compound was identified as a strigolactone (Akiyama et al. 2005), previously found to be a seed germination factor for parasitic weeds such as *Striga* and *Orobanche*.

Various studies have analyzed flavonoid functions in interactions with AMF. Flavonoids were shown to stimulate spore germination, hyphal branching and root colonization (Kikuchi et al. 2007; Scervino et al. 2005, 2007; Siqueira et al. 1991; Steinkellner et al. 2007). Among flavonoids in tomato root exudates, chrysin, luteolin and morin increased the number of entry points and the root colonization of *Gigaspora rosea*, *Gigaspora margarita*, *Glomus mosseae*, and *Glomus intraradices*, whereas kaempferol, isorhamnetin, and rutin showed no effect (Scervino et al. 2007). In addition, a *M. truncatula* mutant with coumestrol accumulation was found to be hyperinfected by AMF (Morandi et al. 2009). These results suggest that flavonoids have positive effects on mycorrhizal symbiosis, but are not considered essential in mediating symbiosis with AMF, inasmuch as root exudates of maize mutants deficient in chalcone synthase exhibited branching activity on hyphae of germinating spores similar to wild type, with both equally colonized with AMF (Becard et al. 1995; Buee et al. 2000). Various phytochemicals, not only flavonoids, may have critical functions in AMF, depending on the plant species. During infection, flavonoids are thought to regulate plant defense reactions, but flavonoid pathway induction during AMF infection was lower than that observed during pathogen infection (Morandi 1996). Flavonoids in the white lupin (*Lupinus albus*), which does not symbiotically interact with AMF, were shown to inhibit interactions with AMF. Moreover, pyranaisoflavones such as licoisoflavone B, sophoraisoflavone A, alpinumisoflavone were found to be strong inhibitors of hyphal branching and germ tube growth (Akiyama et al. 2010).

Interactions with plant growth promoting rhizobacteria

Rhizospheres contain several microorganisms that promote plant growth and health through direct or indirect mechanisms. Beneficial plant-microbe interactions include symbiosis with rhizobia and AMF, as shown above, forming a special structure in roots.

In some cases, these interactions are associated with a broad range of bacteria colonizing the surface of roots or root tissues, mainly in apoplasts. The latter group of bacteria, which do not form special structures, are called plant growth promoting rhizobacteria (PGPR) and have been found to stimulate the growth and health of host plants via various mechanisms, such as phosphate solubilization, siderophore production, phytohormone production, inhibition of pathogen infection, and induction of plant defenses (Vacheron et al. 2013). Many plants, both monocots and dicots, act as hosts for these bacteria, and many bacterial genera, such as *Pseudomonas*, *Azospirillum* and *Bacillus*, and even those containing pathogenic bacteria, function as PGPR. PGPR flourish around the roots due to the exudation of plant metabolites and mucilages. Because plants secrete large amounts of sugars, organic acids and amino acids into the rhizosphere, PGPR utilize these metabolites as nutrients. In addition, PGPR such as *Pseudomonas* and *Bacillus* utilize flavonoids as carbon sources. In *Pseudomonas putida*, quercetin is metabolized to form phloroglucinol and 3,4-dihydroxy cinnamic acid, with the latter further metabolized to protocatechuic acid (Pillai and Swarup 2002). These catabolites and intermediates are secreted by these bacteria, making them secondary metabolites supplied to the rhizosphere.

Pseudomonas fluorescens CHA0 produces the antifungal compounds 2,4-diacetylphloroglucinol and pyoluteorin (Keel et al. 1992; Maurhofer et al. 1994), which contribute to the biocontrol activity of this bacterium. Flavonoids in root exudates have been reported to modulate the expression of *phlA* and *pltA*, genes involved in the synthesis of 2,4-diacetylphloroglucinol and pyoluteorin, respectively. Of 63 plant compounds, including flavonoids and other phenolic compounds, 25 were found to alter the expression of *phlA*, the 2,4-diacetylphloroglucinol biosynthesis gene, and 27 compounds modulated the expression of *pltA*, the pyoluteorin biosynthesis gene (de Werra et al. 2011). These results suggest that phenolic compounds in root exudates are important in regulating the production of antifungal compounds and biocontrol activity.

Relatively little is known about the impact of PGPR on the metabolism of host plants; however, several reports showed that the presence of PGPR altered flavonoid profiles in root exudates (for review see Shaw et al. 2006). For example, *Chryseobacterium balustinum* Aur9 is a PGPR that promotes nitrogen fixation by rhizobia and increases total nitrogen concentration in *Lupinus albus* and soybeans (Garcia et al. 2004; Manero et al. 2003). Under sterile conditions, various flavonoids, such as apigenin, quercetin, naringenin, daidzein, genistein and isoliquiritigenin, were detected in the root exudates of soybeans; however, quercetin and naringenin were not detected in root exudates of soybeans inoculated with

C. balustinum (Dardanelli et al. 2010). Inoculation of *C. balustinum* may have altered the expression of genes involved in the synthesis, accumulation and secretion of flavonoids into the rhizosphere, whereas the disappearance of these flavonoids may indicate their catabolism by *C. balustinum*. However, the ability of *C. balustinum* to utilize these flavonoids has not yet been determined. *C. balustinum* inoculation of the common bean resulted in the disappearance from these exudates of quercetin and isoliquiritigenin, but not of naringenin (Dardanelli et al. 2012). In contrast, apigenin, which was absent from the root exudates of control plants, was present after *C. balustinum* inoculation.

Interactions with pathogens

Plants produce antimicrobial compounds in response to attacks by pathogenic microorganisms. These compounds are called phytoalexins, with many of these compounds being flavonoids. In legume plants, most phytoalexins are isoflavonoids, such as pterocarpan and isoflavanes (Aoki et al. 2000). For example, pea (*Pisum sativum*) produces maackiain and pisatin, which have antifungal activity against *Nectria haematococca*. Maackiain produced by

alfalfa and pea also inhibited the oomycete pathogen *Pythium graminicola* (Jimenez-Gonzalez et al. 2008) and the fungal pathogen *Rhizoctonia solani* (Guenoune et al. 2001; Pueppke and Vanetten 1974). *L. japonicus* contains the phytoalexin vestitol, with most of the genes involved in its synthesis identified (Shimada et al. 2007). In addition to its activity against microbial pathogens, vestitol was secreted into the rhizosphere upon the intrusion of parasitic weed (*Striga hermonthica*), acting as a chemical barrier (Ueda and Sugimoto 2010).

Glyceollins, a family of prenylated pterocarpan derived from daidzein, are specifically induced in soybeans response to pathogens such as *Phytophthora sojae* and *Macrophomina phaseolina* (Ng et al. 2011). The crucial step in glyceollin biosynthesis is the prenylation of glycinol, a reaction catalyzed by the prenyltransferases G2DT and G4DT (Akashi et al. 2009). Glyceollin I is biosynthesized through prenylation at the 4 position, and glyceollins II and III are biosynthesized through prenylation at the 2 position. Yeast extract treatment of cultured soybean cells induced the accumulation of glyceollins I and III, as well as the precursor glycinol, and induced the expression of the genes involved in glyceollin

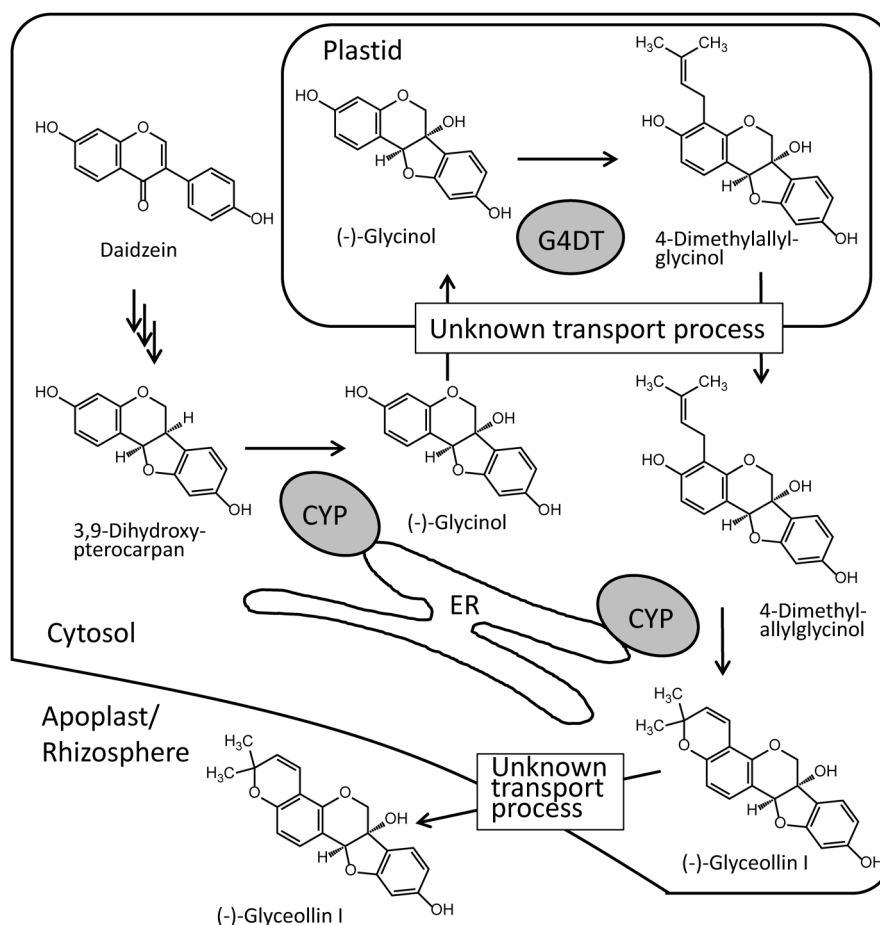


Figure 3. Biosynthesis of glyceollin I in soybean cells. Putative transport processes are also shown. CYP represents cytochrome P450. Additional CYP is involved in the 2'-hydroxylation of daidzein to form 2'-hydroxydaidzein, but not included in this figure due to space limitation.

biosynthesis (Akashi et al. 2009). G4DT was shown to localize at the plastids (Akashi et al. 2009), similar to other flavonoid prenyltransferases identified to date (Yazaki et al. 2009). However, glycinol is biosynthesized by 3,9-dihydroxypterocarpan 6a-hydroxylase, a cytochrome P450 localized at the ER (Schopfer et al. 1998), suggesting an as yet uncharacterized mechanism of glycinol transport into the plastids. Moreover, 4-dimethylallylglycinol, the product of G4DT, is cyclized by another cytochrome P450 localized at the ER (Welle and Grisebach 1988). These different localizations of successive biosynthetic enzymes suggest possible intracellular movement of intermediates between the ER and plastids (Figure 3), a movement that may also occur during the synthesis of sophoraflavanone G in *S. flavescent* (Sasaki et al. 2008; Yamamoto et al. 2000, 2001). Recently, using mutants in tocopherol biosynthesis and the transorganellar complementation approach, in which plastid-localized enzymes were retargeted to the ER, non-polar substrates in plastids were found to be accessed by ER-localized enzymes (Mehrshahi et al. 2013). These observations suggest interorganellar metabolic pathways, with a variety of compounds including pterocarpan and prenylated flavonoids present in one organelle modifiable by enzymes localized to another organelle.

Phytoalexins are synthesized in response to pathogen infection and exhibit anti-pathogen effects, such as inhibiting the elongation of fungal germ tubes and mycelial growth and inducing hypersensitive response-mediated cell death (Ahuja et al. 2012; Hassan and Mathesius 2012). In addition, several phytoalexins are secreted into the rhizosphere, or at least into the medium. For example, infection of soybean hairy roots with *Fusarium solani*, a soil-born fungal pathogen of soybeans, was found to induce glyceollin secretion (Lozovaya et al. 2004), and fungal infection of the hairy roots of peanuts (*Arachis hypogaea*) was found to induce trans-resveratrol secretion (Medina-Bolivar et al. 2007). However, the induction of phytoalexin secretion may not be a general phenomenon, because the phytoalexin medicarpin was induced in *M. truncatula* but the secretion into the medium was not induced (Farag et al. 2008). Thus, the mechanisms underlying phytoalexin secretion in these plants are tightly regulated, with transporters or other transport mechanisms, such as vesicle transport, being among the most prominent targets in future research.

Interactions with nematodes

Plant-parasitic nematodes like root knot and cyst nematodes cause tremendous damage to agricultural crops. Upon infection by these nematodes, galls or cysts are formed in root tissues, which show characteristic multiple cell divisions and endoreduplication. The involvement of flavonoids in these interactions with

nematodes are not clearly understood, but various phenolic compounds, especially flavonols such as kaempferol and quercetin and their glycosides, were shown to inhibit the chemotaxis and motility of nematodes such as *Meloidogyne incognita* (Wuyts et al. 2006). Invasion with root knot and cyst nematodes was also shown to induce genes involved in flavonoid biosynthesis, with the accumulation of these flavonoids regulating auxin transport (Grunewald et al. 2009; Jones et al. 2007), although conflicting results have been observed using mutants deficient in flavonoid biosynthesis. For instance, none of the flavonoid-deficient mutants of *Arabidopsis* tested, such as *tt4*, *tt5*, *tt6* and their double mutants, showed reductions in the number of infecting nematodes or adult female nematodes in infected roots. Furthermore, several mutant lines produced significantly higher numbers of adult female nematodes, suggesting that these lines permitted the development of more syncytia that supported female development (Jones et al. 2007). In contrast, flavonoid-deficient mutants of *M. truncatula* showed smaller gall formation and fewer cell divisions, although the number of galls was not reduced (Wasson et al. 2009). Although the impact of flavonoids secreted into the rhizosphere on plant-nematode interactions is not completely understood, other plant metabolites secreted into the rhizosphere have significant effects on plant-nematode interactions. Interestingly, for example, β -caryophyllene released from maize roots in response to feeding by a maize pest, the larvae of the beetle *Diabrotica virgifera virgifera*, resulted in the attraction of an entomopathogenic nematode to these maize roots (Rasmann et al. 2005). In contrast to volatile terpenoids, which can move away from plant roots due to their volatility, flavonoids remain in the vicinity of plant roots, suggesting their involvement in attachment and/or infection, rather than in attraction.

Interactions with other plants

Allelopathy is defined as the suppression or inhibition of plant growth and/or development by other plants through the release of organic compounds. In addition to well-characterized allelochemicals, including momilactones A and B from rice and juglone from black walnut (*Juglans nigra*) (Kato-Noguchi and Peters 2013; Soderquist 1973), flavonoids have been implicated as allelochemicals in the rhizosphere. *Dittrichia viscosa* is a ruderal plant, aggressively occupying disturbed areas with allelopathic effects. The effect of flavonoids secreted from the roots of *D. viscosa* on the growth of lettuce seedlings was investigated in detail, with apigenin, 6-methoxy-kaempferol, rhamnetin and isorhamnetin found to reduce the root length of lettuce seedlings, whereas kaempferol-7-methyl ester, 6-methoxy-kaempferol and taxifolin reduced root biomass (Levizou

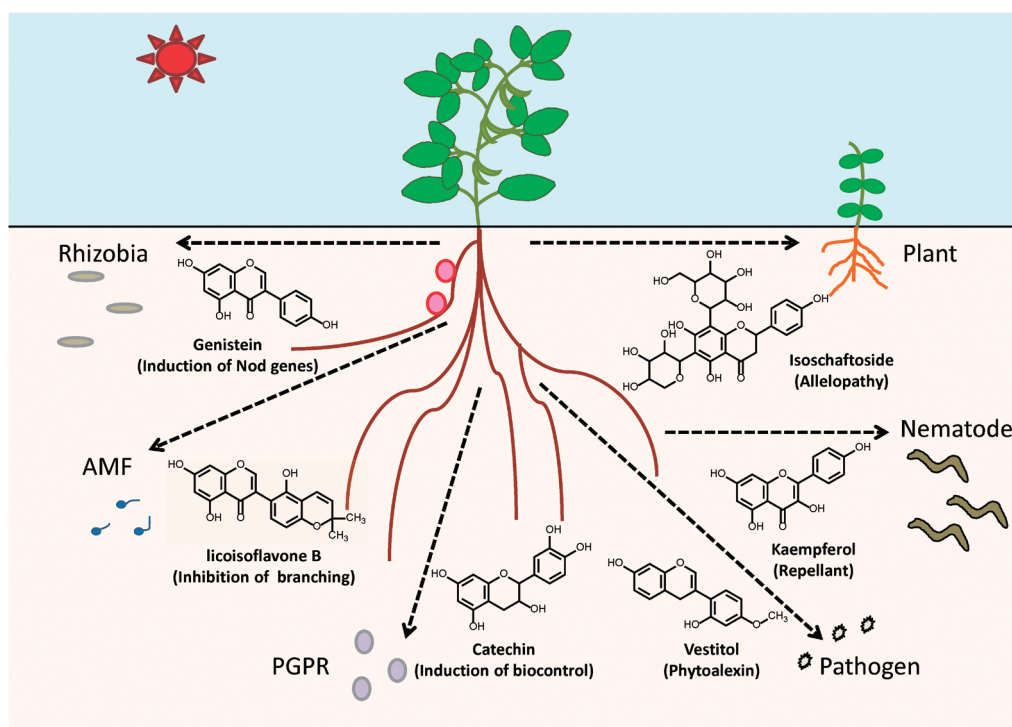


Figure 4. Schematic overview of the functions of flavonoids in the rhizosphere. Representatives of biologically active flavonoids mediating various biological communications are shown. Pink circles represent nodules.

et al. 2004). In contrast, several flavonoids found in root exudates of *D. viscosa* stimulated lettuce root growth (Levizou et al. 2004). Many types of compounds, including alkaloids, phenolics, cyanoglucosides, polyamines and hydroxyamic acids, have been reported to function as allelochemicals in barley (*Hordeum vulgare*) (Kremer and Ben-Hammouda 2009). The precise functions of barley flavonoids in allelopathy have not yet been determined, although flavonoids such as cyanidin and catechin may inhibit the germination and cell growth of other plants, as well as interfering with auxin function (Kremer and Ben-Hammouda 2009).

As an example of the agricultural application of allelopathic flavonoids, a legume plant, *Desmodium uncinatum*, was used to protect cereal crops from infection by Striga, a parasitic weed that causes tremendous crop damage in Africa (Khan et al. 2010). The roots of *D. uncinatum* secrete a C-glycosylflavonoid, isoschaftoside, into the rhizosphere (Hopper et al. 2010). C-glycosylflavonoids are rather unusual metabolites compared with the more common O-glycosylflavonoids. In contrast to O-glycosylflavonoids, which are rapidly hydrolyzed in soil, C-glycosylflavonoids are more stable in soil, better inhibiting Striga infection (Hooper et al. 2010).

Conclusion and future perspective

Large gaps exist in our knowledge of flavonoid biosynthesis and their function in the rhizosphere, including the mechanisms of secretion, the rate

of secretion, and the fate and degradation of these compounds. In particular, few genes responsible for the secretion of flavonoids into the rhizosphere have been identified, although various flavonoids are actively secreted by roots. Relatively little is currently known about the kinetics of flavonoid degradation and the fate and further catabolism of these degradation products. Although the mechanism by which flavonoids induce *nod* genes in rhizobia have been well characterized, the mechanisms involved in flavonoid interactions with other organisms are not clearly understood. It is highly probable that the degradation compounds of flavonoids, not the flavonoids themselves, are the key regulators in these interactions. The half-lives of flavonoids in soils are very short and, to our knowledge, the functions of their catabolites in the rhizosphere have not been determined. Meta-metabolome analysis in rhizosphere soil is of particular importance in future research, in addition to metagenome and metatranscriptome analyses. Chemical analyses of the types and amounts of flavonoids and their degradation products are technically difficult, as judged by previous conflicting results, in which different extraction methods, soil types, and microbial communities were used. Therefore, future research should include systematic approaches to soil analysis, especially of microbial communities, in addition to measurements of flavonoids and their degradation products. Determination of the mobility, space distribution and structural changes of flavonoids in correlation with

soil types and microbes in the rhizosphere is crucial in understanding the functions of these plant-derived compounds in biological communications.

Harnessing the beneficial interactions between plants and other organisms to manage nutrition and disease, thus improving the crop yields, is of particular importance in sustainable agriculture, reducing the use of chemical fertilizers and pesticides (Figure 4). Transgenic plants and natural variations with altered flavonoid accumulation and exudation can be used to study the effects of flavonoid exudation in the rhizosphere. Flavonoid accumulation was recently shown to enhance plant tolerance to drought stress (Nakabayashi et al. 2014). Thus, molecular breeding methods that increase flavonoid accumulation and/or alter flavonoid composition are promising for crop improvements. The rhizosphere is recognized as being a frontier in plant breeding (Wissuwa et al. 2009), as shown by the soybean breeding program in Brazil, in which the disuse of nitrogen fertilizers led to a *Bradyrhizobium* strain more efficient in nitrogen fixation (Drinkwater and Snapp 2007). Manipulation of plant metabolites, especially those secreted into the rhizosphere, could be the basis for the next-generation rhizosphere breeding program for better utilization of rhizosphere biological communication.

Plants can be grown under various conditions, including axenic culture in the laboratory; in vermiculites; in commercial or field soils in pots, either in growth chambers or greenhouses; or in fields. The biological interactions observed in tightly controlled, or even highly artificial, conditions cannot be readily reproduced under field conditions, with this being one of the biggest obstacles in applying scientific insights to agriculture. To pave the way towards applying molecular components to mediate the beneficial interactions, basic mechanisms discovered in studies performed in well-controlled environments should further be tested under greenhouse conditions, followed by tests in the field. The *raison d'être* of plant specialized metabolites was first proposed as being aerial biological communication (Fraenkel 1959). Half a half century later, substantial evidence has accumulated to show that their *raison d'être* is biological communications in rhizospheres, suggesting their potential application in agricultural practice.

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