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REVIEW PAPER

The role of flavonoids in root–rhizosphere signalling: opportunities and challenges for improving plant–microbe interactions

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Abstract

The flavonoid pathway produces a diverse array of plant compounds with functions in UV protection, as antioxidants, pigments, auxin transport regulators, defence compounds against pathogens and during signalling in symbiosis. This review highlights some of the known function of flavonoids in the rhizosphere, in particular for the interaction of roots with microorganisms. Depending on their structure, flavonoids have been shown to stimulate or inhibit rhizobial *nod* gene expression, cause chemoattraction of rhizobia towards the root, inhibit root pathogens, stimulate mycorrhizal spore germination and hyphal branching, mediate allelopathic interactions between plants, affect quorum sensing, and chelate soil nutrients. Therefore, the manipulation of the flavonoid pathway to synthesize specifically certain products has been suggested as an avenue to improve root–rhizosphere interactions. Possible strategies to alter flavonoid exudation to the rhizosphere are discussed. Possible challenges in that endeavour include limited knowledge of the mechanisms that regulate flavonoid transport and exudation, unforeseen effects of altering parts of the flavonoid synthesis pathway on fluxes elsewhere in the pathway, spatial heterogeneity of flavonoid exudation along the root, as well as alteration of flavonoid products by microorganisms in the soil. In addition, the overlapping functions of many flavonoids as stimulators of functions in one organism and inhibitors of another suggests caution in attempts to manipulate flavonoid rhizosphere signals.

Key words: Flavonoids, mycorrhizae, nodulation, rhizosphere.

Introduction

The flavonoid pathway is one of the best studied biosynthetic pathways of specialized metabolites. Flavonoids are phenylpropanoid metabolites, most of which are synthesized from *p*-coumaroyl-CoA and malonyl-CoA and share their precursors with the biosynthetic pathway for lignin biosynthesis (Stafford, 1990). However, some rare flavonoids are synthesized from CoA esters of substrates such as cinnamic acid or dihydro-coumaric acid, (Friederich *et al.*, 1999). To date, >10 000 flavonoids have been identified in plants, and their synthesis appears to be ubiquitous in plants (Ferrer *et al.*, 2008). Their diversity stems from the generation of a number of basal flavonoid structures that include flavones, flavonols, flavan-3-ols, flavanones, isoflavonoids, isoflavans, and pterocarpans (Fig. 1). The flavonoid skeleton can be modified by glycosylation, malonylation, methylation, hydroxylation, acylation, prenylation, or polymerization, leading to the diversity of end-products (Winkel-Shirley, 2001). These substitutions have important effects on flavonoid function, solubility, mobility, and degradation.

The synthesis of flavonoids is in general well understood and the majority of enzymes have been identified, often from multiple species (Dixon and Steele, 1999; Winkel-Shirley, 2001; Du *et al.*, 2010). Flavonoid synthesis starts on enzyme complexes located on the cytosolic side of the endoplasmic reticulum (Jorgensen *et al.*, 2005). Some of the enzyme complexes localize to the tonoplast where they might channel flavonoid intermediates for subsequent glycosylation reaction and storage in the vacuole (Aoki *et al.*, 2000;

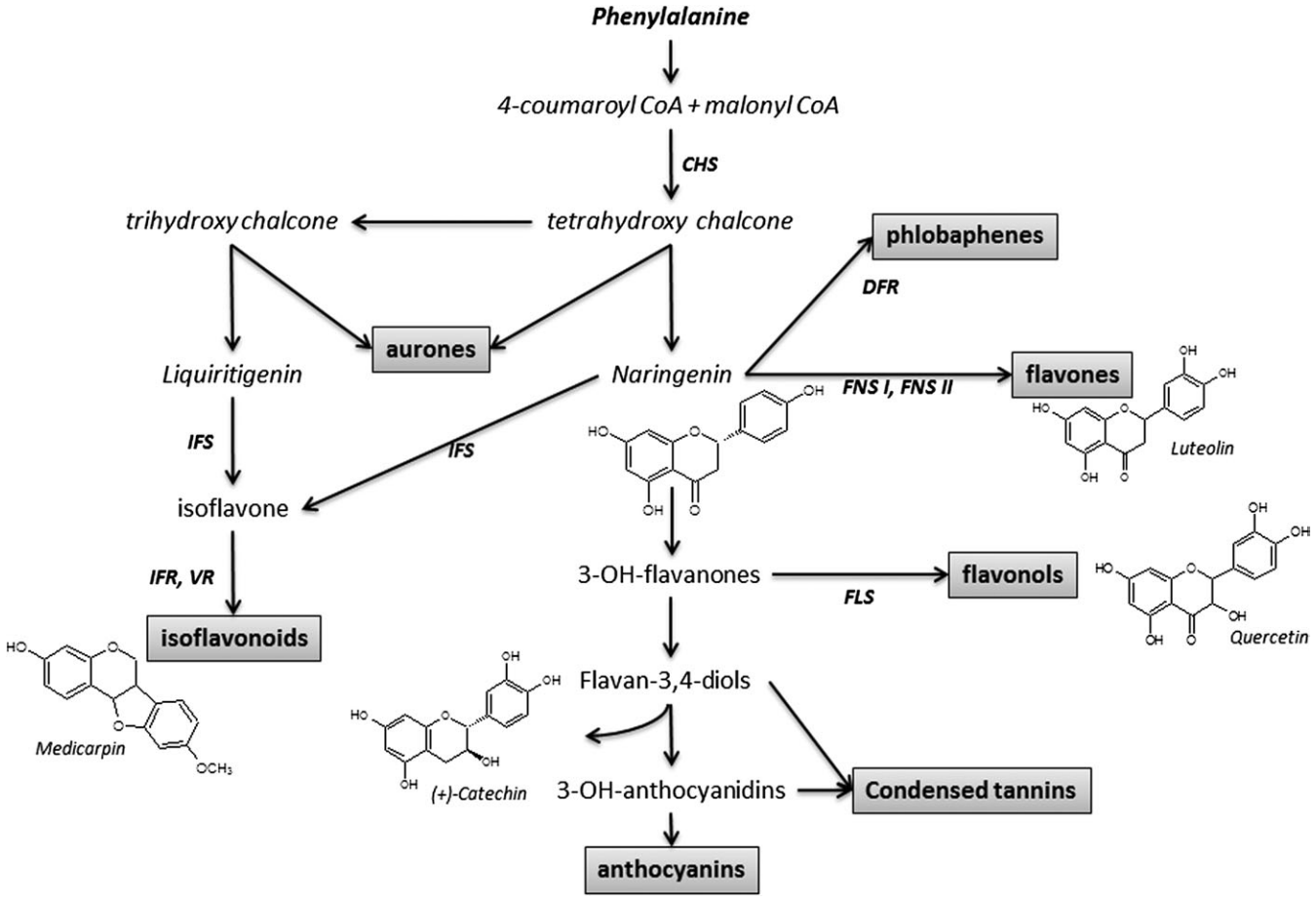

Fig. 1. Major branches of the flavonoid biosynthesis pathway. Some of the critical enzymes are highlighted in bold and are abbreviated as follows: CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; FS I/II, flavone synthase I/II; FLS, flavonol synthase; IFS, isoflavone synthase; IFR, isoflavone reductase; LCR, leucoanthocyanidin reductase; VR, vestitone reductase. Examples of a few structures of compounds discussed in the text are provided. Major classes of end-products are emphasized in grey boxes.

Winkel, 2004). Flavonoid synthesis and accumulation is often very specific for certain cell types. For example, along the length of a root, flavonoids are often accumulated at the root tip and in root cap cells (Fig. 2A). Specific flavonoid end-products are also localized to specific cell types (Fig. 2B) where they could have functions in regulating development (Mathesius *et al.*, 1998*a*; Mathesius, 2001). Within the cell, flavonoids also show specificity for their location. Flavonoids have been localized to the nucleus, the vacuole, the cell wall, cell membranes, and the cytoplasm (Fig. 2B–D) (Hutzler *et al.*, 1998; Erlejman *et al.*, 2004; Saslowsky *et al.*, 2005; Naoumkina and Dixon, 2008). Flavonoid localization and synthesis in different cell types and in response to environmental stimuli can be regulated by a number of transcription factors, in particular of the MYB and bHLH families (Koes *et al.*, 2005; Quattrocchio *et al.*, 2006). In many cases, the regulation of cell specificity is unknown.

Flavonoids can also be transported within and between cells and tissues. Within the cell, flavonoids are likely to move via vesicle-mediated transport or through membrane-bound transporters of the ABC (ATP binding cassette) or MATE (multidrug and toxic extrusion compound) families (Zhao and Dixon, 2009). Flavonoid transport into vacuoles can be achieved by conjugation of glutathione with flavonoids in the cytoplasm, followed by ATP-driven transport via glutathione *S*-transferase pumps (Marrs *et al.*, 1995; Mueller *et al.*, 2000; Goodman *et al.*, 2004). Long-distance transport of flavonoids is less well understood but has been demonstrated in *Arabidopsis*, where application of flavonoids to the root or the shoot led to their transport towards distal tissues (Buer *et al.*, 2007). Application of transporter inhibitors showed that the long-distance transport is likely to be mediated by members of the ABC transporter families and is also altered by glutathione, which is likely to act as a transport vehicle for flavonoids after binding. To date, the exact mechanisms of flavonoid transport out of cellular organelles and out of the cell, as well as long-distance transport, remain poorly understood.

Flavonoids in the rhizosphere

Flavonoids are not only found within the plant but constitute a large part of root exudates (Cesco *et al.*, 2010). Flavonoid exudation into the rhizosphere is not well understood, although some progress has been made towards

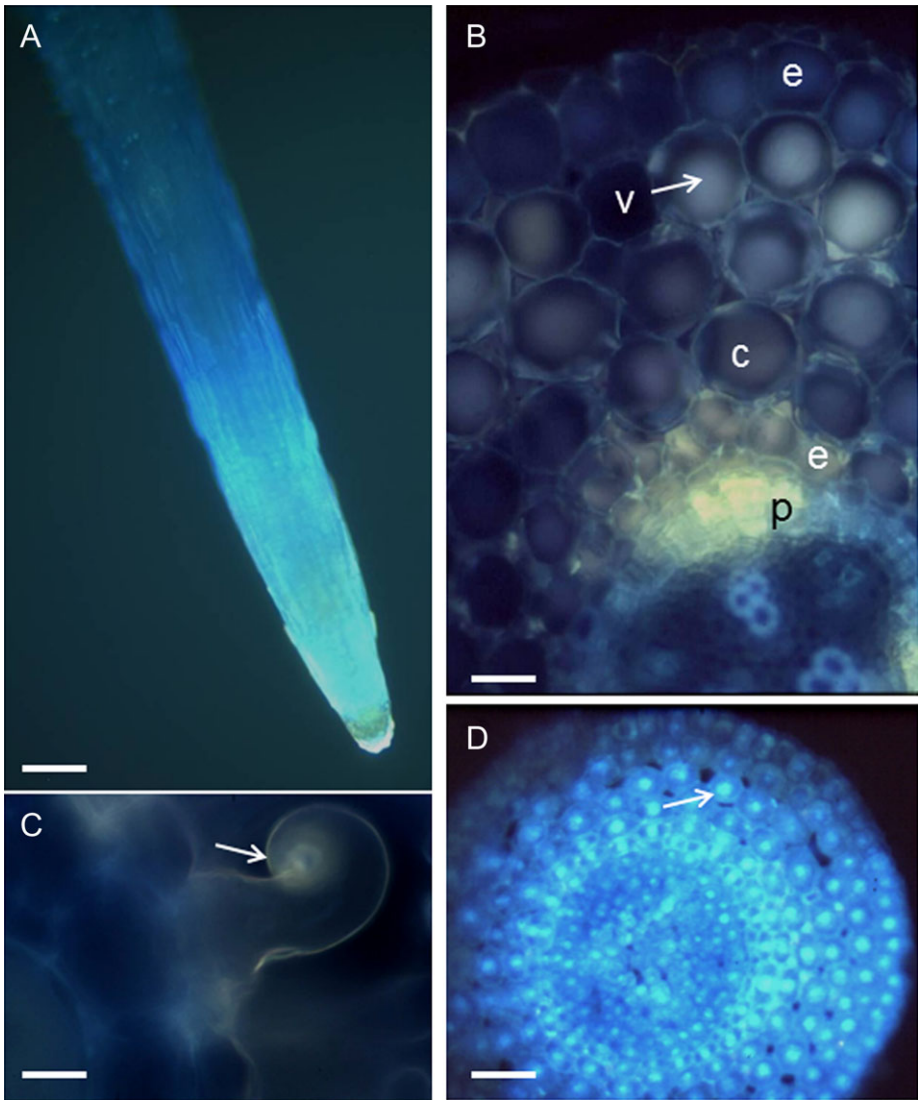

Fig. 2. Spatial differences in flavonoid accumulation within and between cells. (A) Flavonoid accumulation (yellow fluorescence) at the root tip of a *Medicago truncatula* root. (B) Specific flavonoids (fluorescing in different colours) accumulate in different cell types of white clover (*Trifolium repens*), for example pericycle (p), endodermis (e), cortex (c), and epidermis (e). Flavonoids are located in vacuoles (v) of cortical cells. (C) Flavonoids (fluorescing orange) in the cell wall and/or membrane of a curled root hair of Medicago truncatula in response to *Sinorhizobium meliloti* infection. (D) Nuclear localization of flavonoids in a cross-section through the root tip of white clover. All photos were taken under UV excitation (365 nm) and are from vibratome sections of fresh roots stained with 0.5% diphenylboric acid-2-aminoethyl ester. Bars represent 500 μm in A, 50 μm in B, 25 μm in C, and 75 μm in D.

the identification of transporters. Flavonoids are likely to be actively exuded from roots, often in response to elicitors (Schmidt *et al.*, 1994; Armero *et al.*, 2001). ABC transporter mutants of *Arabidopsis* were shown to have altered root exudate profiles, although they probably affect multiple compounds (Badri *et al.*, 2008). Exudation of the isoflavonoid genistein from soybean root plasma membrane vesicles was ATP dependent and most probably catalysed by an ABC-type transporter (Sugiyama *et al.*, 2007). Several phenylpropanoid exudates were affected in the ABC transporter mutant *abcg30*, although it was not shown whether this transporter directly transports the altered phenolics (Badri *et al.*, 2009). Altogether, most of the transporters responsible for flavonoid exudation into the rhizosphere, their location, or regulation are so far unknown. Flavonoids can also be released passively from decomposing root cap and border cells (Hawes *et al.*, 1998; Shaw *et al.*, 2006). Apoplastic β-glucosidases have been found to release isoflavones from their conjugates in soybean roots, and this could be an important mechanism for releasing active flavonoid aglycones during root–microbe interactions (Suzuki *et al.*, 2006).

Many studies have determined types and concentrations of flavonoids in root exudates (summarized in Cesco *et al.*, 2010), although most of these were from plants grown in solution. Both aglycones and glycosides of flavonoid can be found in root exudates. Their concentrations vary widely and depend on plant growth conditions, sampling

techniques, nutrient supply, and plant species (Cesco *et al.*, 2010). In general there is only little information on actual flavonoid concentrations in soil and how these concentrations change in space and time. In addition, there are large differences in exudation of flavonoids along the root, with larger amounts being reported to be exuded from the root tip (Graham, 1991; Hawes *et al.*, 1998); see also Fig. 2A. During cluster root formation in lupins, isoflavonoid exudation, together with citrate release, is spatially and temporally regulated to coincide with maturation of the cluster roots (Weisskopf *et al.*, 2006; Tomasi *et al.*, 2008). Solid phase root zone extraction with the use of micro-tubes that can be placed along the root could be used in future studies to determine spatial and temporal changes in flavonoid exudation along roots grown in soil (Mohney *et al.*, 2009; Weidenhamer *et al.*, 2009).

Once in the rhizosphere, the fate of flavonoids depends on various conditions in the soil. Flavonoids can be absorbed to the cell wall and to soil particles with cationic binding sites, thus becoming unavailable (Shaw and Hooker, 2008). Depending on their modifications, flavonoid solubility and mobility in the soil varies. While glycosylation improves their solubility in water, it is likely that flavonoid glycosides are quickly deglycosylated by microorganisms and plant exoenzymes, leaving the more hydrophobic aglycone (Hartwig and Phillips, 1991). Flavonoid persistence in the soil varies and can be <72 h, depending on the structure (Shaw and Hooker, 2008). Persistence in non-sterile soil can be much shorter than in sterile soil, suggesting degradation by microorganisms. Some bacteria metabolize flavonoids as a carbon source; others specifically modify flavonoids. For example, rhizobia can modify *nod* gene-inducing flavonoids by partial breakdown to produce flavonoids more or less active as *nod* gene inducers (Rao and Cooper, 1995).

Flavonoids also alter the soil by acting as antioxidants and metal chelators. Chelation and reduction of metals can alter nutrient concentration in the soil, and this might have importance especially for the availability of phosphorus and iron. For example, an isoflavonoid identified in *Medicago sativa* (alfalfa) root exudates was able to dissolve ferric phosphate, thus making both phosphate and iron available to the plant (Masaoka *et al.*, 1993). Flavonoids, including genistein, quercetin, and kaempferol, can also alter iron availability by reducing Fe(III) to Fe(II) and by chelating iron otherwise unavailable in iron oxides (Cesco *et al.*, 2010).

Flavonoids can be synthesized and released specifically in response to abiotic and biotic signals in the rhizosphere (Dixon and Paiva, 1995). For example, flavonoid synthesis is affected by phosphorus (Juszczuk *et al.*, 2004) and nitrogen supply (Coronado *et al.*, 1995) in the soil. Flavonoids are specifically induced by symbionts and pathogens (see below), and also respond to purified signalling molecules of these organisms. The following sections highlight some examples for the diverse function of flavonoids in the rhizosphere along with some of the opportunities for using flavonoids as regulators of rhizosphere functions (Fig. 3).

Multiple roles for flavonoids in nodulation

Most legumes have the ability to form root nodules that house symbiotic nitrogen-fixing bacteria or rhizobia. In addition, members of several families of non-legumes, so-called actinorhizal plants, form symbioses with nitrogen-fixing actinomycetes, in particular *Frankia* species. The rhizobia fix atmospheric nitrogen into a form that the plant can use to fulfil its nitrogen requirement while the plant provides the bacteria with a source of carbon. Nodules are root organelles that are developed through signal exchange between the plant roots and the bacteria. In this symbiosis, flavonoids act as chemoattractants, inducers of nodulation (*nod*) and other genes, determinants of host specificity, developmental regulators, and regulators of phytoalexin resistance in rhizobia (Cooper, 2004).

One well-studied role of root-exuded flavonoids is their action as regulators of *nod* genes in rhizobia. A number of *nod* gene products are necessary to synthesize species-specific Nod factors, lipochitin oligosaccharides required for nodule formation in the host (Spaink, 1995). *Nod* gene transcription is regulated by NodD, a transcription factor of the LysR family of transcriptional regulators. Binding of an appropriate flavonoid to NodD is thought to enhance the access of RNA polymerase and improve transcriptional ability of the *nod* genes at the site in the promoter where NodD is localized (Peck *et al.*, 2006; Li *et al.*, 2008). The perception of flavonoids by rhizobia is also linked to elevation in concentrations of intracellular calcium in rhizobia that subsequently induces NodD proteins for Nod factor expression (Moscatiello *et al.*, 2010). The first flavonoids to be discovered to act as *nod* gene inducers were luteolin, isolated from *M. sativa*, and 7,4' dihydroxyflavone (DHF) from *Trifolium repens* (white clover) (Peters *et al.*, 1986; Redmond *et al.*, 1986). Since then many other flavonoids have been discovered to have *nod* gene-inducing roles (summarized by Cooper, 2004). Most of these flavonoids are active as *nod* gene inducers at nanomolar to low micromolar concentrations. It has been suggested that a mixture of flavonoids is more effective in inducing *nod* genes as opposed to a single compound (Bolanos-Vasquez and Warner, 1997; Begum *et al.*, 2001). The specific exudation of flavonoid (mixtures) from legume hosts together with the specific perception of flavonoids by NodD proteins of different rhizobia is partially responsible for the host specificity of the symbiosis.

Interestingly, some flavonoids also show *nod* gene-repressing activity for certain rhizobia. For example, the isoflavonoids medicarpin and coumestrol have been shown to control Nod factor production negatively in *Sinorhizobium meliloti* (Zuanazzi *et al.*, 1998). The *nod* gene activators and repressors together are thought to maintain an optimal level of Nod factor production and prevent elicitation of defence responses by the plant (Savouré *et al.*, 1997; Zuanazzi *et al.*, 1998).

Flavonoid exudation from the root changes during the symbiosis. Altered flavonoid exudates have been found in rhizobia-inoculated roots of several legumes. This alteration

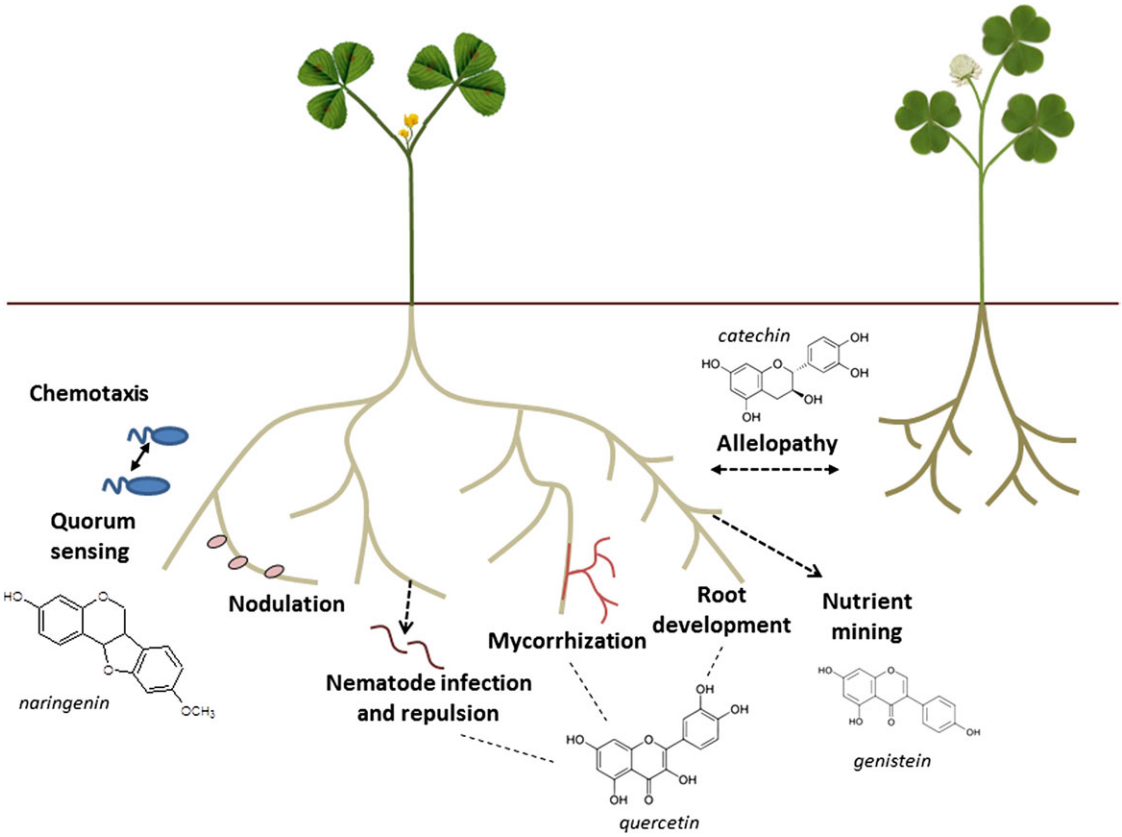

Fig. 3. Schematic overview of flavonoid functions in the rhizosphere. Flavonoid functions in the rhizosphere range from *nod* gene inducers and chemoattractants in rhizobia, stimulators of mycorrhizal spore germination and hyphal branching, possible quorum-sensing regulators in bacteria, repellents for parasitic nematodes, nutrient mining, and as allelochemicals in plant–plant interactions. They can also affect root development. Examples of biologically active flavonoids mediating the different interactions are shown.

in flavonoid profiles could fine-tune Nod factor synthesis during different stages of symbiosis (Dakora *et al.*, 1993; Schmidt *et al.*, 1994). Altered flavonoid exudate profiles could also be the result of flavonoid metabolism by rhizobia themselves, causing changes in the activity of flavonoids as *nod* gene inducers or repressors (Rao and Cooper, 1995).

Some flavones and flavonones that induce *nod* genes, such as luteolin and apigenin, have also been shown to evoke a strong chemoattractant response from the rhizobia, with different flavonoids attracting different *Rhizobium* species (Aguilar *et al.*, 1988; Dharmatilake and Bauer, 1992). These responses occur at flavonoid concentrations of 10^{-6} to as little as 10^{-10} M, a much lower minimum concentration than that reported for flavonoid activity as *nod* gene inducers.

In actinorhizal symbioses, flavonoids might also play a role in host specificity and selection of rhizobia, even though no canonical *nod* genes have been found in *Frankia* (Normand *et al.*, 2007). Flavonoids were found to accumulate inside actinorhizal nodules (Laplaze *et al.*, 1999). Flavonoids from seeds of actinorhizal plants were shown to enhance or inhibit symbiosis, although the flavonoids were not identified in this study (Benoit and Berry, 1997). Flavonoids extracted from fruits of the host *Myrica gale* had positive effects on growth and nitrogen fixation in compatible, but negative effects in incompatible *Frankia* strains (Popovici *et al.*, 2010), suggesting that flavonoids could play a role in selection of compatible rhizobia by the host. This is similar to the situation in legumes, where it has been shown that the phytoalexin medicarpin produced by clover and medic species inhibits the growth of incompatible but not that of compatible strains (Pankhurst and Biggs, 1980). This effect might be due to the fact that certain (iso)flavonoids can induce resistance to phytoalexins in rhizobia at micromolar concentrations (Parniske *et al.*, 1991), thus enabling host plants simultaneously to exude phytoalexins in order to inhibit pathogens while still allowing rhizobial infection.

Flavonoids have also been shown to regulate a number of other *Rhizobium* genes, including those for exopolysaccharide synthesis, which is important for regulating defence responses in the host. For example, genistein at 1 μM concentration altered exopolysaccharide concentration and composition in *Rhizobium fredii* cultures (Dunn *et al.*, 1992). In addition, type III secretion systems, which play a role in nodulation in some rhizobia, as well as the production of exported proteins, can be induced by flavonoid exudates (Krishnan *et al.*, 2003). Proteome analysis also found a number of other proteins in response to host flavonoids, many of which await characterization (Guerreiro *et al.*, 1997).

Nod factors are important in inducing the initial response of root cortical cell division and root hair curling. Certain

flavonoids act as negative regulators of auxin transport and could thus cause accumulation of auxin at the nodule initiation site to stimulate cell division and nodule organogenesis (Mathesius *et al.*, 1998*b*; Boot *et al.*, 1999; Wasson *et al.*, 2006). Exactly how flavonoids redirect auxin transport during nodule initiation is not known, but the perception of Nod factors by the plant is thought to induce endogenous flavonoids that could locally inhibit auxin transport (Mathesius *et al.*, 1998*a*). In *Medicago truncatula*, silencing of different branches of the flavonoid pathway showed that flavonols such as kaempferol are the most likely candidates for auxin transport inhibitors (Zhang *et al.*, 2009). Whether auxin transport regulation occurs during nodulation of legumes forming determinate nodules, for example soybean, is still unclear, but it is likely that other flavonoids, possibly isoflavonoids, are involved (Subramanian *et al.*, 2006).

Changes in auxin accumulation could also be due to auxin breakdown by peroxidases, which can be modulated by flavonoids. The isoflavonoid formononetin, which accumulates in the nodule primordia of white clover, accelerated auxin breakdown. In contrast, a derivative of DHF and free DHF, which accumulate in the vacuoles of the cortical cells that later form the nodule primordia, were shown to inhibit auxin breakdown (Mathesius, 2001). The local changes in auxin may be critical in regulating cell divisions during nodule development.

One of the opportunities in nodulation research could be the expression of appropriate flavonoid synthesis pathways in non-legumes in future efforts to extend nodulation beyond legumes. In addition, altered branches of the flavonoid pathway could be engineered to extend legume host ranges to non-specific rhizobia.

Effects of flavonoids on quorum sensing-regulated behaviours

Many behaviours of rhizosphere bacteria are coordinated by cell-to-cell signals called quorum-sensing signals (Fuqua *et al.*, 2001). Quorum-sensing signals are synthesized by most bacteria and the so-far best studied signals belong to the class of acyl homoserine lactones (AHLs), which are used by many Gram-negative bacteria. Quorum-sensing signals diffuse into and out of bacterial cells and can bind to receptors inside the bacteria once their concentration exceeds a certain threshold (Fuqua *et al.*, 1994). This activates the expression of hundreds of bacterial genes, many of which are important in plant–microbe interactions, including genes responsible for biofilm formation, nitrogen fixation, synthesis of degradative enzymes, exopolysaccharides, and toxins, as well as motility and conjugation (Gonzalez and Marketon, 2003; von Bodman *et al.*, 2003).

In the past years, a number of molecules have been identified that interfere with quorum sensing, including halogenated furanones produced by red algae (Manefield *et al.*, 1999). A number of land plants have been shown to synthesize quorum-sensing mimics, which can both inhibit and stimulate AHL-dependent genes in various reporter strains, although most of these compounds remain unidentified (Teplitski *et al.*, 2000; Gao *et al.*, 2003). The first mimic signal identified from plants was lumichrome, a riboflavin derivative (Rajamani *et al.*, 2008). Another potential AHL mimic is the phenolic compound *p*-coumaric acid, a lignin precursor that can be exuded by roots into the soil (Bodini *et al.*, 2009). *p*-Coumaric acid can also be produced by breaking down flavonoids from root exudates (Rao and Cooper, 1995). In addition, *p*-coumaric acid can form *p*-coumaroyl-homoserine lactone (HSL), a distinct quorum-sensing signal used by some bacteria (Schaefer *et al.*, 2008). Thus *p*-coumaroyl-HSL could have two functions, to sense the presence of a host plant and to control density-dependent bacterial behaviors. A flavonoid identified from the medicinal tree *Combretum albiflorum*, catechin, which also occurs in many other plant species, also showed activity as a quorum-sensing mimic, although at rather high concentrations of between 0.125 mM and 4 mM (Vandeputte *et al.*, 2010). Catechin can also be present in the rhizosphere of plants, for example as an exudate of spotted knapweed, where it acts as a potent allelochemical (Weir *et al.*, 2003). Another flavonoid with inhibitory effects on quorum sensing-regulated reporters is naringenin, which was shown to inhibit quorum sensing in *Escherichia coli* and *Vibrio fischeri* at concentrations of 20–360 μM (Vikram *et al.*, 2010) as well as in *Pseudomonas aeruginosa* at 4 mM (Vandeputte *et al.*, 2011). Naringenin is exuded by some legume roots and also acts as a *nod* gene inducer in rhizobia (Novak *et al.*, 2002). Therefore, it would be interesting to test the effect of naringenin on quorum sensing-regulated genes in rhizobia.

Interestingly, the flavonoid pathway is activated in legumes by exposure to quorum-sensing signals from rhizobia, and it has also been shown that bacterial AHLs (at 50 μM concentrations) can stimulate production of AHL mimics by *M. truncatula* (Mathesius *et al.*, 2003). These results strongly suggest a link between AHL perception by plants, activation of the flavonoid pathway, and possible feedback on bacteria by production of possible AHL mimics. However, effective concentrations of potential flavonoid mimics in the rhizosphere will have to be established. A recent study found that *Nod* gene-inducing flavonoids increased AHL synthesis in three species of rhizobia at low micromolar concentrations, concomitant with enhanced expression of AHL synthesis genes (Perez-Montano *et al.*, 2011). This suggests coordination between *Nod* gene induction and quorum sensing, possibly to enhance symbiotic behaviours of rhizobia. If flavonoids indeed act as quorum-sensing mimics and activators in plant-associated bacteria at relevant concentrations, this could be explored as an avenue to alter the ability of bacteria to colonize and infect host plants.

Mycorrhizal symbioses

Mycorrhizal fungi are important symbionts of the majority of land plants that contribute primarily to plant phosphorus nutrition. Mycorrhizal symbioses are stimulated under

phosphorus deficiency in the soil. Mycorrhizal fungi germinate from spores and form hyphae in the soil which branch in response to root exudates which attract the hyphae to a host root. Hyphae then penetrate the host root tissue and form ecto- or endomycorrhizal invasion structures (Harrison, 2005). Some of the host exudates that stimulate spore germination, hyphal branching in the soil, and root colonization, often in a symbiont-specific manner, have been identified as flavonoids (Siqueira *et al.*, 1991; Scervino *et al.*, 2005, 2007; Kikuchi *et al.*, 2007; Steinkellner *et al.*, 2007). Most of these studies reported active flavonoid concentrations of between 0.5 μM and 20 μM. Not surprisingly, some of the flavonoids enhancing mycorrhizal infection are induced under phosphorus stress (Akiyama *et al.*, 2002). The isoflavonoid coumestrol has been identified as a particularly active stimulator of hyphal growth (Morandi *et al.*, 1984), and an *M. truncatula* mutant hyperaccumulating coumestrol was also found to be hyperinfected by its mycorrhizal symbiont (Morandi *et al.*, 2009).

Flavonoids are also likely to play a role during fungal invasion and arbuscule formation inside the root. Infection of roots with vesicular arbuscular mycorrhizal fungi was shown to induce the flavonoid pathway in a number of host species, in particular in infected cells (Harrison and Dixon, 1994).

Flavonoid accumulation starts before the onset of infection and was shown to vary with different stages of infection and in response to different symbionts (Harrison and Dixon, 1993; Larose *et al.*, 2002). One of the roles of flavonoids inside the root could be to regulate defence reactions, and it has been hypothesized that mycorrhizal invasion triggers a temporary defence response in the root that involves induction of flavonoid phytoalexins (Harrison and Dixon, 1993). However, compared with the induction of phytoalexins in response to pathogens, mycorrhizal induction of these flavonoids is relatively low (Morandi, 1996). Flavonoids may also be responsible for an autoregulation of mycorrhization at later stages of the symbiosis (Larose *et al.*, 2002). Split-root studies have demonstrated that formononetin and its glycoside are down-regulated systemically by either rhizobia or mycorrhizae, concomitant with autoregulation of both symbioses, and that their external application restored the symbioses (Catford *et al.*, 2006).

Interestingly, pyranoisoflavones produced by white lupin, which is not a host for mycorrhizal fungi, inhibited hyphal branching of mycorrhizal fungi, suggesting that flavonoids could play both stimulating and inhibitory roles on fungal symbionts in the soil (Akiyama *et al.*, 2010). However, inhibitory activity of flavonoids on hyphal branching was also reported from host plants (Tsai and Phillips, 1991), and thus it is likely that host and non-host plants can modulate the establishment of symbiosis by altering the profile of flavonoid exudates. While flavonoids clearly appear to enhance mycorrhization through stimulation of spore germination, hyphal branching, and host infection, their presence in the host is not essential for the symbiosis. Experiments in flavonoid-deficient carrot and maize plants have shown that mycorrhizal infection was not abolished (Becard *et al.*, 1995).

Mycorrhial fungi can also protect plants from pathogens (Whipps, 2004) and enhance symbiosis with rhizobia. One possibility is that the flavonoids induced by mycorrhizal fungi also stimulate Nod factor synthesis. For example, daidzein is induced by mycorrhizal fungi in soybean (Morandi *et al.*, 1984), where it also acts as a *nod* gene inducer for *Bradyrhizobium japonicum* (Kosslak *et al.*, 1987). On the other hand, coumestrol is induced by mycorrhizal symbionts in *M. truncatula* (Harrison and Dixon, 1994), where it could act as a *nod* gene inhibitor in *S. meliloti* (Zuanazzi *et al.*, 1998). It is possible that combinations of flavonoids, rather than single compounds, need to be tested in more detail for their combined effects on multiple symbionts. The tripartite symbiosis between soybean and its mycorrhizal and rhizobial symbionts was shown to enhance nodulation compared with inoculation of plants only with rhizobia. In the co-inoculated plants, flavonoid profiles changed specifically in response to both symbionts, although, interestingly, flavonoid accumulation was largely inhibited by the symbionts, including the flavonoids with activities as *nod* gene and hyphal branching inducers (Antunes *et al.*, 2006). Therefore, enhanced symbiosis in tri-partite interactions might be due to enhanced nutrient uptake rather than, or in addition to, stimulation of flavonoids.

The protection of the host plant from pathogens by mycorrhizal fungi has also been partially attributed to the enhanced synthesis of flavonoid (phytoalexins) in response to the mycorrhizal symbiont (Morandi, 1996), although data are still scarce and the protective effect could have multiple causes (Borowicz, 2001). While some studies have shown increased phenolic content of roots co-inoculated with mycorrhizal fungi and pathogens (Dehne and Schonbeck, 1979; Cordier *et al.*, 1998), others found decreased phytoalexin accumulation in co-inoculated plants compared with plants only inoculated with mycorrhizal symbionts (Carlsen *et al.*, 2008).

Flavonoids are involved in defence against root pathogens

Flavonoids and other phenolics have been found to inhibit a range of root pathogens and pests, ranging from bacteria to fungi and insects (Makoi and Ndakidemi, 2007). This has been attributed to their role as antimicrobial toxins (Cushnie and Lamb, 2011) and anti- or pro-oxidants (Jia *et al.*, 2010). Their role within the plant as antioxidants is suspected to be protective, although clear evidence is lacking (Hernández *et al.*, 2009). Some of the major gaps in knowledge pertain to the fact that because of their highly oxidative nature, the transport and storage of flavonoids is tightly regulated. Hence, the mechanism by which these compounds may become available to prevent oxidative stress in case of a pathogen attack is unknown.

The challenge from a pathogen can lead to *de novo* synthesis of flavonoid phytoalexins that exhibit antifungal

and antibacterial activities. These molecules can also be stored in an inactive form to function as broad-spectrum phytoanticipins to mount a quick defence against future attacks (Lattanzio *et al.*, 2006).

Isoflavonoids represent a major class of phytoalexins in legumes. Using a promoter:GUS (β-glucuronidase) fusion, it was shown that the expression of isoflavone synthase (Fig. 1) was elevated when elicited with salicylic acid and cyst nematodes (Subramanian *et al.*, 2004), suggesting their direct involvement in plant defence. Derivatives of isoflavonoids called pterocarpans such as medicarpin, pisatin, and maackiain are known to have antimicrobial properties (Naoumkina *et al.*, 2010). Maackiain also inhibited the oomycete *Pythium graminicola* at a concentration of 20 μg l^{-1} (Jimenez-Gonzalez *et al.*, 2008). Medicarpin from alfalfa and pea protects the plants from the pathogenic fungus *Rhizoctonia solani* (Pueppke and Vanetten, 1974; Kapulnik *et al.*, 2001) possibly by having inhibitory activities on the elongation of fungal germ tube and mycelial growth (Higgins, 1978; Blount *et al.*, 1992). There is also genetic evidence that pisatin from pea contributes to resistance against the fungus *Nectria haematococca* (VanEtten and Wu, 2004), as a knockdown of enzymes responsible for pisatin biosynthesis reduced its concentration from 28 μg g^{-1} dry weight by one-third and increased the susceptibility of pea roots towards the fungal infection.

The mechanism of resistance against fungal infection through pterocarpanoid phytoalexins is suspected to be through a hypersensitive response (HR)-mediated cell death (Heath, 2000). The cell death through this pathway incorporates an initial oxidative burst with an influx of Ca^{2+}, followed by alkalinization of the apoplast through K^+/H^+ exchange leading to the depolarization of the membrane and an extended period of the oxidative state. Isoflavonoids are thought to be oxidized during this process, leading to generation of toxic free radicals that may cause cell death. Alternatively, it is also suggested that some pterocarpans target the membrane ATPase and mitochondrial electron transport for degradation, leading to cell death (Graham *et al.*, 2007).

It is also interesting to note that in some plant–microbe interactions the pterocarpans may not accumulate within the roots but may be secreted instead. When chickpea seedlings were challenged by the endogenous elicitor glutathione, an increase in pterocarpan biosynthesis was observed (Armero *et al.*, 2001). It was also shown that these compounds were released by the roots to the surroundings, possibly to cause damage to the pathogens before they can infect the roots.

Flavonols also contribute to resistance against pathogens. One of the most widely distributed flavonols, quercetin, has strong antimicrobial properties. Quercetin binds to the GyrB subunit of *E. coli* DNA gyrase and inhibits the ATPase activity. However, the promotion of DNA cleavage was induced only at concentrations >80 μM (Plaper *et al.*, 2003; Naoumkina *et al.*, 2010). Quercetin also inhibited the growth of the fungus *Neurospora crassa* (Parvez *et al.*, 2004). The plant carnation (*Dianthus caryophyllus*) mounts a defence against *Fusarium* attacks through increasing the concentration of the fungitoxic flavonol triglycoside of kaemferide at concentrations as low as 50 nM (Curir *et al.*, 2005).

Global gene expression studies have also shown that elevation of flavonoid synthesis occurs when *M. truncatula* plants were challenged by *Phymatotrichopsis omnivora*, cause of cotton root rot disease (Uppalapati *et al.*, 2009). In order to colonize the plant successfully, virulent strains of pathogens such as *P. omnivore*, *N. haematococca*, and *R. solani* have 'learned' to evade many of these flavonoids (Denny and Vanetten, 1981, 1982; Padmavati *et al.*, 1997; Pedras and Ahiahonu, 2005).

An appealing opportunity to enhance plant protection would be to engineer plants with increased, or inducible expression of effective flavonoid phytoalexins and phytoanticipins; however, it might be necessary for the plant to synthesize a range of active antimicrobial flavonoids to avoid emergence of resistance by pathogens. In addition it would be imperative to test whether ectopic expression of these flavonoids would cause any harm to beneficial rhizosphere organisms.

Flavonoids in nematode interactions

Plant parasitic nematodes, including root knot, cyst, and root lesion nematodes, constitute some of the major root pathogens. Many of these pathogens exhibit a wide host range, often of thousands of plant species. Sedentary endoparasitic nematodes cause the formation of feeding structures that are characterized by multiple cell divisions and endoreduplication in root tissues, leading to the formation of galls or cyst (Goverse *et al.*, 2000; Gheysen and Mitchum, 2011). Invasion of roots with root knot and cyst nematodes induces the flavonoid pathway in infection structures (Hutangura *et al.*, 1999; Jones *et al.*, 2007), and it has been hypothesized that the flavonoids could act as regulators of auxin transport and accumulation during gall formation (Hutangura *et al.*, 1999; Grunewald *et al.*, 2009). In *M. truncatula* plants deficient in flavonoids, gall formation still occurred, although galls were smaller and showed fewer cell divisions (Wasson *et al.*, 2009). In flavonoid-deficient *Arabidopsis* and tobacco mutants, reproduction of several species of nematodes was not affected (Wuyts *et al.*, 2006*a*; Jones *et al.*, 2007). However, flavonoids did have an effect on nematode behaviour, for example certain flavonoids acted as repellents for specific nematode species and inhibited their motility and hatching at millimolar concentrations (Wuyts *et al.*, 2006*b*). Therefore, while flavonoids do not seem to be essential for feeding site development in the host plant, flavonoids exuded into the rhizosphere could alter nematode attraction to the roots. This could be exploited for designing nematode trap plants that could be intercropped to reduce the infection of crop roots.

Flavonoids can cause allelopathy

Allelopathy, the inhibition of plant growth and germination by other plants, plays an important role in parasitic and

invading plants and can have far-reaching ecological consequences. In some cases, flavonoids have been implicated as allelochemicals in the rhizosphere. The parasitic weed *Striga* constitutes one of the major problems in African agriculture, with yield losses up to 100% in large parts of sub-Saharan Africa, thus inhibiting its germination would be an important achievement (Gressel *et al.*, 2004). In a search for intercropping plants, the forage legume *Desmodium uncinatum* was found to inhibit post-germination and attachment of *Striga* significantly, and this inhibition was mimicked by several (iso)flavonoids identified from its root exudates (Hooper *et al.*, 2010; Khan *et al.*, 2010). In addition, at least one of the isoflavones stimulated germination, which could be used to cause 'suicidal' germination of the weed. Thus the use of *Desmodium* as a 'push–pull' intercrop plant has been a cheap and successful strategy for smallholder farmers to control *Striga* infestations (Khan *et al.*, 2006; Hooper *et al.*, 2009).

The success of some invasive weeds has also been attributed to flavonoid allelochemicals. For example, spotted knapweed (*Centaurea maculosa*), which has been invading large parts of North America, exudes (–)-catechin, which can induce reactive oxygen species in susceptible species that lead to cell death and the demise of the root system (Bais *et al.*, 2003). A racemic mixture of catechin can also have detrimental effects on legume nodulation at high (~3 mM) concentrations (Alford *et al.*, 2009). (–)-Catechin has also been suggested to inhibit germination and growth of native species, but it has been questioned whether soil concentrations of (–)-catechin would be high enough to be effective (Blair *et al.*, 2005; Duke *et al.*, 2009).

Flavonoid metabolic flux engineering

The biosynthesis of flavonoids involves several branches of pathways to which multiple strategies could be applied to alter the metabolic flux. The production of secondary metabolites requires the plant to invest its energy, and therefore the pathways are tightly regulated. The initial committed step for flavonoid biosynthesis is catalysed by chalcone synthase (CHS). Silencing of CHS transcripts has been demonstrated to cause a dramatic decrease in flavonoid accumulation within the plant (Wasson *et al.*, 2006; Zhang *et al.*, 2009).

Many strategies can be adopted for engineering the flux of metabolites through the biosynthesis pathways. Some of those included are indicated below.

(i) Changes in enzyme specificity through changes in the active site configuration. By studying the crystal structure of an enzyme's active site, novel methods to manipulate it may be developed to affect substrate specificity. Jez *et al.* (2002) demonstrated that the substrate specificity could be altered by inducing single point mutations at the active site of CHS.

(ii) Since flavonoid biosynthesis is an energy-consuming process, the plants utilize control points for each branch. Knockouts or knockdowns of these critical enzymes would direct the flux into alternative branches. Some of the enzymes that may be directed with this strategy include isoflavone synthase, flavone synthase II, and vestitone reductase. Zhang *et al.* (2009) confirmed that knockdown of isoflavone synthase and flavone synthase led to the generation of roots deficient in isoflavonoids and flavones, respectively. The authors were then able to demonstrate that plants can nodulate even in the absence of isoflavonoids that are thought to play an essential role in the process.

(iii) Transporters. Flavonoids are synthesized in the cytoplasm but often have to be transported for exudation into the rhizosphere or for storage. Similar to enzymes, the expression of transporters would also have an impact on the direction of the flux. This was recently demonstrated when a MATE-type transporter was knocked down to generate proanthocyanidin-rich plants (Zhao and Dixon, 2009; Zhao *et al.*, 2011).

(iv) Modification enzymes. Transport, activity, and storage of flavonoids often require modifications of their structure. Changes in abundance or activity of enzymes responsible for these modifications may increase or decrease generation of the end-product. An example of such an enzyme is *O*-methyl transferase (OMT) that is responsible for 4'-*O*-methylation and 3'-*O*-methylation of isoflavonoids in the generation of phytoalexins. Silencing of OMTs in pea affects the flux in the biosynthetic pathway, leading to a reduction in the phytoalexin pisatin (Liu *et al.*, 2006). A similar strategy may be applied to other modification enzymes, although it may be challenging to find enzymes that are not catalytically promiscuous, allowing control of a single pathway.

(v) Transcription factors. Altered expression of transcription factors regulating different parts of the flavonoid pathway could be an approach to altering activity of more than one enzyme. Members of the MYB, bHLH, and WD40 transcription factors play an important role in regulating the flavonoid pathway (Koes *et al.*, 2005; Du *et al.*, 2010). For example, it has been shown that ectopic expression of MYB transcription factors can significantly increase anthocyanin synthesis in tomato (Butelli *et al.*, 2008). In soybean, isoflavonoid synthesis was increased by a combination of ectopic expression of maize transcription factors and inhibition of the competing anthocyanin pathway (Yu *et al.*, 2003).

Challenges in manipulating the flavonoid pathway

While it appears opportunistic to modify the flavonoid pathway in order to manipulate root–rhizosphere interactions, the many interactions between plants, flavonoids, and microorganisms demand a cautionary approach. First, while most studies on the function of flavonoids have been done under laboratory conditions, their demonstration in real rhizosphere conditions remains to be carried out to determine how effective some of the flavonoid functions remain under conditions of breakdown, adsorption, metabolism, and altered solubility in the soil. Secondly, there are

likely to be unforseen effects of flavonoids on non-target organisms. Whereas certain flavonoids could enhance nodulation or mycorrhization of host plants, they could also indirectly affect bacterial quorum sensing, plant–plant interactions, and soil biochemistry. For example, catechin could inhibit quorum sensing in host-related soil bacteria, while it may also suppress plant growth as a potent allelopathic signal. Similarly, naringenin, which induced *nod* gene expression in several rhizobia, could have an effect on quorum sensing regulation in non-target bacteria. Exudation of isoflavonoids from soybean can stimulate attraction of its symbionts *Bradyrhizobium japonicum* as well as the devastating pathogen *Phytophthora sojae* (Morris *et al.*, 1998). In addition, metabolism of flavonoids by rhizosphere bacteria could alter their activity and availability in the soil and could affect other bacteria (Shaw *et al.*, 2006). Thirdly, altering concentrations or forms of flavonoids inside the plant tissue is likely to have effects on plant development which could be either beneficial or detrimental for the plant host. Flavonoids, via their effect on auxin transport (Brown *et al.*, 2001), have been shown to alter cell morphology, root growth, gravitropism, and light responses (Buer and Muday, 2004; Ringli *et al.*, 2008; Buer and Djordjevic, 2009).

Flavonoid exudation is also likely have effects on microbial community structure in the soil because it could increase species that use flavonoids as a carbon source while inhibiting the growth of others such as phytoalexins (Rao and Cooper, 1994; Walker *et al.*, 2003). Increased exudation of phenolics in the *Arabidopsis abcg30* mutant was shown to have wide-ranging effects on bacterial and fungal community structure, although this mutation also affected other exudates (Badri *et al.*, 2009).

Alteration of flavonoid synthesis is feasible and has been demonstrated in many studies in various plants, and both overexpression and inhibition of the flavonoid pathway using RNA interference have been successful (Wang *et al.*, 2011). In some cases, the transfer of a single gene encoding flavonoid enzymes might be sufficient to have an effect. For example, transfer of stilbene synthase from grapevine to tobacco resulted in increased resistance to *Botrytis cinerea* (Hain *et al.*, 1993). Altering glycosylation or targeting vacuolar transporters are other options that would allow the release of stored flavonoids from the vacuole for subsequent export (Weisshaar and Jenkins, 1998; Zhao *et al.*, 2011). One aspect of the manipulation of flavonoid synthesis or glycosylation is that it would be most efficient if it was targeted in the appropriate tissues (Fig. 2), for example the root epidermis for subsequent exudation. This would require the use of epidermal-specific promoters, which are currently not available for most crop plants.

An important drawback in the manipulation of specific branches of the flavonoid biosynthesis pathway could be that it alters fluxes through other branches of the flavonoid pathway or related pathways (Liu *et al.*, 2002; Wang *et al.*, 2011). This has been cited as one reason why sufficient flavonoid accumulation for large-scale production in engineered plants has not been successfully achieved (Fowler and Koffas, 2009). For example, mutants that show changes in the flavonoid pathway have been shown to have altered lignin biosynthesis, and vice versa, as both pathways share the same precursors. In the *cra* (*compact root architecture*) mutant of *M. truncatula*, alterations in the flavonoid pathway are accompanied by altered lignin biosynthesis with effects on root growth (Laffont *et al.*, 2010). Similarly, silencing of lignin synthesis in transgenic *Arabidopsis* plants resulted in increased flavonoid accumulation, and this increase was hypothesized to reduce plant growth (Besseau *et al.*, 2007). However, this was refuted in a more recent study that showed that a double mutant defective in *p*-coumaroyl shikimate 3'-hydroxylase (showing reduced lignin biosynthesis) and chalcone synthase (unable to synthesize flavonoids) showed similar growth to the single *p*-coumaroyl shikimate 3'-hydroxylase mutant (Li *et al.*, 2010).

The challenge of manipulating the flavonoid pathway in order to affect rhizosphere biology will involve more detailed information on the regulation of flavonoid transport and exudation than is currently available. While some flavonoid transporters are known, to the authors' knowledge none has definitively been demonstrated to be specific for exudation of flavonoids into the rhizosphere. Increased exudation of specific flavonoids into the rhizosphere would involve (i) alteration of specific branches of the flavonoid pathway to overexpress, newly express, or inhibit synthesis or to alter glycosylation; (ii) up-regulation of flavonoid exudation; and (iii) coupling of altered expression to rhizosphere signals that would specifically induce the desired pathways. The latter would require detailed knowledge of promoters and transcription factors that are specific for flavonoid induction by the correct trigger (Grotewold, 2008). Since flavonoid storage is compartmentalized within the cell and between different cell types (Fig. 2), altering flavonoid synthesis without control of their final destination could result in storage of flavonoids in the vacuole without release or release from the wrong region of the root where the target microorganisms are not found. For example, rhizobia only infect roots close to the root tip so flavonoid exudation would be most effective in that region.

As an alternative to genetic manipulation of the flavonoid pathway, it will be useful to exploit the huge diversity of flavonoids synthesized in different plant species (Dakora, 1995). As described above, the selection of intercropping plants producing *Striga*-inhibiting flavonoid exudates is one example that has shown success in making actual improvements to crop yields for farmers in sub-Saharan Africa.

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