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# Flavonoids as Signaling Molecules and Regulators of Root Nodule Development

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## ABSTRACT

Flavonoids are a diverse class of secondary plant metabolites, synthesized from phenylpropanoid precursors, which play an array of important functions in plants, ranging from floral pigments for the attraction of insect pollinators to antioxidants and auxin transport inhibitors. Many plant species also use flavonoids as signal molecules for beneficial microorganisms in the root rhizosphere, and as antimicrobial defense compounds in their interactions with pathogenic microbes. In legumes, flavonoids also play a critical role in promoting nitrogen-fixing symbiosis with rhizobia. Legume root-exuded flavonoids act both as chemo-attractants for symbiotic rhizobia, and signaling molecules for the activation of the expression of rhizobial *nod* genes, which are responsible for the synthesis of Nod factors, the bacterial signals that are necessary for the initiation of a new plant organ, the nodule. In addition, flavonoids also play a key role in enabling the initiation of differentiation of nodule primordia by inhibiting auxin transport in roots in response to rhizobial Nod factors. This review reports our present level of understanding on the role of flavonoids in the establishment of legume-*Rhizobium* symbiosis. Also described are the limits of our knowledge in this area of research, and how functional genomic strategies will help in clarifying the regulatory roles of individual flavonoids in mediating *nod* gene induction in rhizobial cells and auxin transport inhibition in the legume roots during the course of the development of legume-*Rhizobium* symbiosis.

**Keywords:** Auxin transport inhibition, flavonoids, legumes, *nod* gene induction, nodule development, nonlegumes, rhizobia, symbiosis
**Abbreviations: 4CL**, 4-coumarate CoA ligase; **C4H**, cinnamic acid 4-hydroxylase; **CHI**, chalcone isomerase; **CHR**, chalcone reductase; **CHS**, chalcone synthase; **DFR**, dihydroflavonol 4-reductase; **F3H**, flavanone 3-hydroxylase; **FLS**, flavonol synthase; **FNR**, flavone 4-reductase; **FNS**, flavone synthase; **FS**, Flavonol synthase; **IFS**, isoflavone synthase; **LCR**, leucoanthocyanidin reductase; **Nod factors**, lipo-chitin oligosaccharide nodulation factors; ***nod* genes**, nodulation genes; **NPA**, *N*-(1-naphthyl)phthalamic acid; **PAL**, phenylalanine ammonia lyase; **TIBA**, 2,3,5-triidobenzoic acid

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## INTRODUCTION

Flavonoids are a diverse group of secondary plant metabolites which are derived, via phenylpropanoid biosynthetic pathway, from condensation of 4-coumaroyl CoA and malonyl CoA. Chalcone synthase (CHS), a key enzyme in the phenylpropanoid metabolism facilitates this reaction, the first committed step of the flavonoid pathway, leading to the synthesis of a chalcone derivative, 4,2',4',6-tetrahydroxychalcone, from which a variety of the flavonoid end products are derived (Stafford 1990; Yu *et al*. 2006; **Fig. 1**). This vast group of compounds comprises of chalcones, flavanones, flavones, flavonols, flavandiols (leucoanthocyanidins), anthocyanidins, condensed tannins (proanthocyanidins), aurones and coumarins. In addition, some plant species, primarily legumes such as soybean, synthesize specialized flavonoids known as isoflavones. The basic structure of flavonoid compounds is the flavan nucleus consisting of 15 carbon atoms arranged in three rings named A, B and C. The different classes of flavonoids vary in the level of oxidation and pattern of decoration of the C ring, while individual compounds within a class differ in the configuration of substitution on the A and B rings (**Fig. 2**).

Many studies link flavonoids to a vast array of biological functions (Dixon and Steele 1999; Pietta 2000; Winkely-Shirley 2002 and references therein). Flavonoids act as catalysts during the light reaction of photosynthesis. They also confer protection to leaf cells from photo-oxidative damage by scavenging reactive oxygen species produced by the photosynthetic electron transport system as well as UV radiation. In addition, variously colored flavonoids such as anthocyanin pigments serve functions in plant reproduction by acting as visual signals for recruiting pollinators and seed dispersers. Because of their toxic/astringent properties,

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Invited Review

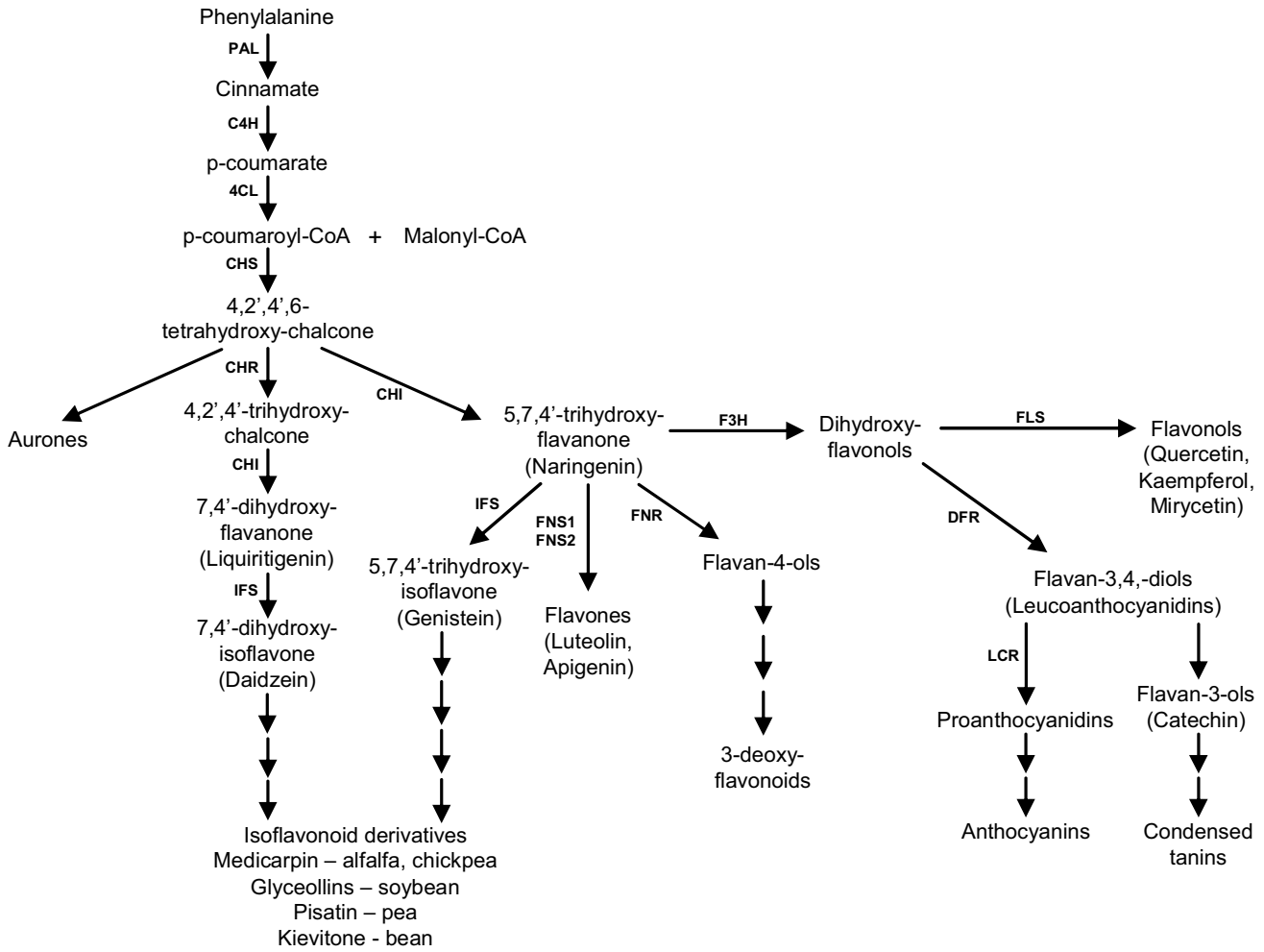


**Fig. 1 A partial diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in flavoniod biosynthesis.** 4CL, 4-coumarate CoA ligase; C4H, cinnamic acid 4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavone 3-hydroxylase; FLS, flavonol synthase; FNR, flavone 4-reductase; FNS, flavone synthase; IFS, isoflavone synthase; LCR, leucoanthocyanidin reductase; PAL, phenylalanine ammonia lyase.

some flavonoids form a defense system against phytopathogenic microorganisms and insect pests. In contrast to their role in defense against phytopathogenic microorganisms, flavonoids also participate in promoting mutually beneficial symbiotic relationship between legume plants and rhizobia leading to the formation of nitrogen-fixing root nodules.

Leguminous plants form symbiosis with soil bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Allorhizobium*, usually known as rhizobia (Hirsch 1992; Gage 2004). In addition, some tropical legumes are also capable of entering into symbiotic partnership with certain species of *Methylobacterium*, *Burkholderia* and *Ralstonia* (Chen *et al*. 2003). This plant-microbe interaction leads to the formation of specialized organs called nodules, in the roots of their host legumes, where the atmospheric nitrogen is converted into ammonium. Even if there are many examples of plants and rhizobia that can enter into symbiosis with more than one partner, only certain combinations of symbionts produce nitrogen-fixing nodules. Indeed, the specificity among compatible partners reduces the risk of infections and the formation of unproductive associations. This is to a large extent determined by two major sets of signals exchanged between the legume host and its microsymbiont partner. Legumes excrete, into their rhizosphere, various metabolites (carbohydrates, organic acids, vitamins, amino acids, phenolic substances, etc.) that aid interactions with rhizobia. Of these, the most important signaling molecules from the symbiotic perspective are flavonoids. Flavonoids act as chemotactic signals and metabolites to enable the congregation of rhizobia in the vicinity of root-hairs in the potential infection zone of the legume roots (Rao and Cooper 1994; Cooper *et al*. 1997). In addition, the exuded flavonoids function as key signalling molecules that specifically trigger the expression of nodulation (*nod*) genes in rhizobia promoting the synthesis of lipochitooligosaccharide compounds, also known as Nod factors, which are bacterial signal molecules for nodule organogenesis in legumes (Zaat *et al*. 1988; Stafford 1997; Broughton *et al*. 2000; Perret *et al*. 2000). Nod factors elicit, in a host-specific manner, many of the plant responses observed during early stages of nodule formation. These responses include changes in free calcium levels and ion balance, alterations in cytoskeletal organization and reorientation of the root hair cell wall growth leading to the formation of curled root-hairs around the invading rhizobia, the development of tubular structures known as infection threads to facilitate the bacterial entry into the plants, the initiation of cortical cell divisions, and the formation of nodule primordia, as well as nodules (Gage 2004; Oldroyd and Downie 2004; Mulder *et al*. 2005). In addition to stimulating Nod factor production, flavonoids (whose synthesis is upregulated in plants, upon perception by the *Rhizobium*-derived Nod factors) also play a part in promoting cortical cell divisions leading to nodule initiation in roots through their action on the plant hormone auxin (Hirsch 1992; Mathesius *et al*. 1998a, 2003; Wasson *et al*. 2006).

This review will cover the various processes/mechanisms mediated by flavonoids that enable the establishment of *Rhizobium* symbiosis and nodule development in leg-

umes. In addition, at the end, a brief discussion is included on the effects of flavonoids on root colonization by rhizobia in nonleguminous plants, and how the amendment of flavonoid biosynthetic pathway in them could pave a way for enhancing such plant-microbe interactions.

## FLAVONOIDS ACT AS CHEMO-ATTRACTANTS, AND GROWTH REGULATORS OF RHIZOBIA

Interaction of plants with microbes in the rhizosphere is contingent upon on the ability of roots to communicate with microbes, and the microbes rely on their associations with plants that are often mediated by carbon compounds released through root exudates. Among the various carbon compounds released from the roots, organic acids, amino acids, sugars, phenolic substances including flavonoids, etc., form important components. A major role described for flavonoids in legume root nodulation is as inducers of *nod* gene expression in rhizobia (see below). However, studies with *Sinorhizobium meliloti* revealed that certain plant-derived flavonoids also serve as chemo-attractants for rhizobia in a species-specific manner (Aguilar *et al*. 1988; Armitage *et al*. 1988; Dharmatilake and Bauer 1992) to promote bacterial movement towards the roots for establishing contact, paving way for subsequent colonization and infection leading to nodule development (Phillips and Tsai 1992). Interestingly, mutations in *nodD1* and *nodA*, besides abolishing *nod* gene expression, were also found to eliminate the chemotactic response of *S. meliloti* to the flavonoid luteolin; thus alluding to the possibility of a genetic connection between luteolin chemotaxis and *nod* gene induction (Caetano-Anolles *et al*. 1988). Subsequent studies, however, evidenced that even though *nod* gene induction and flavonoid chemotaxis have same biochemical specificity, these two functions appear to have independent receptors or transduction pathways (Dharmatilake and Bauer 1992).

In spite of the ability exhibited by the flavonoids to function as chemo-attractants, extensive studies revealed that the chemotactic responses of rhizobia to various *nod* gene inducing flavonoids was found to be weak and variable; typically only in the range between 2 to 5 times higher than background, in contrast to the 10- to 100-fold response elicited by organic and amino acids (Bauer and Caetano-Anolles 1990). Thus, comparing to the chemotactic property of organic and amino acids in root exudates, flavonoids appear to play a minor role in attracting rhizobia to the root surface.

Several lines of investigation indicated that plant flavonoids also act as growth regulators of rhizobia (Phillips and Tsai 1992). For example, daidzein, a *nod* gene inducing isoflavonoid from soybean, and luteolin-7-*O*-glucoside and quercetin-3-*O*-galactoside from alfalfa were found to enhance growth of *Bradyrhizobium japonicum* (D'Arcy-Lameta and Jay 1987) and *S. meliloti* (Hartwig *et al*. 1991), respectively. It is likely that growth effects of flavonoids help regulate the rhizobial colonization of roots, thus paving way for the establishment of nodular symbiosis in legumes. Apparently, similar to chemotactic responses, the growth altering effect of flavonoids is also controlled independently from *nod* gene induction, because a strain of *S. meliloti* mutated in all *nodD* genes still grew faster with luteolin and quercetin (Hartwig *et al*. 1991).

## FLAVONOIDS REGULATE *NOD* GENE EXPRESSION IN RHIZOBIA

Early studies using *in vivo* bioassays involving legume seedlings/plantlets grown in the presence of *nod-lacZ* tagged rhizobial cells showed that the exudates secreted from roots are able to influence *nod* gene expression in rhizobia (Innes *et al*. 1985; Redmond *et al*. 1986; Djordjevic *et al*. 1987; Peters and Long 1988; Goethals *et al*. 1989, 1990). Detailed examination of the *in vivo* plate induction assays revealed that the exudates behaved as either stimulators or inhibitors of *nod* gene expression depending on the root zone from which they were released (Redmond *et al*. 1986; Djordjevic *et al*. 1987; Peters and Long 1988). Subsequently it was demonstrated that the flavonoids present in the root exudates are the key compounds responsible for *nod* gene expression in rhizobia (Firmin *et al*. 1986; Peters *et al*. 1986; Redmond *et al*. 1986; Djordjevic *et al*. 1987).

Different legumes release various sets of flavonoid compounds, and *in vitro* studies have demonstrated that these compounds are perceived as either inducers or inhibitors of the expression of nodulation (*nod*) genes in rhizobia living in the rhizosphere (Firmin *et al*. 1986; Peters *et al*. 1986; Redmond *et al*. 1986; Djordjevic *et al*. 1987; Peters and Long 1988; Phillips and Kapulnik 1995; Zuanazzi *et al*. 1998; **Table 1; Fig. 2**). For example, analysis of the extracts from white clover (*Trifolium repens*) seedlings revealed three particularly active compounds: one was the inducer compound 4',7-dihydroxyflavone, which could induce *nod* gene expression in *Rhizobium leguminosarum* bv. *trifolii*, while the other two bioactive substances, present at 3-4 times the concentration of the active flavone, were formononetin and umbelliferone, which were inhibitory to the *nod* gene expression from being induced by the stimulatory molecule 4',7-dihydroxyflavone (Redmond *et al*. 1986). The production of the *nod* gene-inducing and -inhibiting flavonoids is tightly regulated by the developmental stage of the root. There are evidences to show that the *nod* gene inducer production is localized to the part of the elongating root hair zone that is maximally receptive to rhizobial infection. In white clover, the inducer flavonoid (4',7-dihydroxyflavone) for *nod* gene induction in its microsymbiont *R. leguminosarum* bv. *trifolii* is chiefly excreted from the cells behind growing root tip in the zone of emerging root hairs, while the release of the anti-inducers (formononetin and umbelliferone) occurs in the region between this zone and the root tip, and elsewhere on the root (Djordjevic *et al*. 1987). Also in *Medicago sativa* (alfalfa) the production of the flavonoids 4',7-dihydroxyflavone and methoxychalcone that trigger *nod* gene induction in its symbiont *S. meliloti* is largely restricted to the newly developing root hair zone from which most nodules later develop (Peters and Long 1988; Zuanazzi *et al*. 1998). In addition to the developmental regulation, nitrogen status of the plant also contributes to root flavonoid production in legumes. Studies with alfalfa showed that nitrogen limitation increases the production of all root-secreted flavonoids, particularly those that promote the *S. meliloti nod* gene inducing activity (Coronado *et al*. 1995). The secretion of flavonoids is indispensable for the establishment of *Rhizobum*-legume symbiosis. And recently it has been evidenced in soybean that a membrane localized ATP-binding ABC-type transporter facilitates the secretion of flavonoids from roots (Sugiyama *et al*. 2007).

Many bacterial genes are important for the establishment of symbiosis between plants and rhizobia. Among them are *nod*, *nol* and *noe* genes that play a very critical role. The *nod* genes are involved in the synthesis of extracellular Nod factors, which perform a central role in determining the fate of the symbiotic interaction (Dénarié *et al*. 1996; Long 1996; Spaink 2000; Broughton *et al*. 2000; Miklashevichs *et al*. 2001; D'Haeze and Holsters 2002). *In vitro* studies demonstrated that the regulation of *nod* gene expression in rhizobia is mediated by plant derived flavonoids that act as inducers of gene expression in a mechanism requiring a *nodD* gene product (see Schlaman *et al*. 1998; **Fig. 3**). In all rhizobial species the induction of *nod* gene expression is almost invariably mediated by NodD, even though the type of inducer flavonoid required for the induction of the gene activity varies from one species to another (**Table 1**). The NodD proteins are constitutively expressed in all rhizobia that have been examined. NodD proteins belong to a family of LysR-like transcriptional regulators that bind to highly conserved 49-bp 5' DNA elements, referred to as *nod* box, present in the promoter regions of many nodulation genes, and promote their expression (Rostas *et al*. 1986; Fisher *et al*. 1988). Even though NodD proteins are able to bind to *nod* boxes even in the absence of an

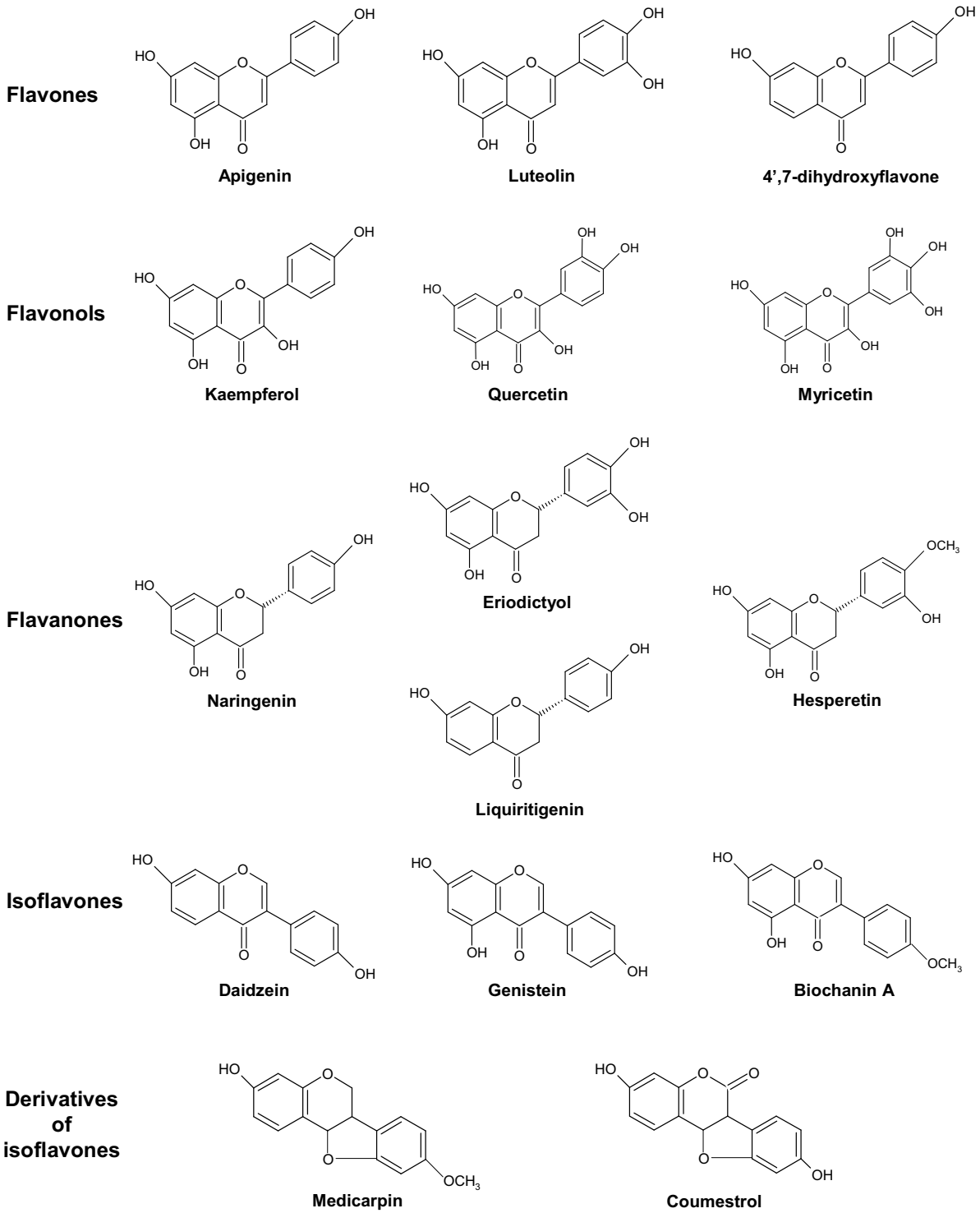


**Fig. 2 Structures of representative legume-secreted flavonones, isoflavones, flavones and flavonols that participate in *nod* gene induction in rhizobia.**

inducer, interaction with flavonoids is essential for the expression of *nod* genes (Fisher *et al*. 1988; Fisher and Long 1993; Györgypal and Kondorosi 1991; Goethals *et al*. 1992; Peck *et al*. 2006).

Initial interaction between NodD protein and flavonoid seem to occur in the cytoplasmic membrane, since both molecules are localized in this compartment. In *R. leguminosarum* bv. *viciae*, the inducing flavonoid naringenin was found to accumulate at the cytoplasmic membrane (Recourt *et al*. 1989) where NodD proteins are localized (Schlaman *et al*. 1989). It has also been suggested that, in the cases where NodD is in the cytosol, as in *S. meliloti*, when the appropriate inducer flavonoid is supplemented to the cell (Kondorosi *et al*. 1989), NodD migrates towards the cytoplasmic membrane to facilitate the interaction with the flavonoid. Computational analysis has predicted a hydrophobic domain harbouring proline residues that could facilitate the presumed membrane-anchoring of NodD (Schlaman *et al*. 1989).

Although it has not been possible to show any direct interaction between flavonoids and NodD, many studies support this theory: First, by comparing the structures of NodD with several nuclear receptors, it has been noticed that both types of proteins share conserved ligand-binding regions at the C-terminal end of their respective DNA binding domains (Györgypal and Kondorosi 1991). Second, through *in vitro* studies it was shown that the GroEL chaperonin co-purifies with NodD1 in *S. meliloti*, and that it is only in the presence of GroES, ATP and luteolin (which has been reported to be an inducer flavonoid in *S. meliloti* 1021;

**Table 1** Inducibility of *nod* gene expression in rhizobia by legume-derived flavonoids.

| Bacterium | Host Plant | Flavonoids | |
|---|---|---|---|
| | | **Inducers** | **Anti-inducers** |
| *Sinorhizobium fredii* USDA191 | *Glycine max* | Daidzein[1] ++++ | |
| | | Coumestrol[1] ++++ | |
| | | 7-hydroxyisoflavone[1] ++++ | |
| | | 5,7-dihydroxyisoflavone[1] ++++ | |
| | | 4',7-dihydroxyflavone[1] +++ | |
| | | Genistein[1] +++ | |
| | | Apigenin[1] ++ | |
| | | Luteolin[1] ++ | |
| | | Naringenin[1] ++ | |
| *Sinorhizobium meliloti* 1021 | *Medicago sativa* | Luteolin[2, 3] ++++ | Coumestrol[6] |
| | | Apigenin[4] +++ | Medicarpin[6] |
| | | 4,4'-dihydroxy-2'-methoxychalcone[5] +++ | |
| | | 4',7-dihydroxyflavone[5] ++ | |
| | | Liquiritegenin[5] + | |
| | | Eriodictyol[4] + | |
| *Rhizobium* sp. NGR234* | *Sesbania grandiflora* | 4',7-dihydroxyflavone[3] ++++ | |
| | *Phaseolus leptostachyus* | Apigenin[3] +++ | |
| | *Glycine tabacina* | Naringenin[3] +++ | |
| | *Desmodium* | Daidzein[3] ++ | |
| | *dichotomum* | Liquiritegenin[3] ++ | |
| | | Genistein[3] + | |
| *Rhizobium leguminosarum* bv. *viciae* | *Pisum sativum* | Hesperetin[7, 8] ++++ | Daidzein[8] |
| Rlv 300 | *Lens culinaris* | Eriodictyol[8, 9] ++++ | Genistein[8] |
| | | Naringenin[7, 8] +++ | Kaempferol[8] |
| | | Apigenin[7, 8] +++ | |
| | | Luteolin[7, 9] ++ | |
| | | 7-hydroxyflavone[9] + | |
| *Rhizobium leguminosarum* bv. *trifoli* ANU843 | *Trifolium repens* | 4',7-dihydroxyflavone[10] ++++ | Umbelliferone[11] |
| | | 7-hydroxyflavone[9] ++++ | Formononetin[11] |
| | | Geraldone[10] +++ | |
| | | Luteolin[9] +++ | |
| | | Naringenin[9] ++ | |
| | | 4'-hydroxy-7-methoxyflavone[10] + | |
| *Rhizobium leguminosarum* bv. *phaseoli* CIAT956 | *Phaseolus vulgaris* | Genistein[12] ++++ | |
| | | Naringenin[12] ++++ | |
| | | Isoliquiritigenin[13] +++ | |
| | | Liquiritigenin[13] +++ | |
| | | Daidzein[13] ++ | |
| | | Coumestrol[13]+ | |
| | | Eriodictyol[12] + | |
| *Rhizobium tropici* CIAT899 | *Phaseolus vulgaris* | Apigenin[14] ++++ | |
| | *Leucaena esculenta* | Naringenin[14] ++++ | |
| *Bradhyrizobium japonicum* USDA110 | *Glycine max* | Genistein[1, 15, 16] ++++ | Chrysin[17] |
| | | Daidzein[1, 15, 16] ++++ | Luteolin[17] |
| | | Isoliquiritigenin[15] ++++ | Apigenin[17] |
| | | 5,7-dihydroxyisoflavone[1] ++++ | 4',7-dihydroxyflavone[17] |
| | | 7-hydroxyisoflavone[1] +++ | 7-hydroxyflavone[17] |
| | | 4',7-dihydroxyflavone[1, 3] ++ | Naringenin[17] |
| | | Coumestrol[1, 15] + | Umbelliferone[17] |
| | | Apigenin[1, 3] + | Coumestrol[17] |
| *Azorhizobium caulinodans* ORS571 | *Sesbania rostrata* | Naringenin[18, 3] ++++ | 7,2'dihydroxyflavanone[19] |
| | | Liquiritigenin[19, 3] ++++ | |
| | | Isoliquiritigenin[19] ++ | |

*NGR234 was found to nodulate 112 genera of legumes (Pueppke and Broughton 1999). *nod* gene inducing ability of flavonoids: +, weak; ++, moderate; +++, good; ++++, strong. [1]Kosslak *et al*. 1987; [2] Peters *et al*. 1986; [3]Sreevidya *et al*. 2006; [4]Peters and Long 1988; [5] Maxwell *et al*. 1989; [6] Zuazzani *et al*. 1998; [7]Begum *et al*. 2001; [8]Firmin *et al*. 1986; [9]Peck *et a*l. 2006; [10]Redmond *et al*. 1986; [11]Djordevik *et al*. 1987; [12] Hungria *et al*. 1991; [13]Bolaños-Vásquez and Werner 1997; [14] Sousa *et al*. 1993; [15]Kape *et al*. 1992; [16]Banfalvi *et al*. 1988; [17]Kosslak *et al*. 1990; [18] Goethals *et al*. 1989; [19] Messens *et al*. 1991.

**Table 1**) that NodD1 is able to bind the *nod* boxes (Yeh *et al*. 2002). Therefore, Yeh *et al*. (2002) hypothesized that adding GroES and ATP to the NodD1-GroEL complex helps NodD1 to fold with luteolin, which allows the protein to bind the *nod* boxes. This suggested that the GroESL system together with the appropriate inducer flavonoid is essential to effect conformational changes in NodD1 to augment its role as a transcriptional regulator. Having this background, the DNA binding activity of purified NodD1 has been determined, treating the protein, *in vivo* and *in vitro*, with luteolin, to understand the biochemical effects of the protein-flavonoid interaction (Peck *et al*. 2006). It was observed that preincubation of GroES and ATP with high levels of NodD1 lead to the formation of the NodD1-*nod* box complex; however, adding luteolin during the preincubation stimulated dramatically the formation of the complex. An enhanced binding of *nod* box DNA domain by NodD in the presence of the flavonoid inducer suggested that NodD acts as a specific receptor for flavonoids. Deciphering the 3-dimensional structure of NodD by X-ray crystallography may provide an insight into the possible features of the protein that facilitate NodD-flavonoid interaction.

Studies with other flavonoids that do not enable the activation of NodD proteins, such as naringenin, daidzein and eriodicytol for NodD1 in *S. meliloti*, revealed that even with the noninducer flavonoids the NodD DNA binding affinity

at *nod* gene promoters reaches similar levels to those attained when the inducer flavonoid is added (Peck *et al*. 2006). According to these results the elevated capacity to bind DNA in the promoter region of the *nod* genes is not specific for the inducer flavonoid. Earlier it was hypothesized that anti-inducer flavonoids compete with inducer compounds and occupy the inducer binding site on NodD, leading to a very poor or even null NodD activity, and hence resulting in the diminished *nod* gene induction (Djordjevic *et al*. 1987; Peters and Long 1988; Zuanazzi *et al*. 1998). Consistent with this hypothesis, in *S. meliloti*, it was observed that luteolin-mediated NodD1-dependent *nodC* expression decreased under the conditions of increasing amounts of noninducing naringenin, daidzein and eriodicytol compounds in the presence of luteolin (Peck *et al*. 2006). In contrast, increasing amounts of inducer luteolin was found to overcome the inhibitory effect produced by the noninducing compound such as naringenin. These results clearly evidenced that noninducer flavonoids compete for the interaction domains with the inducer flavonoids, to prevent the activation of NodD1 from activating *nod* gene transcription. It seems that the negative regulation of *S. meliloti nod* gene expression by some flavonoids may contribute to an optimal level of *nod* gene expression. A net positive interaction between the flavonoid compounds in the root exudates and NodD appears to be crucial for efficient nodulation to occur. Generally, the concentrations required for antagonistic effect of inhibitory flavonoids are in the range of 1 – 10 orders of magnitude greater than the concentration required for *nod* gene induction by the most potent of the activating flavonoid (Firmin *et al*. 1986; Djordjevic *et al*. 1987). Thus it is the composition and ratio of inducer:anti-inducer flavonoids liberated from the plant that ultimately determines the *nod* gene activity. Therefore the production of nonactivating flavonoids by legumes could be another mechanism to regulate the nodulation process, including the control of nodule number.

The nodulation process requires a very strict regulation of the Nod factor production, since high and persistent levels of these compounds, promote defense-like responses in the plant, which interrupt the infection process. Besides, it is known that a strong or ectopic *nod* genes expression, leads to a defective nodulation. This is why rhizobia have evolved different mechanisms to control the transcription of the nodulation loci. It is remarkable that the response of rhizobia to different flavonoids, as reflected by the activity of NodD proteins, is specific for each species (Spaink *et al*. 1987; Fellay *et al*. 1995). In addition, as in the case of *S. meliloti*, even various NodD homologues (NodD1, NodD2 and NodD3) in the same strain may have different flavonoid preferences (Hartwig *et al*. 1990; Györgypal and Kondorosi 1991; Phillips *et al*. 1992); NodD1 and NodD2 are activated by plant derived inducer flavonoids (Peters *et al*. 1986), but NodD3 is independent of flavonoid requirement (Honma *et al*. 1990; Mulligan and Long 1989). Several lines of evidence suggest that NodD controls the response of rhizobia to flavonoids in a species-specific manner (Horvath *et al*. 1987; Bender *et al*. 1988). In order to clarify this hypothesis, Spaink *et al*. (1987) introduced in to *R. leguminosarum* bv. *trifolii* the plasmids carrying *nodD1* from *S. meliloti*, *nodD* from *R. leguminosarum* bv. *trifolii* or *nodD* from *R. leguminosarum* bv. *viciae*, and measured the induction of the *nod* genes in the presence of a spectrum of flavonoids. These studies demonstrated that the profile of flavonoids needed to induce the *nod* genes shifted depending upon the source of NodD. In an analogous study, Peck *et al*. (2006) expressed the open reading frames of *nodD1* from *S. meliloti* and *nodD*s form *R. leguminosaum* bv. *viciae* and *R. leguminosaum* bv. *trifolii*, under the control of a constitutive promoter in *S. meliloti* (which carried insertional mutations in it's *nodD1*, *D2* and *D3* genes), and measured the induction *nod* gene expression using the reporter construct *nodC-lacZ*. When the native NodD1 from *S. meliloti* was expressed, there was a very strong induction mediated by luteolin, while in the other two cases, the induction occurred, in addition to luteolin, with eriodictyol (with *R. leguminosaum* bv. *viciae* NodD) as well as 4',7-dihydroxyflavone (with *R. leguminosaum* bv. *trifolii* NodD). These results confirmed the earlier findings that changing the source of NodD alters the response of the host cell to a set of flavonoid inducers. The ability of the NodD proteins to differentially sense inducers and to activate the transcription of the *nod* genes functions as a key determinant of host specificity.

In *Bradyrhizobium japonicum*, the flavonoid-dependent activation of the *nod* genes is controlled by the two-component system NodV/NodW. Although NodD1 is crucial for the expression of the *nod* genes, mutants in *B. japonicum* for this factor are able to nodulate the host, but in a lower rate compared to the wild type (Göttfert *et al*. 1992). This apparent contradiction is explained with the regulatory system NodVW, which upon activation by the isoflavone genistein induces the expression of the *nod* genes in *B. japonicum*. The phosphorylation of both NodV and NodW was shown to be essential for inducing the expression of the *nod* genes (Loh *et al*. 1997). It has been demonstrated that phosphorylation of NodW is genistein-induced, and depends on both acyl phosphate and the activity of NodV kinase.

Different flavonoids interact with NodD to induce a signal transduction cascade, which controls the nodulation process. There seems to be a correlation between the spectrum of flavonoids that interact with NodD and the host range that rhizobia infect. For example, NodD proteins from species with a few hosts, like *S. meliloti*, *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae*, respond to a small number of flavonoids (Zaat *et al*. 1988), while other species such as *Rhizobium* sp. NGR234 with a wide range of host plants respond to a large number of molecules that, besides flavonoids, include aromatic compounds such as vanillin and isovainillin (Bassam *et al*. 1988; Le Strange *et al*. 1990). In addition, there are synergistic interactions between flavonoids in some species. In the strain *R. leguminosarum* pIJI477, a combination of naringenin and hesperetin induces the transcription of the *nod* genes at a higher level than any of these flavonoids individually (Begum *et al*. 2001). In *S. meliloti,* 4,4'-dihydroxy-2'-methoxychalcone interacts synergistically with luteolin (Hartwig *et al*. 1989). Similar to the synergistic effects produced by two strong inducers, weak inducers can also act synergistically to promote enhanced *nod* gene induction in rhizobia. For example, coumestrol is a weak inducer of the *nod* genes in *R. leguminosarum* bv. *phaseoli*, followed by daidzein, liquiritigenin, isoliquiritigenin, naringenin and genistein (arranged according to their efficacy). When increasing amounts of liquiritigenin or isoliquiritigenin were added, together with daidzein, the expression of the *nod* genes was dramatically induced, showing a synergistic event (Bolaños-Vásquez and Werner 1997). The synergism produced by a chalcone and luteolin, a flavone, in *S. meliloti*, is comparable to the case of isoliquiritigenin and daidzein, an isoflavone, in *R. leguminosarum* bv. *phaseoli*. Similar results have been obtained in *R. leguminosarum* bv. *phaseoli* (Hungria *et al*. 1992) with eriodictyol and naringenin combined with genistein. Therefore, it can be concluded that chalcones act synergistically with flavones and isoflavones. Possibly interaction of isoflavonoids and flavanones, or flavones and chalcones, at the same binding site in NodD, likely contribute to enhanced *nod* gene expression.

It is worthwhile to mention here that the studies conducted on rhizobial *nod* gene expression have so far focused only on a few known compounds of flavonoids. However, examination of the metabolite profiles derived from various legume root exudates/extracts (for example, Redmond *et al*. 1986; Peters *et al*. 1986; Graham 1991; Wasson *et al*. 2006) reveal a range of unidentified compounds that may have effects on *nod* gene induction. It is possible that many of the unidentified flavonoids, which await analysis, play a critical role, in a *Rhizobium*-specific manner, in regulating nodulation processes.

In addition, besides flavonoids, some non-flavonoid compounds such as betaines released from alfalfa seeds

were found to act as *nod* gene inducers in *S. meliloti* (Phillips *et al.* 1992). Similarly, erythronic acid and tetronic acids found in root exudates and seed effusates of *Lupinus albus* were also shown to induce *nod* gene expression in *R. lupini* and *S. meliloti* (Gagnon and Ibrahim 1998). Interestingly, it has been observed that tetronic acid is able to produce synergistic effect on *nod* gene induction in *S. meliloti*, when added together with luteolin. In contrast, organic acids such as such as malate, succinate and α-ketoglutarate (common carbon sources used by bacteroids in nodules) were found to inhibit *nod* gene expression in *B. japonicum* (Yuen and Stacey 1996). These results evidenced a role for nonflavonoid compounds as well, in modulating *nod* gene expression in rhizobia.

## RHIZOBIA AND NOD FACTORS PROMOTE FLAVONOID SYNTHESIS IN LEGUME ROOTS

Several studies demonstrated that flavonoid gene expression and flavonoid production are developmentally regulated during plant growth even prior to rhizobial infection and nodule formation. In legumes, production of flavonoid compounds, both qualitatively and quantitatively, varies for different regions of the developing root (discussed above; Djordjevic *et al.* 1987; Rolfe 1988). Fluctuations in the synthesis of these compounds in the plant roots are perhaps governed by cell- or tissue-specific developmental activities *vis-à-vis* cellular and environmental stimuli. There is ample of evidence showing that flavonoid synthesis is up regulated in legumes during interaction with rhizobia, leading to a change in the internal flavonoid pool of the root (Recourt *et al.* 1992; Lawson *et al.* 1994). It has been demonstrated that indeed the production of various flavonoids is both qualitatively and quantitatively increased in root exudates in response to the influence of homologous rhizobia (Dakora *et al.* 1993a, 1993b; Bolaños-Vásquez and Werner 1997). In fact, quantification of the flavonoids naringenin, isoliquiritigenin, liquiritigenin, genistein, daidzein and coumestrol found in the root exudates of *Phaseolus vulgaris* inoculated with the various strains of its symbionts *R. etli*, *R. leguminosarum* bv. *phaseoli* and *R. tropici* revealed strain-specific effect on flavonoid production in the legume roots (Bolaños-Vásquez and Werner 1997). For instance, *R. etli* CIAT 2510 and *R. leguminosarum* bv. *phaseoli* CIAT 956 were able to markedly elevate the levels of naringenin, isoliquiritigenin and daidzein in the root exudates as compared to *R. etli* TAL182. Soybean roots showed increased levels of isoflavone production in response to *B. japonicum* treatment (Cho and Harper 1991a, 1991b). Using *Vicia sativa*, van Brussel *et al.* (1990) demonstrated that the application of Nod factors alone is sufficient for increasing the exudation of *nod* gene-inducing flavonoids by the root.

Flavonoids are synthesized by the enzymes of the phenylpropanoid pathway (**Fig. 1**). In order for the upregulation of flavonoid production, it is essential that the genes specific for encoding the enzymes required for flavonoid synthesis should be activated first. Indeed inoculation of various legumes with their homologous microsymbionts was found to promote rapid induction of chalcone synthase (*CHS*) and other genes (isoflavone synthase, etc.) involved in flavonoid biosynthesis in roots prior to (Estabrook and Sengupta-Gopalan 1991; Lawson *et al.* 1994; Krause *et al.* 1997; McKhann *et al.* 1997a; Subramanian *et al.* 2004) and during nodule development (Yang *et al.* 1992; Charrier *et al.* 1995). Analysis utilizing isoflavone synthase 1 (*GmIFS1*) promoter–*gusA* fusion in *Glycine max* (soybean) and flavone synthase II-2 (*MtFNSII-2*) promoter-*gusA* fusion in *M. truncatula*, showed that inoculation with the respective homologous rhizobial strains (*B. japonicum* or *S. meliloti*) promoted the expression of *IFS1* and *MtFNSII-2* activity in vascular tissues and root hairs in the infection competent root zone (Subramanian *et al.* 2004; Zhang *et al.* 2007). Expression studies with the Pr*CHS3-gusA* reporter gene in white clover revealed that upon inoculation with the compatible *R. leguminosarum* bv. *trifolii* strain, chalcone synthase promoter activity is upregulated specifically in the cortical

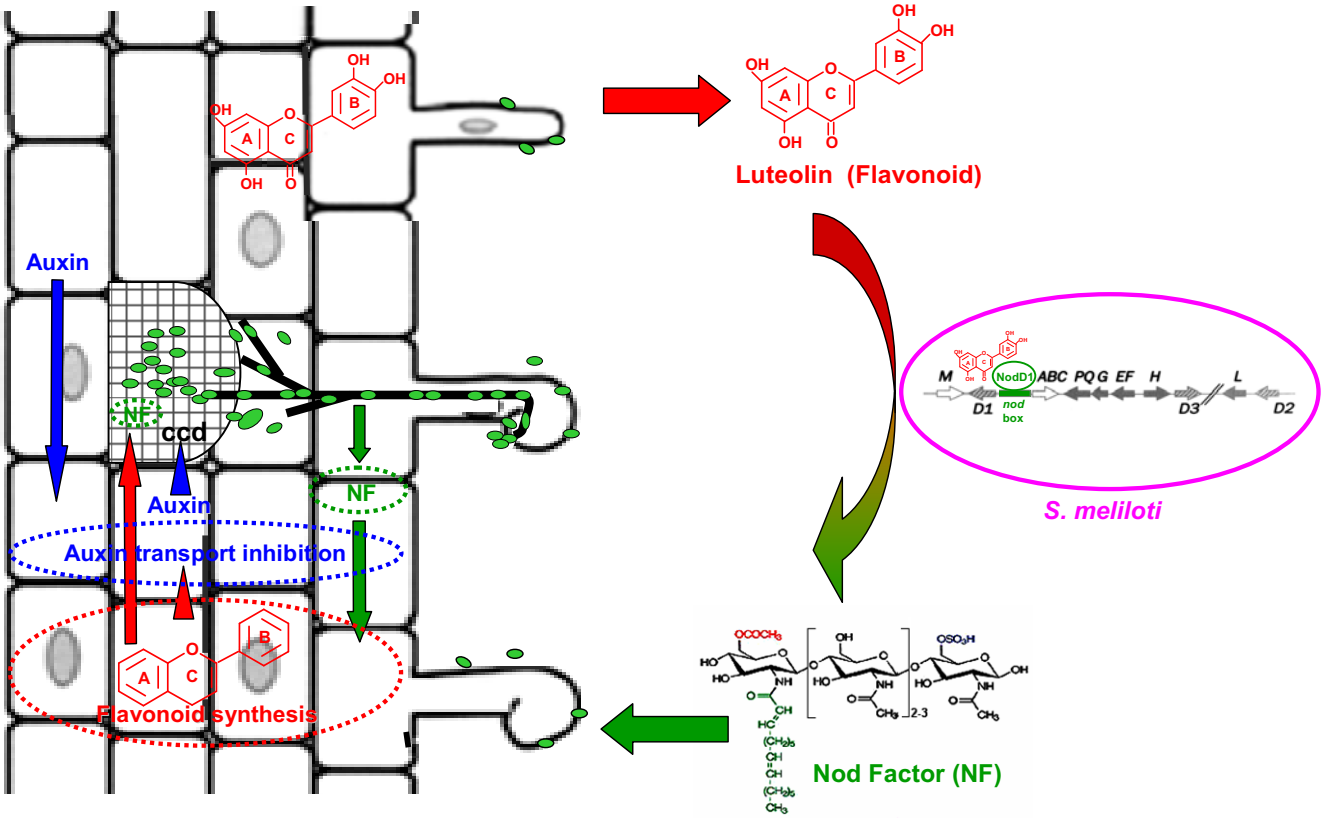


**Fig. 3 Flavonoid-mediated processes during the development of *Rhizobium*-legume symbiosis and nodulation.** As an example, flavonoid-activated symbiotic responses in *Sinorhizobium meliloti* and *Medicago truncatula* are represented in this diagram. ccd: Cortical cell divisions; Arrows and arrowheads – Red: Flavonoid secretion and Flavonoid-elicited processes; Green: Nod factor production and Nod factor-triggered flavonoid synthesis; Blue: Auxin transport and auxin-promoted cortical cell divisions. See text for details.

cells located in the infection competent susceptible zone of the root, and developing nodule primordia, alluding to the suggestion that the *Rhizobium*-triggered flavonoid production takes place at the earliest stages of nodule initiation (Mathesius *et al*. 1998a, 2000). Further detailed *in vivo* analysis of transgenic white clover confirmed that rhizobial inoculation indeed elicits *de novo* synthesis of flavonoids in nodule-competent inner cortical cells even prior to the initiation of cortical cell divisions, which eventually lead to the development of nodule primordium and nodule (Mathesius *et al*. 1998a). Studies with other legumes have further shown that purified Nod factors of rhizobia are responsible and sufficient for the promotion of *CHS* expression (Savouré *et al*. 1997; Krause *et al*. 1997) and flavonoid accumulation (Schmidt *et al*. 1994; Mathesius *et al*. 1998a). These findings indicated that Nod factors mediate the signal transduction cascade that leads to the induction of *CHS*, and thereby flavonoids, in the legume roots (**Fig. 3**).

In addition to the *de novo* activation of flavonoid biosynthesis during symbiotic interaction, flavonoid synthesis is also triggered in legumes as a defense response against pathogens (Nicholson and Hammerschmidt 1992; Dieter 2006). Indeed there is ample evidence to show that *PAL* genes involved in flavonoid biosynthesis are activated in plants during pathogen attack. However, several studies in legumes showed that the gene activation leading to flavonoid biosynthesis triggered by rhizobial symbionts differs from that produced by the action of pathogens. For example, investigations on soybean-*B. japonicum* symbiosis have evidenced that the symbiotic-specific *PAL* and *CHS* genes activated during the early stages of symbiosis are clearly distinct from other gene family members induced by pathogens (Estabrook and Sengupta-Gopalan 1991). Using microarray analysis, Lohar *et al*. (2006) demonstrated that in *M. truncatula* the expression of the genes involved in flavonoid biosynthesis is altered from 6 hours onwards after inoculation (hai) with *S. meliloti*: some members of the chalcone synthase family are up-regulated from 6 to 72 hai compared to control roots, while others are up-regulated transiently, and some are down-regulated. In an analogous study, McKhann *et al*. (1997b) working with alfalfa observed that although inoculation with *S. meliloti* triggers some of the *PAL* genes induced during an interaction between a host and a pathogen, the expression of these genes in the *Rhizobium*-legume interaction is at a very low level, suggesting that rhizobia have evolved a mechanism(s) to avoid triggering the host's defense responses. The cascade of plant responses leading to nodule development is triggered by the recognition of rhizobia-produced Nod factors by a family of LysM receptor kinases, which include NFR1 and NFR5 in *L. japonicus*, and NFP, LYK3 and LYK4 in *M. truncatula* (see Oldroyd and Downie 2004, and references therein). It will be interesting to investigate whether the interaction of Nod factor with these receptors mediates a mechanism(s) that suppresses defense responses that are normally activated during pathogen attack.

## ROLE OF FLAVONOIDS ON AUXIN TRANSPORT INHIBITION AND NODULE DIFFERENTIATION

Several lines of evidence evoke a role for the plant hormone auxin in nodule development. Synthetic auxin transport inhibitors, such as the *N*-(1-naphthyl)phthalamic acid (NPA) and 2,3,5-triidobenzoic acid (TIBA), induce the formation of structures, called pseudonodules, on legume roots that share similarities, including the expression of early nodulin genes, with *Rhizobium*-induced nodules (Allen *et al*. 1953; Torrey 1986; Hirsch *et al*. 1989). Based on these results Hirsch *et al*. (1989) proposed that treating the legume roots with auxin transport inhibitors lead to an endogenous hormone imbalance which is manifested by cell divisions and the formation of pseudonodules. Similar to NPA, application of purified Nod factors was shown to impede auxin transport in legume roots (Mathesius *et al*. 1998b; Boot *et al*. 1999; Pacios-Bras *et al*. 2003). At the same time Nod factors were also shown to have the ability to promote cortical cell divisions leading to the formation of nodule primordia, as well as nodules (Truchet *et al*. 1991; van Brussel *et al*. 1992). Therefore, it could be suggested that the Nod factors produced by rhizobia are able to mimic the activity of auxin transport inhibitors (Hirsch 1992; Mathesius *et al*. 1998b). However, since Nod factors do not share any structural similarities with known auxin transport inhibitors, it was suggested that an intermediate signaling molecule may be involved in the regulation of auxin transport (Wasson *et al*. 2006). Thus, the apparent ability of rhizobia/Nod factors to interfere with auxin transport is probably mediated through the generation of secondary signaling molecules such as flavonoids (Hirsch 1992), as it has been reported that flavonoids act as endogenous auxin transport inhibitors (Jacobs and Rubery 1988; also see Mathesius *et al*. 1998b). In concordance with this suggestion, Nod factors in fact were found to induce local increment of flavonoid concentration in the roots leading to transient disruption in polar auxin transport, which results in a localized auxin accumulation, triggering the activation of cell division and subsequent development of nodule primordia and nodules (Mathesius *et al*. 1998a, 1998b; **Fig. 3**). It was evidenced in white clover that the *de novo* synthesis and localized rise in the flavonoid concentration in root cortical cells commence within 24 h upon Nod factor application (Mathesius *et al*. 1998a), and this phenomenon is simultaneously followed by the transient disruption in polar auxin transport (Mathesius *et al*. 1998b). Thus, in roots, the inhibition of acropetal auxin transport at the site of the elevated flavonoid content results in basipetal accumulation of auxin, leading to the induction of cortical cell divisions and initiation of the development of nodule primordium in the vicinity of Nod factor application within 72 h (Mathesius *et al*. 1998b). Numerous studies have shown that indeed rhizobia and their Nod factors stimulate the synthesis of flavonoids in roots prior to and during nodule organogenesis (see the previous section for details).

Recently, Wasson *et al*. (2006) by silencing the genes encoding CHS (the enzyme that catalyzes the first committed step of the flavonoid biosynthesis pathway) using RNA interference (RNAi) provided genetic evidence that root flavonoids play a critical role in nodule initiation by interfering with auxin transport in *M. truncatula*, a legume that produces indeterminate nodules. It was shown that *CHS*-silenced roots produced decreased amounts of flavonoids, and failed to form nodules when inoculated with *S. meliloti*. However, supplementation of the growth medium with the exogenous flavonoids liquiritigenin and naringenin (precursors of a vast majority of flavonoids) restored nodule formation in these roots. In an analogous study with soybean, which forms determinate nodules, it was found that silencing of isoflavone synthase (*IFS*) genes encoding the key enzyme in the biosynthesis of isoflavones also severely reduced nodulation (Subramanian *et al*. 2006). In addition, silencing of *IFS* drastically reduced isoflavone (both daidzein and genistein) production, and allowed increased polar auxin transport in soybean roots similar to the phenomenon observed in the *CHS*-silenced roots in *M. truncatula*. On the contrary, unlike in *M. truncatula*, exogenous application of isoflavones (daidzein or genistein) failed to rescue nodulation indicating a critical role for endogenously produced isoflavones in the development of determinate nodules. Subramaniam *et al*. (2006) further reported that the application of genistein-hypersensitive *B. japonicum* or its purified Nod factors restored nodulation in *IFS*-silenced roots, thereby suggesting that isoflavone-mediated inhibition of auxin transport is not critical to nodulation in soybean. It appears that flavonoids trigger differential nodulation responses in legumes producing indeterminate and determinate nodules (Subramanian *et al*. 2007). Nevertheless, it is yet to be deciphered, in a given *Rhizobium*-legume (homologous) interactions, whether the same flavonoids are involved in triggering the *nod*-gene induction in *Rhizobium*, and auxin transport inhibition in the host plant roots.

Mathesius *et al*. (1998b) demonstrated that structure of flavonoids affected the ability to influence auxin transport in white clover roots. For example, exogenous application of the flavonols quercetin, fisetin and kaempferol, the flavone apigenin and the flavonone naringenin caused local down regulation of *GH3-gusA* (auxin responsive reporter gene, whose expression positively correlates with auxin levels in plant tissues), whereas the isoflavonoid genistein and the flavonoid glucosides like quercetin-3-glucoside, kaempferol-3-glucoside, apigenin-7-rhamnoglucoside and naringening-7- rhamnoglucoside failed to alter *GH3-gusA* expression in white clover roots. Recent studies with RNAi–mediated suppression of *MtFNSII* genes (that encode enzyme involved in flavone synthesis) demonstrated a drastic depletion of endogenous dihydroxyflavone level in roots, and led to reduction in nodulation in *M. truncatula* when inoculated with *S. meliloti* (Zhang *et al*. 2007). However, the effect of dihydroxyflavone on auxin transport inhibition in roots was not investigated. It will be interesting to identify specific endogenous flavonoids that act as auxin transport inhibitors during nodule organogenesis (**Fig. 3**). This can be achieved through RNAi-mediated gene silencing of individual genes encoding the enzymes that mediate the synthesis of specific flavonoids.

## ROLE OF FLAVONOIDS IN THE RHIZOBIA-NONLEGUME INTERACTIONS

In the previous sections of this review, we presented a treatment on the role of flavonoids in rhizobia-legumes interactions. The possibility of establishing association with rhizobia is potentially available in nonlegumes, since many of the phenylpropanoid pathway compounds that could interact with rhizobia are also present in nonlegume roots. Indeed there are some cases in which the flavonoid-assisted rhizobial interactions include nonlegume hosts as well. *Parasponia*, a genus of Ulmaceae, is the only nonlegume known to establish a nitrogen-fixing nodular symbiosis with rhizobia such as *Bradyrhizobium* RP501/CP279 (microsymbionts of *Parasponia*), and the broad host-range *Rhizobium* sp. NGR234 (Trinick and Hadobas 1988; Scott and Bender 1990). Nodulation genes responsive to flavonoids have been localized in these rhizobial strains (see Scott and Bender 1990 and the references therein). It has been demonstrated that seedling extracts from *Parasponia* are able to induce *nod* gene expression in *R. leguminosarum* bv. *trifolii* harboring *nodD1* gene from *Rhizobium* sp. NGR234 (Bender *et al*. 1988), indicating that *Parasponia* is capable of producing *nod* gene inducing compounds. Even though there is no information on the nature of *nod* gene inducing compounds released by *Parasponia* roots, in *Bradyrhizobium* infection of *Parasponia*, fluorescent compounds, whose properties are consistent with those of flavonoids, accumulate in dividing cells during nodule development (Rolfe *et al*. 2000).

Several studies demonstrated that rhizobia can interact with non-nodulating nonlegumes such as rice (Reddy *et al*. 1997; Webster *et al*. 1997; Yanni *et al*. 1997; Prayitno *et al*. 1999; Gopalaswamy *et al*. 2000; Jain and Gupta 2003), wheat (Webster *et al*. 1997, 1998), potato (Spencer *et al*. 1994), *Arabidopsis* (Gough *et al*. 1997; Stone *et al*. 2001) and oilseed rape (O'Callaghan *et al*. 2000), and establish endophytic association by colonizing intercellular spaces and xylem vessels in roots. Extracts derived from seedlings of the nonlegumes rice, wheat, maize, sunflower, cotton and *Trema* were shown to have the ability to induce low to moderate levels of *nod* gene expression in rhizobia (Bender *et al*. 1988; Reddy *et al*. 2000a, 2000b; Rolfe *et al*. 2000; Sreevidya *et al*. 2006). In wheat it was found that the simple phenolic compounds vanillin and isovanillin present in the seedling extracts interact with the *nodD1* gene from *Rhizobium* strain NGR234 to express *R. leguminosarum* bv. *trifolii nod* genes (Le Strange *et al*. 1990). Extended studies with several rice varieties indicated that the roots of only a limited number of rice cultivars are actually able to exude compounds that are capable of inducing, that too only to a low extent, the transcription of the rhizobial *nod* genes (Reddy *et al*. 2000b). Above results suggested that, like legumes, nonlegumes also produce (at least to a limited extent) activators of *nod* gene expression, although it is yet to be deciphered whether any of these unknown compounds belong to the flavonoid family. Recently it has been found that rhizobia are able to induce phenylpropanoid biosynthetic pathway in rice leading the production of phenolic compounds (Mishra *et al*. 2006).

In spite of the ability of nonlegumes to induce *nod* gene expression, investigations with rice, wheat and *Arabidopsis thaliana* revealed that unlike in legumes, the infection/invasion of roots of the nonlegumes by rhizobia is a *nod*-gene independent phenomenon (Reddy *et al*. 1997; Gough *et al*. 1997; Webster *et al*. 1997, 1998; Stone *et al*. 2001; Jain and Gupta 2003). Nevertheless, exogenous application of specific flavonoids such as naringenin was found to induce rhizobia-mediated root hair curling in oat (Terouchi and Syōno 1990) and promote root colonization in rice (Webster *et al*. 1997; Gopalaswamy *et al*. 2000; Jain and Gupta 2003), wheat (Gough *et al*. 1996; Webster *et al*. 1997, 1998), *Arabidopsis* (Gough *et al*. 1997) and oil seed rape (O'Callaghan *et al*. 2000), indicating differences in the mechanisms operating in the induction of nodulation by flavonoids in legumes and the stimulation of colonization by these molecules in nonlegume plants.

## PROSPECTS FOR AMMENDING FLAVONOID BIOSYNTHETIC PATHWAY TO PROMOTE PLANT-RHIZOBIA INTERACTIONS IN NONLEGUMES

Extension of symbiotic nitrogen fixation to nonlegumes requires the establishment of an effective association between the plants and rhizobia. To devise general strategies for promoting stable symbiotic association between rhizobia and nonleguminous plants, exploratory studies have been initiated with rice, a nonlegume model crop plant (Reddy *et al*. 1997, 2000a, 2000b; Sreevidya *et al*. 2006). Rice varieties have very low capacity to induce *nod* genes in rhizobia, presumably because of lack or limited ability to synthesize *nod* gene inducing flavonoids (Reddy *et al*. 2000b; Rolfe *et al*. 2000; Sreevidya *et al*. 2006). As the production of appropriate flavonoids in roots is a prerequisite for legume plants for entering into a symbiotic relationship with rhizobia, and because rice lacks the biosynthetic pathway to produce isoflavones from flavanones such as naringenin, Sreevidya *et al*. (2006) transferred a legume isoflavone synthase (*IFS*) gene to test whether rice could be conferred with the ability to synthesize isoflavonoids needed for the induction of *nod* genes in rhizobia. The exploratory study evidenced that the expression of *IFS* gene enabled rice plants to synthesize the isoflavone genistein, and conferred the rice plants the ability to produce appropriate flavonoids that are able to induce *nod* gene expression in different rhizobia. However, it is yet to be determined whether the augmentation of isoflavonoid biosynthesis promotes rhizobial interaction and colonization in rice roots.

Above studies demonstrated that amendment of flavonoid biosynthetic pathway can enable nonlegume, non-nodulating plants to generate flavonoids that could promote positive interactions with rhizobia. It is established that the expression of genes that code for the transcription factors such as C1 myb-type transcription factor of maize and the Myb305 of *Antirrhinum majus* can regulate the induction of a variety of genes that encode enzymes of the phenylpropanoid pathway (Grotewold *et al*. 1998; Sablowski *et al*. 1994). Transcription factors such as these may be expressed in host plant cells to activate expression of genes in the flavonoid biosynthetic pathway thereby increasing the encoded enzyme activities and the flux of compounds (Jung *et al*. 2003) suitable for *nod* gene induction in rhizobia, and for promoting cortical cell divisions needed for nodule development.

## CONCLUDING REMARKS

Establishment of the *Rhizobium*-plant symbiosis is the culmination of elaborate signaling-communications between the symbionts. In legume-*Rhizobium* symbiosis, flavonoids act as chemo-attractants and metabolites for rhizobial symbionts and signalling molecules for rhizobial *nod* gene expression (**Fig. 3**). In addition, flavonoids mediate the action of Nod factors via localized perturbation of the auxin balance in roots, thus playing a critical role in facilitating nodule differentiation. Considerable information is available on the mechanism of *nod* gene induction in rhizobia, and the action of flavonoids in mediating these reactions. In comparison, very little is known about the systems that perceive the flavonoids, and promote the flavonoid-regulated nodule development. Plant molecular biology and genomic approaches provide new tools to clarify genetic and biochemical mechanisms that mediate the action of flavonoids in nodule development. Understanding of the genetic programs involved in flavonoid biosynthetic pathway that promote root endosymbiosis should allow the possibility of exploring and improving existing symbiotic genetic programs in legumes, and extending such symbiotic genetic networks to nonlegumes. Availability of gene silencing technologies such as RNAi may in fact hasten the process of dissecting out the genetic pathways involved in flavonoid-mediated developmental processes during nodule organogenesis. In addition, gene silencing technologies can also be used to alter phenylpropanoid pathway to facilitate the synthesis of appropriate *nod*-gene inducing flavonoid compounds or inhibit the synthesis of anti-inducers, in order to encourage intimate and beneficial rhizobial interactions in roots, both in nodulating and non-nodulating plants.

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