

Chapter 8

Plant Flavonoids: Key Players in Signaling, Establishment, and Regulation of Rhizobial and Mycorrhizal Endosymbioses

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Abstract Plants belonging to family fabaceae play an imperative role in restoring soil fertility, with the remarkable ability to engage endosymbiotically with both rhizobia and arbuscular mycorrhiza (AM). Establishment of both symbioses is based on a finely regulated molecular dialogue between two partners. Plant roots secrete an assortment of flavonoids, competent to shape rhizosphere microflora by amplifying chemotactic surface motility of beneficial microorganisms while combating pathogenic ones. Flavonoids potentially regulate transcriptional activity of many microbial genes, e.g. *nod* genes, and fungal hyphal branching and initiate the production of microsymbiont signal molecules (Nod/Myc factor). The perception of these lipo-chito-oligosaccharides at epidermis stimulates partly analogous downstream signal transduction cascade to activate symbiosis-related genes and consequently enable successful penetration of both microsymbionts in the host. In response to host-specific microbe, selective accumulation of flavonoids drives suppression of plant innate immunity as well as cortical cell dedifferentiation into symbiosome. High degree of coordination between root cortical cell machinery and rhizobia/AM results in the formation of symbiotic interfaces—nodules/arbuscules respectively, where harboring bacteroids and arbuscules deliver macronutrients (nitrogen and phosphorus) to host in exchange for photosynthates. Flavonoids cross-link with plant proteins to form an O₂- diffusion barrier in the symbiosome membrane and serve as a checkpoint for nitrogenase efficiency. Under nutrient-rich conditions, plants regulate flavonoid fluxes to prevent an excessive establishment of metabolically expensive symbioses. Therefore, understanding these selective forces that govern host selection of beneficial rhizomicrobiome, followed by underlying establishment and regulation of symbioses in legumes, is crucial for agrobiologists to achieve sustainable agriculture.

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8.1 Introduction

Among plant nutrients, nitrogen (N) and phosphorus (P) are the most limiting nutrients worldwide whose continued supply as fertilizers is necessary if world food needs are to be fulfilled. Modern agriculture has been highly reliant on industrial fertilizers, and the vast amount of resources spent on their production is leading to a substantial carbon footprint (exploiting ~50% of fossil fuel) of this industrial sector (Jensen et al. 2012). However, the rising fossil fuel cost is making chemical fertilizers dramatically expensive; CO₂ emission during fossil fuel combustion is contributing to the greenhouse effect, and leaching of applied fertilizers (~30–50%) is leading to major environmental problems, e.g. eutrophication (Crutzen et al. 2007). Thus, efforts must be directed to enhance the exploitation of rhizospheric soil microorganisms which can effectively ensure substantial uptake of essential nutrients by plants and thus recuperate agricultural fields with nutrient-poor soils (Bonfante and Genre 2008; Parniske 2008; Vieira et al. 2010). Two of the most widespread rhizospheric interactions, the *Rhizobium*–legume (RL) and the arbuscular mycorrhiza (AM)–plant symbioses, have particular significance as natural mini-fertilizer factories in land ecosystems (Venturi and Keel 2016).

Rhizobium–legume (RL) symbiosis is almost entirely limited to economically important family Fabaceae which evolved only about 60 Mya (Manchanda and Garg 2007; Delaux et al. 2015). On the other hand, since 500 million years ago (Mya), ubiquitous soil AM fungi establish a symbiotic relationship with the roots of more than 90% of all higher plants (Smith and Read 2008; Wilde et al. 2009). RL interaction leads to the formation of symbiotic root nodules, where *Rhizobium* bacteria acquires vast fitness output from host carbon and energy (Peix et al. 2015) and legumes gain access to otherwise unavailable soil nitrogen (replenishing approximately 200 million tons of N₂ annually) (Ferguson et al. 2010; Kondorosi et al. 2013). In mycorrhizal association, vegetative growth and reproductive spore production of biotrophic fungal symbionts rely on reduced carbon of living plant tissue (4–20% of their photosynthate) and in return provide various benefits, including—but not limited to—nutrient and water uptake (Parniske 2008).

Legumes have the ability to host N₂-fixing bacteria and AM at the same time (Antunes et al. 2006), and mycorrhizal symbiosis associated with legumes is an essential link for effective phosphorus (P) nutrition leading to enhanced N₂ fixation that advocates a synergistic tripartite association (Geneva et al. 2006). In most cases investigated, especially when both nitrogen and phosphate are limiting factors, rhizobia and AM fungi appear to act synergistically since combined inoculation with rhizobia and mycorrhiza enhances plant growth and reproduction more than inoculation with either microsymbiont alone and also mutually increases each other's establishment (Gould and Lister 2005). Striking similarities between two symbionts have been reported with respect to mutual recognition, infection process, and genetic and hormonal regulation (Mukherjee and Ané 2011). Exchange of molecular signals between the host plant and microsymbiont in the form of cell-to-cell inter-organismal communication is an important stage for the initiation of

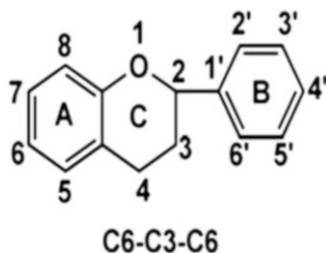
effective symbiosis (Mapope and Dakora 2013), and during N_2 fixation, a wide variety of host FLAVONOIDS have been shown to attract compatible rhizobia for symbiosis, by their *nod* gene inducing activity (Shaw et al. 2006; Mandal et al. 2010). Apart from the function of flavonoids in RL interaction, they also act as signaling compounds in the host communication with AM (Catford et al. 2006; Steinkellner et al. 2007; Shaw and Hooker 2008). Moreover, plant flavonoids vary qualitatively and quantitatively with root endosymbiont colonization, indicating dependability of nodulation and mycorrhization on flavonoids beyond signaling (Carlsen et al. 2008; Zhuang et al. 2013). Thus, this prompts in-depth review on diverse role of flavonoids in the establishment of legume symbioses with rhizobia and AM.

8.2 Flavonoid: A Versatile Compound

8.2.1 Structural Diversity and Subcellular Distribution

Flavonoids (derived from the Latin word for yellow, *favus*), the low-molecular-weight secondary metabolites, are biologically active polyphenolic compounds and are widespread throughout the plant kingdom, ranging from mosses to angiosperms (Williams and Grayer 2004). Diverse flavonoid molecules share the same core carbon framework of phenyl-benzopyran functionality, the flavan nucleus (Fig. 8.1), consisting of two aromatic rings with six carbon atoms (rings A and B) interconnected by a heterocyclic benzopyrano (chromano) C ring with three carbon atoms (Saito et al. 2013; Cheng et al. 2014). The position of B aromatic ring linkage to the benzopyrano moiety allows a broad separation of these compounds into flavonoids (2-phenyl-benzopyrans, e.g., kaempferol, apigenin), isoflavonoids (3-phenyl-benzopyrans, e.g., genistein), and neoflavonoids (4-phenyl-benzopyrans) (Winkel-Shirley 2001; Dixon and Pasinetti 2010). These groups usually share a common chalcone precursor and therefore are biogenetically and structurally related (Marais et al. 2006). A number of divergent chalcones and flavonoid structures are formed from the extensive modification (rearrangement, alkylation, oxidation, and glycosylation) of the basic molecules (Halbwirth 2010). To date, more than 10,000 different flavonoids have been identified in plants (broadly

Fig. 8.1 Flavan nucleus of flavonoids



classified into flavonols, flavones, flavan-3-ols, flavanones, anthocyanins, iso-flavones) and the number is still increasing (D'haeseleer et al. 2010; Hassan and Mathesius 2012; Cheynier et al. 2013) and even within the same species a number of different flavonoids have been identified (Martens and Mithöfer 2005). Typically, flavonoids are stored in their glycoside forms (more stable than the free form); however, some flavones and flavonols can be found naturally as the aglycone (Birt and Jeffery 2013). The chemical diversity, size, three-dimensional shape, and physical and biochemical properties of flavonoids allow them to interact with targets in different subcellular locations to influence biological activity in plants, animals, and microbes (Taylor and Grotewold 2005; Buer et al. 2010).

Consistent with their diverse physiological functions, flavonoids are found in most plant cell compartments, including cytosol, vacuole (anthocyanins, flavonol and flavone glycosides), ER, chloroplast (quercetin and kaempferol glycosides), nucleus (isoflavonoids coumestrol and 4',7-dihydroxyflavone), pollen surface and small vesicles, as well as the extracellular space (Saslowsky et al. 2005; Lepiniec et al. 2006; Hsieh and Huang 2007; Naoumkina et al. 2007; Hernández et al. 2009). In the plant, flavonoids function as developmental regulators, antioxidants, pigments, UV sunscreens, nutrient acquirer, energy escape valve, auxin transport regulators, defense compounds against pathogens (signal for jasmonate-induced mobilization of vacuolar isoflavonoid glucosides for phytoalexin biosynthesis), and signals during symbiosis (Buer and Muday 2004; Naoumkina and Dixon 2008; Cheynier et al. 2013). However, their importance in plant biology goes beyond their specific functions within the plant (Dixon and Pasinetti 2010). Flavone and flavonolglycones have been detected in root exudates from numerous species, where isoflavones are particularly secreted by legume roots into the rhizosphere (Zhao and Dixon 2010).

8.2.2 *Flavonoids in the Rhizosphere*

In response to elicitors, both aglycone and glycoside flavonoids are often exuded into the rhizosphere in order to fulfill some of their ecological roles as mediators of belowground interactions, for example, in order to attract compatible rhizosphere-dwelling rhizobia, stimulate or inhibit rhizobial *nod* gene expression, inhibit root pathogens, stimulate mycorrhizal spore germination and hyphal branching, affect quorum sensing, and chelate soil nutrients (Broughton et al. 2003; Cooper 2004; Martens and Mithöfer 2005). Exudation of flavonoids is ATP dependent and catalyzed by an ABC-type transporter as has been suggested by the study of isoflavonoid genistein exudation from soybean root plasma membrane vesicles (Sugiyama et al. 2007). This was also supported by the study of Badri et al. (2009) where ABC transporter mutants of *Arabidopsis* like *abcg30* had altered root exudate profiles. Besides ABC-type transporters, flavonoids can also be released passively in the rhizosphere from decomposing root cap and border cells (Shaw et al. 2006). Another important mechanism for releasing active flavonoid aglycones during

root–microbe interactions is through apoplastic β -glucosidases, which release isoflavones from their conjugates in soybean roots (Suzuki et al. 2006). Recently, Sugiyama et al. (2016) reported that the expression of gene encoding isoflavone conjugates hydrolyzing beta-glucosidase (ICHG) coordinately peaked at vegetative stages with the higher secretion of daidzein. However, under nitrogen-deficient conditions, besides daidzein, genistein was also highly secreted, with no induction of ICHG. Thus, their study suggested that two pathways for isoflavone secretion in soybean roots are expected to have distinct physiological roles, i.e., ICHG-mediated secretion during vegetative growth and ATP-dependent transport during nitrogen deficiency.

Once in the rhizosphere, the fate of flavonoid persistence in the rhizosphere (likely to be from hours to days) varies with environmental conditions, flavonoid structure, and the presence of soil microbes, some of which can metabolize or modify flavonoids. 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 structural modifications, the solubility and mobility of flavonoids in the soil varies. For example, genistein is described as “practically insoluble in water,” whereas genistin, the glucoside, is “sparingly soluble in water” (O’Neil et al. 1996). Thus, conjugated forms are expected to be less adsorbed to the soil matrix, more mobile, and therefore more bioavailable than the free aglycone form. 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 (Sosa et al. 2010; Weston and Mathesius 2013). Once present in the aglycone form, new flavonoid structures may be produced during biodegradation of a parent flavonoid, which can be more efficient inducers of *nod* genes than the flavonoids themselves (Anders and Huber 2010; Rose et al. 2012). Moderate bioavailability and mobility of flavonoids (organic carbon normalized partition coefficients, i.e., logKoc of 3.12 for formononetin and 3.19 for naringenin), makes ecological sense, as it would be evolutionarily favorable for a plant root to produce a chemical signal that is sufficiently bioavailable to allow interaction with its intended microbial target, but, at the same time, not so mobile that it will diffuse rapidly to the outer reaches of the rhizosphere. Thus, bioavailability, mobility and persistence of a flavonoid will determine the degree and outcome of flavonoid–microbe interaction with important consequences for plant nutrition (Shaw and Hooker 2008; Weston and Mathesius 2013).

8.3 Flavonoids in *Rhizobium*–Legume (RL) Endosymbiosis

Among the wide range of bacteria that have the ability to reduce N_2 to ammonia (NH_3), the most important are soil-dwelling prokaryotic bacteria collectively called rhizobia, where each legume species has its own cognate *Rhizobium* partner (s) (Gibson et al. 2008; Soyano and Kawaguchi 2014). Legume genomes are at least 50 times larger than those of their microsymbionts; nevertheless, their

respective contributions are probably not vastly different (Irving et al. 2000). Thus, RL symbioses are cross-kingdom collaboration between two vastly different genomes (Yang et al. 2010). The endosymbiosis generally commences within a specific “susceptible” root zone close to the root tip, where initial bacteria–host recognition takes place (Laloum et al. 2014). The bacteria invade the roots of compatible legume plants leading to the development of specialized root structures called nodules, providing a unique ecological niche in which bacteria differentiate into bacteroids and fix N_2 . Although the interaction is beneficial to both partners, it comes with rigid rules that are strictly enforced by both the partners (Oldroyd et al. 2011). Here, we review the various signaling pathways by which the plant allows bacterial infection and promotes the construction of the nodule as well as how intricate transaction of metabolites (especially flavonoids) directs the bacteria into a nitrogen-fixing organelle-like state.

8.3.1 Signaling in Rhizobium–Legume Symbiosis

The succession to the symbiotic affirmation by two originally autonomous, free-living partners is governed by reciprocal generation and perception of signals, which has been described as “molecular dialogue” (De’nari et al. 1993). At least three different sets of symbiotic signals are exchanged between legumes and rhizobia during nodule development: flavonoids, Nod factors, EPS, and extracellular proteins (NOPs = *nodulation outer proteins*) (Broughton et al. 2003).

8.3.1.1 Signals from the Host Plants

A diverse array of compounds is exuded into the rhizosphere, including sugars, aliphatic as well as aromatic acids, amino acids, amines, and many other low-molecular-weight compounds such as flavonoids, isoflavonoids, steroids, alkaloids, vitamins, and growth regulators (Skorupska et al. 2010; Lareen et al. 2016). The “rhizosphere effect,” first described by Hiltner (1904), assumes that many microorganisms are attracted to sugars, acids, and amino acids exuded by plant roots, which serve as C and energy sources for microorganisms. However, in addition to providing a C-rich environment, plant roots initiate cross talk with soil microbes by producing signals that are recognized by the microbes, which in turn produce signals that initiate colonization (Bais et al. 2004). Among the myriad of rhizodepositions, normally and continuously exuded by plants into the rhizosphere are phenolic compounds especially flavonoids that mediate signal traffic between roots and beneficial rhizosphere-dwelling rhizobia (Gibson et al. 2008; Skorupska et al. 2010; McNear 2013; Mandal et al. 2016). Although small quantities are excreted continuously, flavonoid concentrations in the rhizosphere increase in response to compatible rhizobia. Niches in the legume rhizosphere are tailored to rhizobial inoculation, as exudation of flavonoids is mostly restricted to the

elongating root hair zone from which most nodules later develop (Zuanazzi et al. 1998). The role of flavonoids as bacterial chemoattractant and transcriptional activator of bacterial *nod* genes points out their central position in modifying rhizobial phenotypes possibly in relation to plant–root association and then symbiotic interaction (Brencic and Winans 2005; Spini et al. 2016). Once exuded, flavonoids, especially aglycone forms, are recognized to diffuse into the rhizobial membrane (Kobayashi et al. 2004; Wang et al. 2012), possibly through porins (Taylor and Grotewold 2005). Further, it activates rhizobia *nod* (nodulation) D gene expression (Ferguson et al. 2010), and this successful liaison between flavonoids and NodD proteins signals the beginning of association (Broughton et al. 2000). The first flavonoid to be discovered to act as *nod* gene inducers was luteolin, isolated from *Medicago sativa* and 7,4'-dihydroxyflavone (DHF) from *Trifolium repens* (white clover) (Redmond et al. 1986). Since then, about 30 *nod* gene-inducing flavonoids (especially flavanones and isoflavonoids) have been isolated from nine legume genera (Limpen and Bisseling 2008; Gholami et al. 2014). Most of these flavonoids are active as *nod* gene inducers at nanomolar to low micromolar concentrations (Begum et al. 2001a; Gholami et al. 2014) and stimulate bacterial *nod* gene expression within minutes. 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 (Gibson et al. 2008; Skorupska et al. 2010; Rose et al. 2012). Hence, point mutations in *nodD* affect recognition of inducing flavonoids and cause extension of host range (Broughton et al. 2000). Generally, the NodDs of broad host range rhizobia, e.g., NGR234, respond to a wider range of flavonoid species (including phenolics that are inhibitors in other rhizobia, e.g., vanillin, *iso*-vanillin, as well as several estrogenic compounds) than those present in restricted host range rhizobia (Peck et al. 2006; Wang et al. 2012). *nodD* products of various *Rhizobium* species respond in different ways to flavonoids, and NodD homologues from the same strain may have various flavonoid preferences. In *R. meliloti*, NodD1 was activated when cells were supplied with a complex plant seed extract or the flavonoid luteolin. NodD2 only derepressed transcription when supplied with the complex extract, not with purified luteolin, while NodD3 apparently modulates the expression of *nod* genes even in the absence of any plant factor (Smit et al. 1992). Recently, Peck et al. (2013) presented a structural model of wild-type NodD1 identifying residues important for inducer binding, protein multimerization, and interaction with RNA polymerase at *nod* gene promoters. Species-specific flavonoids interact with a class of transcriptional activators of the LysR family which have an N-terminal ligand-binding domain that regulates the activity of the associated C-terminal DNA-binding domain, and NodD ligand-binding domain is thought to function as a flavonoid receptor. The perception of flavonoids by rhizobia is linked to elevation in concentrations of intracellular calcium in rhizobia that subsequently induces NodD proteins for Nod factor expression (Moscatiello et al. 2010; Hassan and Mathesius 2012).

Of the large number of available flavanones, flavones, isoflavones, and other related compounds, capability of flavonoid to act as *nod* gene inducers varies with

the variation in host varieties, bacterial strains, and/or signal compounds (Begum et al. 2001a, b). Within the variety of flavonoids, isoflavonoids, and other compounds secreted by *Lupinus albus* roots into the rhizosphere, major proportion is composed of aldonic acids which act as natural *nod* gene inducers of *Rhizobium lupini*, *Mesorhizobium loti*, and *Sinorhizobium meliloti* (Gagnon and Ibrahim 1998). Luteolin, genistein, naringenin, hesperetin, and apigenin are the principal flavonoids involved in *nod* gene expression in *S. meliloti*, *Bradyrhizobium japonicum*, *Rhizobium*, and *R. tibeticum*, respectively (Kapulnik et al. 1987; Graham 1991; Begum et al. 2001b; Belkheir et al. 2001; Novák et al. 2002; Tsvetkova et al. 2006; Brechenmacher et al. 2010; Abd-Alla et al. 2014). Genistein, coumestrol, and daidzein have been reported as important inducers of rhizobial nodulation genes in the early stages of symbiosis between soybean and *B. japonicum* (Antunes and Goss 2005; Miransari and Smith 2009; Tian et al. 2014; Sugiyama et al. 2016). Begum et al. (2001a) suggested that the attachment of the B-ring to C-2 of flavonoids, as found in flavones and flavanones, is of crucial importance for induction. In this regard, Zhang et al. (2007) provided genetic evidence that RNA interference-mediated suppression of *MtFNSII* genes in *Medicago truncatula* resulted in flavone-depleted roots and led to significantly reduced nodulation when inoculated with *S. meliloti*. In addition, hydroxylation at the C-4 and the C-7 positions of flavones is important for this activity (Brencic and Winans 2005; Subramanian et al. 2007).

Interestingly, some flavonoids also show *nod* gene repressing activity for certain rhizobia. Isoflavonoids, medicarpin and coumestrol, have been reported to negatively control Nod factor production in *S. meliloti* (Zuanazzi et al. 1998). Jain and Nainawatee (1999) studied that except quercetin, alfalfa exudates decreased growth and protein content of *R. meliloti* cells. Naringenin induces the expression of *nod* genes in *R. leguminosarum-Pisum sativum*; however, quercetin is an inhibitor of nodulation (Novák et al. 2002). Inducers in one species or strain of *Rhizobium* are frequently anti-inducers in another species; thus, one type of flavonoid can have opposing effects on different bacteria; for example, the isoflavone diadzein induces *nod* gene expression in *B. japonicum* (nodulating soybean), whereas it inhibits that of those from *R. leguminosarum* (nodulating clover or peas) and thus contributes to host specificity (Andersen and Markham 2006). It has been suggested that a mixture of flavonoids is more effective in inducing *nod* genes as opposed to a single compound (Mandal et al. 2010). Both functions (induction or anti-induction) can co-occur in the exudates of the same plant, where different flavonoids in root exudates can act synergistically as *nod* gene inducers, but also in an antagonistic manner as anti-inducers (Cooper 2007; Makarova et al. 2015). Luteolin and 7,40-dihydroxyflavone are inducers, whereas genistein inhibits expression of *S. meliloti nod* genes (Kosslak et al. 1987; Hartwig et al. 1990; Peck et al. 2006). Thus, the ratio of inducers to anti-inducers in root exudates may be involved in determination of host recognition, maintaining an optimal level of Nod factor production and preventing elicitation of defense responses by the plant (Zuanazzi et al. 1998; Cesco et al. 2010). Li et al. (2016) provided a previously unidentified mechanism by which flavonoids in exudates of one crop root can promote N_2 fixation in another crop in a

two-crop intercropping system. In their study, maize root exudates contained significant flavonoids and promoted flavonoid synthesis in faba bean, thus triggering N_2 fixation.

8.3.1.2 Signals from the Microsymbiont

Second set of signals is synthesized when cytoplasmic membrane-bound NodD–flavonoid complexes activate transcription from conserved 49-bp DNA motifs, i.e., “*nod*-box” promoters found in the promoter regions of many nodulation loci (Gibson et al. 2008). Thus, NodD proteins act as both sensors of the plant signal and transcriptional activators of *nod* loci on symbiotic plasmids (Redmond et al. 1986; Downie 2010). The concerted transcriptional activation of common and host-specific *nod* genes leads to the synthesis of Nod factor (NF) which is a key to legume doors (Mulder et al. 2005; Cooper 2007; Remigi et al. 2016). NFs belong to lipo-chito-oligosaccharides (LCOs) family, having an oligosaccharide backbone of four or five β -1-4-linked *N*-acetyl-D-glucosamine units with a terminal nonreducing sugar *N*-acylated by a 16–18 carbon fatty acid (Fauvart and Michiels 2008; Hamel and Beaudoin 2010). Assembly of the chitin backbone is performed by an *N*-acetylglucosaminyltransferase encoded by *nodC*, and the deacetylase NodB removes the *N*-acetyl moiety from the nonreducing terminus of the *N*-acetylglucosamine oligosaccharides. NodG has the enzymatic activity of a 3-oxoacyl-acyl carrier protein reductase and is involved in fatty acid elongation (López-Lara and Geiger 2001). Finally, an acyltransferase coded by *nodA* links the acyl chain to the acetyl-free carbon C-2 of the nonreducing end of the oligosaccharide (Brencic and Winans 2005). In addition to the common *nod*-*DABC* genes that are essential for symbiosis, the rhizobia harbor different combinations of other *nod*, *nol*, and *noe* genes which may have been recruited from paralogues in the course of the evolution, allowing the diversification of NF structures and host ranges (Taurian et al. 2008; Vieira et al. 2010). For instance, *nodV* and *nodW* of *B. japonicum* are essential for the nodulation of *Macroptilium atropurpureum*, *Vigna radiata*, and *V. unguiculata* but contribute only marginally to the symbiosis with *G. max*. *nodH* encodes a sulfotransferase that transfers a sulfate group to the reducing end of NFs of *R. meliloti* and elicits Ca^{2+} spiking (Wais et al. 2002). A *nodF* mutant produces a NF with a modified *N*-acylation on the terminal nonreducing *N*-acetyl glucosamine residue (Demont et al. 1993). NF produced by *nodL* mutants lacks a C6-*O*-acetylation on the terminal nonreducing glucosamine (Ardourel et al. 1995). *nodFL* double mutants trigger Ca^{2+} spiking and root hair deformation but are unable to infect their hosts (Wais et al. 2002; Haney et al. 2011). Thus, a major determinant of host-symbiont specificity is attributed to the different NF substituents (sulphuryl, methyl, carbamoyl, acetyl, fucosyl, arabinosyl, and other groups) attached to the oligosaccharide backbone as well as differences in the structure of the acyl chain (Downie 2010; Ferguson et al. 2010; Kouchi et al. 2010). Nod factors can trigger plant responses like root hair deformation and calcium oscillations (called calcium spiking) at astonishingly low concentrations, i.e., as little as 10^{-13} M Nod factor

(Rose et al. 2012). As Nod factors also stimulate the synthesis and release of flavonoids from legume roots, the response to inoculation is amplified (Broughton et al. 2003).

8.3.2 *Nod Factor (NF) Signaling Pathway in the Root Epidermis*

Symbiosis initiates if the above-stated chemical cross talk between the interacting partners successfully culminates in the production of NFs (Bek et al. 2010). The mechanism, by which plants regulate the intracellular uptake of symbiotic bacteria, depends on physiological and molecular reprogramming that is associated with the perception of NF and resultant downstream signaling (Xie et al. 2012; Liang et al. 2013). Rhizobia have two main ways of entering the plant root: *via* the root hair or through cracks in root epidermal tissue (Oldroyd and Downie 2008; Ribeiro et al. 2015); however, rhizobial entry along the infection threads in root hairs is largely common (Mathesius 2009; Downie 2010). Two receptor-like kinases (RLK) of chitin-binding LysM RLK family, located on epidermal cells, are involved in nod factor binding: LjNFR1 and LjNFR5 in *L. japonicus*, PsSYM2A and PsSYM10 in *P. sativum*, MtLYK3/MtLYK4 and MtNFP in *M. truncatula*, and GmNFR1 α/β and GmNFR5 α/β in soybean (Ferguson et al. 2010; Indrasumunar et al. 2010; Broghammer et al. 2012; Wang et al. 2012). These receptors consist of an intracellular kinase domain, a transmembrane domain, and an extracellular portion having LysM domains (Gough 2003; Mathesius 2009). Interestingly, LjNFR1/PsSYM2A/MtLYK3/MtLYK4/GmNFR1 α/β has a typical serine/threonine kinase domain, while LjNFR5/PsSYM10/MtNFP/GmNFR5 α/β lacks the activation loop (Indrasumunar et al. 2010). The absence of an activation loop in one of the kinase domains suggests that the two LysM RLKs may assemble into a heterodimeric receptor, with the active kinase (NFR kinase) domain triggering downstream signal transduction through phosphorylation (Markmann et al. 2008; Radutoiu et al. 2008; Hamel and Beaudoin 2010; Indrasumunar et al. 2015).

Rhizobial infection has many similarities with pathogenic infection and induction of defense responses accompanies both interactions, but defense responses are induced to a lesser extent during rhizobial infection. Recently, it was evidenced by Ivanova et al. (2015) that a range of plant defense responses like suberization, callose and unesterified pectin deposition, as well as activation of defense genes can be triggered by different single mutations in symbiotic genes (*sym33*, *sym40*, *sym42*) that cause perception of an otherwise beneficial strain of *Rhizobium* as a pathogen. Besides symbiosis, LysM-RLKs have a role in immune signaling, indicating that NF signaling and pathogen chitin-based immune signaling are intertwined. However, Nod factor signal, unlike microbe-associated molecular pattern (MAMP) derived from microbes, suppresses an innate immune response in the host (Tóth and Stacey 2015). Two putative models have been put forward to

explain this evolutionarily conserved dual function: (a) perception of LCOs factors modulates the balance between different LysM-RK receptor complexes, favoring a symbiotic complex at the expense of complexes required for immune responses, or (b) tight regulation of the receptor complexes at the posttranslational level, involving rapid endocytotic turnover, subsequently prevents activation of defense responses (Limpens et al. 2015). NF perception leads to root hair deformation and to changes in the root hair cytoskeleton (within 3–5 min) that are required for root hair curling (the so-called Shepherd's crooks within 1–3 h) and invasion (Weerasinghe et al. 2005; Yokota et al. 2009). Mutations in genes coding for the NF LRR RLK (Leucine-rich repeat receptor-like kinases), the putative ion channels, or the nucleoporins abolish Ca^{2+} spiking and continued nodule development events; however, they maintain the Ca^{2+} fluxes and root hair deformation events (Kanamori et al. 2006; Miwa et al. 2006; Saito et al. 2007; Capoen et al. 2011; Morieri et al. 2013). In contrast, mutations in genes encoding for a calcium and calmodulin-dependent kinase called CCaMK or DMI3 do not affect Ca^{2+} fluxes and Ca^{2+} spiking events but block continued nodule development (Lévy et al. 2004; Miwa et al. 2006). This suggests that the NF LRR RLK, the ion channels, and the nucleoporins act downstream of NF perception, but upstream of Ca^{2+} spiking, whereas the CCaMK acts downstream of Ca^{2+} spiking (Limpens and Bisseling 2008). Within the nucleus, this sustained calcium spiking is decoded by CCaMK, which phosphorylates a transcriptional regulator CYCLOPS/IPD3 (Yano et al. 2008; Kouchi et al. 2010; Singh et al. 2014). CYCLOPS, together with other TFs belonging to the GRAS (NSP1 and NSP2) (Smit et al. 2005; Oldroyd and Downie 2008), ERF (ERN1) (Cerri et al. 2012; Rose et al. 2012), and the nodule inception (NIN) activator (Marsh et al. 2007) families, modulates early symbiotic gene expression (like *ENOD2*, *ENOD40*, *ENOD11*) for infection thread (IT) formation and polar tip growth (Yano et al. 2008; Madsen et al. 2010). Downstream of DMI3 and NIN, members of Nuclear Factor Y family, i.e., NF-YA1 and NF-YA2 (a CCAAT-box-binding heterotrimeric TF complex), act as early symbiotic regulators of *ENOD11* (Cerri et al. 2012; Laloum et al. 2014). The secondary induction of Nod signals by flavonoids inside the roots is thought to be responsible for an additional level of host specificity. Thus, flavonoids play a multitude of roles during the process of nodulation (Subramanian et al. 2007).

8.3.3 Formation of Nodules: The Conjugal Lodging

NF perception in the epidermis activates a series of events, including polarized root hair tip growth, invagination associated with bacterial infection, and the promotion of cell division in the cortex leading to the nodule meristem (Oldroyd et al. 2011; Gourion et al. 2015; Laplaze et al. 2015). Cytoskeletal rearrangements have been reported in pericycle cells of *M. truncatula* within just 16 h of rhizobia inoculation (Timmers et al. 1999), and *ENOD40* expression is reported in cortical cells within just 24 h of rhizobia inoculation (Mulder et al. 2005; Murray 2011). Thus,

coordination between epidermal and nodule organogenesis seems to be crucial for successful nitrogen-fixing nodule formation (Oldroyd and Downie 2008). Although initial bacterial infection events do not require nodule primordia formation, it is subsequently required to direct infection thread growth (Oldroyd et al. 2011; Rose et al. 2012). To achieve such rapid mitotic activity in the underlying cortical cells after exposing the outer root to rhizobia/NF, role of auxin and cytokinin is imperative (Ryu et al. 2012).

8.3.3.1 Rhizobial Invasion

The host plant permits rhizobium to enter root tissues through plasma membrane-derived conduits called infection threads (ITs) (Jones et al. 2007; Fournier et al. 2008). The new growth can result in the root hair curling/bending, which results in NF-producing bacteria becoming entrapped between appressed cell walls, forming so-called infection pocket (Murray 2011; Wang et al. 2012). The rhizobia entrapped in these infection pockets continue to divide, forming colonies that are referred to as infection foci from which root hair ITs start to develop (Oldroyd et al. 2011). This results in elevated Nod factor concentrations, which are thought to be required to reach a Nod factor threshold concentration (Oldroyd and Downie 2008). The invaginating plant cell wall, along with the extended plasma membrane, grows as a hollow tube within the root hair cell and the bacteria multiply within the polar centripetally growing infection threads, where new cell wall and membrane material are being synthesized at their tip (Xie et al. 2012; Haag et al. 2013).

A third set of signals are represented by other rhizobial products necessary for continued infection thread development and/or preventing defense mechanisms (López-Baena et al. 2016). Among them are extracellular lipopolysaccharides (LPS), extracellular polysaccharides (EPS) and related compounds, as well as proteins exported by the type III secretion system (T3SS) (Limpens and Bisseling 2008; Haag et al. 2013). EPS facilitate attachment of bacterial cells to both biotic and abiotic surfaces and biofilm formation due to hydrophobic interactions and heterogeneity of the envelope surface (Janczarek et al. 2015), affect different stages of the organogenesis of nodules (Kelly et al. 2012), and along with LPS also facilitate suppression of defense response (Dalla Via et al. 2016). Further, bacterial effector proteins delivered from rhizobia into the plant cytosol through a T3SS or T4SS can act to either negatively or positively modulate nodulation, i.e., alter the symbiotic state toward pathogenesis or vice versa (Nelson and Sadowsky 2015; Tóth and Stacey 2015). The suppression of the MAMP-triggered immunity through a T3SS constituted the first evolutionary step toward symbiosis (Gourion et al. 2015; Yamazaki and Hayashi 2015; Okazaki et al. 2016). Interestingly, rhizobial NF, T3SS, and T4SS depend on a common regulator activated by legume-secreted flavonoids (Janczarek and Skorupska 2011; Gourion et al. 2015; Smith et al. 2015). Role of flavonoids in protein secretion in ITs suggests that the same keys can unlock different doors (Broughton et al. 2003). Growth in the ITs is critical stage for selection of competitive rhizobia, and this stage provides checkpoint for the plant

because bacteria mutated in cell wall components such as LPS, EPS, as well as BacA either fail to be released from ITs or fail to form mature symbiosomes (Gibson et al. 2008; Janczarek et al. 2015).

Concomitantly, certain cortical cells divide to form nodule primordia, and it is toward these primordia that the infection thread grows. After initiating the symbiotic dialog, flavonoids function as positional signals for cell division and/or growth in nodulating roots. This function was inferred because both the induction of a chalcone synthase-GusA (CHS-GusA) fusion and the accumulation of flavonoids occurred at the site where either purified Nod factor or nodulating rhizobia strains (but not of non-nodulating strains) were applied (Mathesius et al. 1998). Genes encoding phenyl propanoid biosynthesis enzymes including *Chalcone-O-Methyltransferase* (required for the production of the potent *nod* gene inducer 4,4-dihydroxy-2-methoxychalcone) not only express in rhizobially infected root hairs but also in nodule infection zone (not in the nitrogen fixation zone) (Chen et al. 2015). Some intriguing effects of plant phenolics are the ones associated with long-distance polar auxin transport (PAT) streams (Taylor and Grotewold 2005; Peer et al. 2011). Evidence that flavonoids regulate auxin accumulation *in vivo* was obtained using the flavonoid-deficient mutant, *tt4*, where accumulation of [¹⁴C] indole-3-acetic acid in whole seedling was defective as a considerable amount of auxin escaped from the roots. Treatment of the *tt4* mutant with the missing intermediate naringenin restored normal auxin distribution and accumulation by the root (Murphy et al. 2000). Silencing of lignin biosynthetic gene in *Arabidopsis thaliana* led to redirection of metabolic flux into flavonoid synthesis through chalcone synthase activity. The level of plant growth reduction of HCT-deficient plants was correlated with the inhibition of auxin transport, while suppression of flavonoid accumulation by chalcone synthase repression in HCT-deficient plants restored normal auxin transport and wild-type plant growth. Thus, reduced size phenotype of HCT-silenced plants is not due to the alteration of lignin synthesis but to flavonoid accumulation (Besseau et al. 2007). The molecular interplay of flavonoids and Nod factors is likely to occur at several stages during nodule ontogeny (Taylor and Grotewold 2005). Nod factors bring about an immediate and transient inhibition of PAT in a highly localized fashion, possibly through the action of specific flavonoids, resulting in a change in auxin concentrations and subsequent stimulation of cell divisions at the site of nodule initiation (de Billy et al. 2001; Laplaze et al. 2015). Wasson et al. (2006) identified that accumulation of auxin and nodule formation was restored in the naringenin and liquiritigenin-supplemented chalcone synthase (CHS)-silenced root cultures, thereby indicating that the ability of nodule-forming rhizobia to inhibit auxin transport was flavonoid dependent. Zhang et al. (2009) silenced different flavonoid biosynthesis enzymes to generate transgenic *M. truncatula* roots with different flavonoid profiles. Silencing of chalcone synthase led to flavonoid-deficient roots, while silencing of isoflavone synthase and flavone synthase led to roots deficient for a subset of flavonoids, isoflavonoids (formononetin and biochanin A), and flavones (7,4-dihydroxyflavone), respectively. When tested for nodulation by *S. meliloti*, flavonoid-deficient roots had a near complete loss of nodulation, whereas flavone-

deficient roots had reduced nodulation. Isoflavone-deficient roots nodulated normally, suggesting that isoflavones might not play a critical role in *M. truncatula* nodulation, even though they are the most abundant root flavonoids. Supplementation of flavone-deficient roots with 7,4-dihydroxyflavone, a major inducer of *S. meliloti nod* genes, completely restored nodulation. However, the same treatment did not restore nodulation in flavonoid-deficient roots, suggesting that other non-*nod* gene-inducing flavonoid compounds are also critical to nodulation. Supplementation of roots with the flavonol kaempferol (an inhibitor of auxin transport), in combination with the use of flavone-pretreated *S. meliloti* cells, completely restored nodulation in flavonoid-deficient roots. These observations indicated that flavones might act as internal inducers of rhizobial *nod* genes and that flavonols might act as auxin transport regulators during nodulation. However, all flavonoids have not been shown to exhibit auxin transport inhibition. The flavonol subclass in particular, such as kaempferol and quercetin, shows the strongest inhibitory activity (Ng et al. 2015).

Several possible mechanisms by which flavonoids modulate auxin transport have been reported. Acropetal auxin transport in the root, as well as basipetal auxin transport in the inflorescence, hypocotyl, and root, is all elevated in the absence of flavonoids. Flavonoids, such as quercetin, apigenin, and kaempferol, do not directly compete with IAA but are implicated as inhibitors of IAA transport across the plasma membrane by binding to a plasma membrane protein known as NPA receptor (Cooke et al. 2002). Rhizobia-induced flavonoids could bind to the auxin transporters AtMDR (Multidrug resistance), AtAPM (Aminopeptidase M1), and AtPINs (Pin-formed) (Peer et al. 2004) and prevent their intracellular trafficking, i.e., negatively regulate PAT in vivo (Buer and Muday 2004; Taylor and Grotewold 2005). Another possible mechanism is that flavonoids alter either the amount of synthesis or localization of auxin transport proteins, perhaps by phosphorylation of transcription factors that control the synthesis of these auxin carriers (Buer and Muday 2004). In addition to the participation of flavonoids in the polar transport of auxins, flavonoids are involved in the inhibition of auxin breakdown by peroxidases. Monohydroxy B-ring flavonoids are suggested as cofactors of peroxidase functioning as an IAA oxidase (destroys the hormone), whereas dihydroxy B-ring forms act as inhibitors of IAA-degrading activity (Mathesius 2001). Similarly, Agati and Tattini (2010) postulated that the high light-induced biosynthesis of flavonoids may have a role in regulating whole-plant and individual organ architecture. Pollastri and Tattini (2011) confirmed this as high sunlight-induced synthesis of quercetin derivatives fine-tuned auxin gradients as well as local auxin concentrations which represent the actual determinants for different morphological responses.

As IT reaches the base of the root hair cell, the nucleus in the adjacent cortical cells starts to reposition itself and a centrally located cytoplasmic bridge forms to establish pre-IT in this cell (Timmers et al. 1999). This process is repeated at each cell junction, thereby extending transcellularly, through a tip growth-like mechanism, from the epidermis or subepidermal cortex toward a subtending region of dividing cortical cells that have initiated the formation of a nodule primordium

(NP) (Fournier et al. 2008; Haag et al. 2013). NSP1/2 and NIN not only act downstream of CCaMK in the epidermis, but they also act downstream of CCaMK and the cytokinin receptor in the cortex (Marsh et al. 2007; Suzaki and Kawaguchi 2014; van Zeijl et al. 2015). *ENOD11* and *ENOD12* encode hydroxyproline-rich glycoproteins (HyPRPs), with relatively few tyrosine residues, which enhance cell wall plasticity or in components of the infection thread matrix. Hence, very rapidly and even before contact, legume root cells are paving the way for the accommodation of their symbiotic partner by remodeling the cell wall barrier (Rose et al. 2012; comprehensively reviewed by Rich et al. 2014). Cytokinin receptor LjLHK1/MtCRE1 is essential for Nod factor-induced gene expression and subsequent nodule primordium formation. Miri et al. (2016) postulated that cytokinin participates in orchestrating signaling events that promote rhizobial colonization of the root cortex and limit the extent of subsequent infection at the root epidermis by systemic autoregulation of nodulation (AON), thus maintaining homeostasis of the symbiotic interaction. Recent studies have revealed that exogenously applied cytokinin can bypass *Rhizobium* Nod factor signaling and the symbiotic phenotype of the *Mtcre1* mutant can be complemented, at least in part, by the exogenous application of flavonoids, suggesting that flavonoids mimic cytokinin functioning or even act as small secondary molecules downstream of cytokinin (Ng et al. 2015).

8.3.3.2 Nodule Organogenesis into Symbiosome: The Unifying Feature of Endosymbioses

Eventually rhizobia are released from the growing tip of ITs into an infection droplet in the cytoplasm [a subset of nascent nodule primordia (NP) cells], where they undergo differentiation to bacteroids in confined facultative organelle-like compartments called symbiosomes (SMs) (Kereszt et al. 2011). The rapid growth of bacteria at the tip tends to select out a single bacterial strain even if more than one strain becomes entrapped initially. This selection even works with two near-identical rhizobial strains in the infection (Gage 2002) and can select against potential “cheaters,” which will attempt to coinfect the nodule niche without conferring any benefit to the plant (Downie 2010). Through a process resembling endocytosis, the bacteria are surrounded by a plant-derived membrane, called the peribacteroid membrane, which forms SMs (McNear 2013). To accommodate a high number of endosymbionts, extreme plant cell enlargement is the consequence of repeated endoreduplication (ER) of the genome without mitosis (Kondorosi and Kondorosi 2004; Ribeiro et al. 2015). The bacteroids multiply in the growing host nodule cells to a certain cell density, adapt to the endosymbiotic lifestyle and microaerobic conditions, and mature to nitrogen-fixing bacteroids which convert atmospheric nitrogen gas into ammonia, using the nitrogenase enzyme complex (Ferguson et al. 2010). The couple jointly needs to modify their house especially to insulate the microsymbiont from the plant cytoplasm and to lower oxygen tensions for nitrogen fixation. One essential modification to the corridor is seen as the

peribacteroid or symbiosome membrane in infected cortical cells (Broughton et al. 2000). Thus, rhizobia remain outside the plant cytoplasm and are engulfed in a symbiosome membrane, which functions to regulate nutrient exchange between the partners (Mathesius 2009). In nodulating plants, flavonoids have been observed to delimit the border between symbiotic sites and non-symbiotic plant tissue. Another notable feature in these barrier regions is the accumulation of histidine-rich glycoproteins, such as ENOD2, which are capable of cross-linking with flavonoids (Stafford 1997). The existence of this barrier in legumes has been postulated to act as an O_2^- diffusion barrier that helps to prevent inactivation of nitrogenase in bacteroids (Cohen et al. 2001).

Two major morphological types of nodules are determined by the host legumes: determinate and indeterminate. Differences between the two nodule types are the site of first internal cell divisions, maintenance of a meristematic region, and the form of the mature nodules (Oldroyd et al. 2011; Hichri et al. 2016). Different roles of flavonoids in regulating auxin homeostasis for development of determinate and indeterminate nodules have been reported (Wasson et al. 2006; Subramanian et al. 2007; Zhang et al. 2009). Silencing the flavonoid pathway in *M. Truncatula* (forming indeterminate nodules) prevented localized auxin transport inhibition, and thus, flavonoid-deficient roots were unable to initiate nodules, even though normal root hair curling was observed (Wasson et al. 2006). However, Subramanian et al. (2005) silenced isoflavone synthase, the entry point enzyme for isoflavone biosynthesis in soybean (forming determinate nodules). Isoflavonoid-depleted roots of the determinate nodule forming legume soybean were also deficient in both auxin transport inhibition and nodulation. However, nodulation in isoflavone-deficient soybean roots could be restored by using isoflavone-hypersensitive rhizobia cells, suggesting that induction of Nod signal biosynthesis by isoflavones is crucial, whereas auxin transport inhibition by isoflavones is not. Consistently, during indeterminate nodule formation, flavonoid-regulated auxin transport inhibition occurs at the site of rhizobial infection, whereas during determinate nodule formation there is no significant auxin transport inhibition (Subramanian et al. 2007).

8.3.4 Role of Flavonoids in Autoregulation of Nodulation

At times of sufficient nitrogen supply to the legumes, rhizobial symbiosis can be limited by both local and systemic feedback regulatory mechanisms (Mathesius 2009). Since nodulation and the subsequent nitrogen fixation are energy-intensive processes, host plant maintains a balance between cost and benefit by tightly controlling the number of nodules it forms, *via* a complex root-to-shoot-to-root signaling loop called AON (Herder and Parniske 2009; Magori and Kawaguchi 2009). There are various root [e.g., CLAVATA3/ESR-related (CLE) peptide] and shoot (e.g., SDI) derived inhibitors identified in autoregulation of nodulation (Reid et al. 2011). Rossen et al. (1987) recognized four classes of mutations in the *nodD*

gene of *Rhizobium leguminosarum* biovar *viciae* that can affect its ability to autoregulate and/or activate other *nod* genes in the presence of flavonoid inducers. Class I mutations led to defects in both autoregulation and activation of other *nod* genes in the presence of inducers, class II mutations left activation of the transcription of *nodABC* and *nodEF* unaffected but virtually abolished autoregulation, and class III mutations affected the induction of other *nod* genes but not autoregulation. More adversely, class IV mutations showed the activation of other *nod* genes even in the absence of inducer molecules. Some of the anti-inducers also acted as inducers in the strains with class IV *nodD* mutations, thereby pointing toward the direct interaction of *nodD* proteins with the flavonoids. All the mutations led to decrease in nodule number; however, class IV mutations even failed to fix N₂ (even when a wild-type copy of *nodD* was present in the strain). This suggests that continued transcription of *nodABCII* and/or *nodEF* by bacteria in the nodule interferes with their ability to develop in N₂-fixing bacteroids (Rossen et al. 1987). Catford et al. (2006) identified that levels of formononetin and its 7-*O*-glucoside ononin systemically suppressed in response to *S. meliloti* and NFs, suggesting that these isoflavonoids are probably involved in the systemic suppression of nodulation as their application to autoregulated roots again promoted nodulation (Fig. 8.2).

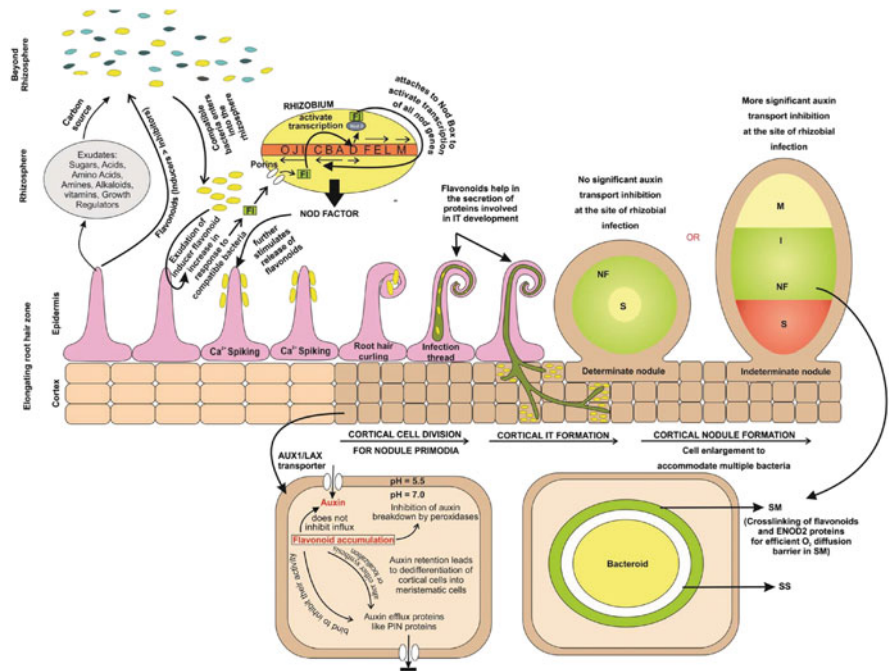


Fig. 8.2 Role of flavonoids in *Rhizobium*–legume symbiosis. Abbreviations: *Fl* flavonoids; *I* infection zone; *M* meristem zone; *NF* nitrogen-fixing zone; *S* senescence zone, *SM* symbiosome membrane; *SS* symbiosome space

8.4 Flavonoids in Arbuscular Mycorrhizal Symbiosis

Phosphorus (P) is the second most important macronutrient, next to nitrogen; however, P is the least accessible and hence most frequently deficient macronutrient limiting growth of crop plants (Balemi and Negisho 2012). Further, excess P supply in the soil, as chemical fertilizers, is another major environmental concern as it increases the risk of P movement to surface and groundwaters (Grant et al. 2005). To this end, arbuscular mycorrhiza (AM) fungi could be of great benefit in enhancing soil P utilization efficiency of the crop and improve nutritional status of the crop (Aggarwal et al. 2011). Unlike rhizobial symbiosis, mycorrhizae are the rule in nature, not the exception. In this mutualistic relationship, mycorrhizal hyphae (up to 100 times longer than root hairs) act as an extension of the root system, extending to soil beyond the P depletion zone (Mohammadi et al. 2011; Balemi and Negisho 2012). Thus, mycorrhization aids roots by providing a more widespread nutrient foraging system to plants, and this foraging provides fungus, an avenue to find new hosts (Denison and Kiers 2011; Bücking et al. 2012). A better understanding of this microbial interaction is therefore a potential key to determine the profitability and sustainability of agricultural systems (Mohammadi et al. 2011). Thus, here we review the mechanisms by which the plant signals (especially flavonoids) allow fungal infection and promote mutualistic symbiosis.

8.4.1 *Unfolding Role of Flavonoids in Signaling Imputed to Arbuscular Mycorrhiza (AM) Development*

Round-shaped thick cell-walled multinucleate fungal spores germinate to form haploid coenocytic hyphae (Hijri and Sanders 2005), when environmental soil conditions such as matric potential, temperature, and CO₂ levels are favorable (Mandal et al. 2010). This phase of growth in the absence of signal from the plant is known as aymbiotic stage (Requena et al. 2007). During this time the fungal colonies, living mainly on its triacylglyceride reserves, lengthen a few centimeters showing a characteristic growth pattern with marked apical dominance and sporadic hyphal branching. AM spores are surprisingly dynamic, and in the absence of a host root, growth ceases where hyphal septation from the apex arrests development and retracts protoplasm back into the spore within 8–20 days, becoming dormant again before consumption of the spore reserves (Bonfante and Genre 2010; Giovannetti et al. 2010). Therefore, even though spores are competent to germinate without the presence of a host, but a switch from the aymbiotic stage of development to an active presymbiotic growth phase triggers only in response to initial recognition by multifaceted fine-tuned reciprocal signaling events (Gachomo et al. 2009). Prior to colonization, significant developmental step in the life cycle of mycorrhizal fungi is host signal interceded increase in hyphal branching and

metabolic activity, which ensures directional growth of hyphal branches toward host root (Pinior et al. 1999).

Germ tubes, with limited growth potential, respond to the presence of “branching factor” in host root exudates in their vicinity, where fungal morphology shifts toward enhanced hyphal growth and extensive hyphal branching (Buee et al. 2000). The branch-inducing factor is present in root exudates of all the mycotrophic plants but absent in those of nonhost plants. Although no directional growth has been observed toward the root, several experiments showed that host-derived signals intensify hyphal ramification, thereby increasing the probability of contact with a host root (Requena et al. 2007). By analogy to the *Rhizobium*–legume symbiosis, some of the potential host exudates that stimulate spore germination, hyphal branching in the soil, and fungal invasion and arbuscule formation inside the root, often in a symbiont-specific manner, have been identified as *flavonoids* (Scervino et al. 2007; Steinkellner et al. 2007). These chemical signals exuded by the plant and the thigmotropic signals from the rhizodermis are possibly recognized by receptor proteins associated with the fungal plasma membrane (Requena et al. 2007). Interestingly, Siqueira et al. (1991b) suggested that flavonoids can stimulate fungal growth directly or remove AM fungal self-inhibition. Pyranosioflavones 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). Martens and Mithöfer (2005) considered the absence of flavone biosynthesis pathway in Brassicaceae as one of the reasons of this family lacking mycorrhizae. Thus, it is likely that host and nonhost plants can modulate the establishment of symbiosis by altering the profile of flavonoid exudates (Hassan and Mathesius 2012).

A variety of flavanones (hesperetin and naringenin), flavones (apigenin), and isoflavones (formononetin) have been reported to stimulate spore germination and hyphal growth of AM fungi *in vitro* (Gianinazzi-Pearson et al. 1989; Tsai and Phillips 1991; Nair et al. 1991). Effect of these compounds on AM fungi is flavonoid type and concentration, AM fungal species and genera, and host specific (reviewed by Vierheilig et al. 1998). At low concentration, different flavonoids can increase AM fungal spore germination, hyphal growth, and hyphal branching, while at high concentration, the same flavonoid turns inhibitory (Nair et al. 1991; Baptista and Siqueira 1994). Siqueira et al. (1991b) reported that isoflavonoids (formononetin and biochanin A) at concentrations of 5 mg l⁻¹ stimulated AM colonization in *T. repens* L., while flavone chrysin increased root colonization at concentration 40–60 mg l⁻¹. Morandi et al. (1992) tested the effect of 2 isoflavonoids (glyceollin I and coumestrol at concentration 0, 0.05, 0.5, 5, and 50 μM) and 1 flavonoid (quercetin at concentration 0, 0.1, 1, and 10 μM) on *in vitro* spore germination of *Gigaspora margarita*. After 5 and 7 days, number of germ tubes per spore was slightly increased by glyceollin I; mycelium length from germinated spores was increased by low concentrations of glyceollin I but was significantly decreased at the highest concentration. A positive correlation was found between coumestrol concentration and mycelium length, while vesicle

number decreased by coumestrol, quercetin, and the highest concentration of glyceollin (but was increased by glyceollin at 0.5 μM). Thus, similar to the case of *nod* gene induction, it is probably not simply the absence or presence of a specific flavonoid in root exudates which determines the signal properties, but rather a tightly controlled concentration-specific release pattern of inducers and anti-inducers.

Bécard et al. (1992) reported that only the flavonols stimulated *in vitro* growth of germinated spores of *Gi. margarita*, while the flavones, flavanones, and isoflavones tested were generally inhibitory. Quercetin (10 μM) prolonged hyphal growth from germinated spores of *Gi. margarita*, while the glycosides of quercetin, rutin and quercitrin, were not stimulatory, thereby indicating the role of structure of flavonoid in AM colonization. In general, at least one hydroxyl group on the B aromatic ring has been found to be necessary for stimulatory effect. Besides this, a hydroxyl on position 3 is also essential to confer stimulatory activity to the flavonol molecule, as the flavones luteolin and apigenin, lacking this hydroxyl, showed no effect (Bécard et al. 1992). Glycosylation at position 3 also promoted the loss of the stimulatory activity, as in quercitrin and rutin. Moreover, it has been suggested that saturation of the 2,3 double bond in flavonols, as seen in flavanones, promoted the loss of activity on hyphal growth of *Gi. margarita* (Chabot et al. 1992). This loss of activity was attributed to the loss of the planar configuration of the flavonol molecule when the double bond disappears; e.g., dihydroquercetin and dihydrokaempferol showed no effect on hyphal growth on *Gi. margarita*. However, these results are in contrast with those of Gianinazzi-Pearson et al. (1989) and Baptista and Siqueira (1994). Naringenin, lacking the 2,3 double bond, was stimulatory with *Gi. margarita* and *Gi. gigantea*, whereas apigenin, differing only by the presence of the 2,3 double bond, showed no effect with *Gi. gigantea*. In general, *Glomus* spp. are stimulated by flavonols as well as by isoflavones (Vierheilig et al. 1998). Whereas some flavonoids such as quercetin exhibit a general stimulatory effect on the hyphal growth of different AMF genera (Chabot et al. 1992, Baptista and Siqueira 1994), data with biochanin A showed a stimulation of *Glomus* (Nair et al. 1991; Vierheilig et al. 1998) but not *Gigaspora* species (Chabot et al. 1992, Baptista and Siqueira 1994).

8.4.2 *Myc Factor Signaling Pathway in the Root Epidermis*

Branched fungal hyphae secrete fungal chitin elicitor to be called “Myc factor” that drive various morphological and physiological changes in the hosts (Requena and Breuninger 2004), to orchestrate the AM infection process by counteracting the plant immune program and upregulating the expression of symbiosis-related genes (Kloppholz et al. 2011). At an early stage of mycorrhizal formation, plant constitutive chitinases may partially cleave these elicitors and thus inducing transient plant defense response (Antunes and Goss 2005; Maillet et al. 2011). However, unlike fungal pathogens, diffusible Myc factors from AM increase lateral root formation, elicit a transient cytosolic calcium elevation within a few minutes, and

induce expression of specific genes in only specific root cells in direct contact with the penetrating fungus (Navazio et al. 2007), while a suppressor activity is induced in non-colonized neighboring cells (Kosuta et al. 2003; Genre et al. 2005).

Several mutants in legumes like *M. truncatula*, *Lotus japonicus*, and pea are deficient for nodulation and AM, indicating the existence of a conserved symbiotic (Sym) pathway required for the establishment of both symbioses (Mitra et al. 2004b; Kistner et al. 2005). One common signaling component is the receptor-like leucine-rich kinase *SymRK* (*MtDMI2/MtSYM2*) that is involved in the direct or indirect transduction of fungal or rhizobial signals through its intracellular kinase domain to the cytoplasm (Stracke et al. 2002). Evolutionarily more recent Nod factors also overlap with Myc symbiotic signals, both structurally and functionally (Laparre et al. 2014; Limpens et al. 2015). Three nucleopore-associated proteins: NUP85, NUP133, and NENA (Saito et al. 2007) act downstream and could be involved in the transport of CASTOR and/or *DMII/POLLUX* (ion channels) to the inner nuclear envelope (Riely et al. 2007). In both the symbioses, these channels lead to calcium oscillations in the nucleus and perinuclear cytoplasm for activating calcium-calmodulin-dependent protein kinase *MtSYMI3/DMI3/CCAMK* (Mitra et al. 2004a). *CCAMK* is known to phosphorylate the last identified SYM gene *CYCLOPS*, encoding IPD3/CYCLOPS protein which interacts with DMI3 (Messinese et al. 2007). In order to activate the appropriate symbiotic program, legumes have to discriminate the two types of symbiotic signals. *CYCLOPS* represents a branch point in the common SYM pathway, as infection threat formation and arbuscular development are *CYCLOPS* dependent, but nodule organogenesis is *CYCLOPS* independent (Yano et al. 2008). Microbial activation of the common symbiosis signaling pathway can also modulate innate immune responses through its effect on the hormonal landscape, where Limpens et al. (2015) speculated a central role for DELLA proteins, in part by influencing the salicylic acid–jasmonic acid balance. However, profound mycorrhization in roots of *cre1* mutant of *M. truncatula* suggested that MtCRE1 (Cytokinin Response 1 required for N₂ fixation) does not belong to the ancestral common symbiotic pathway (Laffont et al. 2015). In addition to the Sym pathway, a parallel pathway exists and mediates AM signaling in non-nodulating eudicots and monocots (Mukherjee and Ané 2011).

Further, hyphal contact with host roots has been reported to induce the flavonoid pathway in a number of host species, in particular in infected cells (Steinkellner et al. 2007; Abdel-Lateif et al. 2012). Harrison and Dixon (1993) reported that in *M. truncatula* + *G. versiforme* interactions, the most striking changes in identified root metabolites included the overall accumulation of formononetin malonyl glucoside (FGM), medicarpin malonyl glucoside (MGM), and daidzein and a transient increase in free medicarpin (the major phytoalexin from MGM) in the early stages of the interaction, as a defense response to fungal elicitors. In mycorrhizal state, either the fungal symbiont may be consuming these phytoalexin precursors as carbon precursors or conjugation of medicarpin may inactivate or remove a potentially toxic metabolite. In contrast to the levels of phenylalanine ammonia lyase (PAL) and CHS transcripts, which remained elevated throughout the interaction, the level of Isoflavone reductase (IFR) transcripts decreased in later stages of the

interaction to 2.5-fold below the level in uninoculated control roots, suggesting that the established mycorrhizal association in *M. truncatula* brought about a specific suppression of this transcript to decline medicarpin levels in these roots. However, in the interaction between *myc*⁻ line and *G. versiforme* the levels of medicarpin and related transcripts remained elevated throughout the interaction suggesting that a defense response was occurring. Further, the inability of *myc*⁻ *M. sativa* line to form a complete interaction may be attributed to the absence of coumestrol and 4',7-dihydroxyflavone (Harrison and Dixon 1993). Studies of Guenoune et al. (2001) and Akiyama et al. (2002) on flavonoids and AM reopened the discussion about the involvement of flavonoids as regulatory compounds during signaling of mycorrhization. Guenoune et al. (2001) demonstrated that medicarpin-3-*O*-glucoside, an isoflavonoid phytoalexin, accumulated both in roots colonized by the pathogenic fungus and in AM-treated roots receiving high P and prevented these roots from being colonized by AMF. Increases in the steady-state levels of chalcone isomerase and IFR mRNAs, as defense responses of alfalfa roots to the pathogenic fungus *Rhizoctonia solani*, were reduced significantly in roots simultaneously infected with the arbuscular mycorrhizal (AM) fungus *Glomus intraradices*. These data suggested that during early stages of colonization by *G. intraradices*, suppression of defense-related properties is associated with the successful establishment of AM symbiosis. Akiyama et al. (2002) detected the C-glycosylflavone— isovitexin 2''-*O*- β -glucoside in non-mycorrhizal, P-deficient melon roots but not in roots with a high P status or in mycorrhizal roots (inoculated with *G. caledonium*). Application of this flavonoid (at concentrations of 20 and 50 μ M) to AM plants not only enhanced root colonization in plants grown under low P conditions, but also in plants grown under high P conditions, thus clearly showing that a high P status or the mycorrhizal status of a plant can reduce the accumulation of a flavonoid in roots which stimulates mycorrhization.

Tsai and Phillips (1991) reported that after 21 days of application, 1.0–2.5 μ M quercetin-3-*D*-galactoside, 4',7-dihydroxyflavone, and 4',7-dihydroxyflavanone promoted spore germination, hyphal elongation, and hyphal branching of *Glomus etunicatum* and *Glomus macrocarpum* *in vitro*. On the other hand, formononetin, an isoflavone that is released from stressed alfalfa roots, inhibited germination of both *Glomus* species. However, flavonoids tested in this study were identified as products from non-AM alfalfa seedlings only, strongly advocating that AM fungi alter flavonoid metabolism in plants and compounds produced as a result of infection have a role in differentiation of vesicles, arbuscules, and/or spores (Tsai and Phillips 1991). Again, application of biochanin A and formononetin in the rhizosphere of AM nonhost plants—*Lupinus polyphyllus* and *Spinacia oleracea* (grown in the presence of *G. mosseae*)—resulted in hyphal attachment and more hyphae around these roots (Vierheilig and Piché 1995). These results could indicate a simple hyphal growth stimulation to be responsible for the enhanced root colonization in AM host plants; however, as soil application also resulted in a colonization of *L. polyphyllus* roots by *G. mosseae* (Vierheilig and Piché 1995; Vierheilig et al. 1996), a role of these compounds in the plant–fungus communication during the colonization stage is suggested (Vierheilig et al. 1998). Vierheilig and Piché (2002)

presented a complex model for the role of flavonoids during the AM association. They proposed that a fundamental molecular dialogue occurs that regulates not only the early development of AM symbiosis but also subsequent colonization which has to be balanced for establishment of genuine mycorrhizal symbiosis.

8.4.3 *AM Hyphal Invasion and Differentiation of Intraradical Hyphae: The Conjugal Lodging*

After hyphal docking to cell surface by means of an appressorium, the fungus enters into root epidermis *via* AM-specific prepenetration apparatus (Bonfante and Genre 2010). AM fungi first grow intercellularly or cross outer cells with linear or simple coiled hyphae (Gianinazzi and Gianinazzi-Pearson 1988). Reaching the inner cortex the fungal symbionts switch to different mode of colonization and penetrate the plant cell, where between cell wall and plant plasma membrane fungal hyphae extensively ramify to form a dichotomously branched haustorium, called “arbuscule” with a high surface to volume area (Gutjahr and Parniske 2013). Although the arbuscule eventually expands to largely fill the cortical cell, the hyphal tree does not enter the plant symplast. This plant–fungal interface consists of the fungal cell wall, the perihyphal or periarbuscular membrane (PAM), and a matrix between the two organisms—periarbuscular space (Pumplin and Harrison 2009; Rich et al. 2014). Hofferek et al. (2014) reported that in the root cortex nodulation and mycorrhization are regulated by *NSP2* whose spatiotemporal expression negatively regulates both types of root endosymbioses through perception of the nutritional status (such as high Pi or N) of the host plant. *VAPYRIN* also promotes intracellular accommodation of endosymbionts by interacting with membranes/cytoskeleton and is thus required for intracellular accommodation of AM fungi and rhizobia in epidermal as well as cortical cells (Feddermann et al. 2010; Gobbato 2015). Shtark et al. (2016) indicated that as compared to wild type (wt), though non-nodulating pea mutants (*sym7*, *sym11*, *sym14*) had considerable increase in root surface colonization by *Rhizophagus irregularis*, a substantial decrease in internal colonization (especially arbuscule formation) was recorded. *sym34* mutants displayed strongly reduced root surface colonization, but 10 days later internal colonization did not differ from wt. While *sym38* mutant did not differ from wt indicating that except *SYM38*, all analyzed genes were essential for both nodule and AM development (Shtark et al. 2016). Various homologues of *MtCBS1/2* gene, to encode protein containing a cystathionine-b-synthase (CBS) domain in ITs, act downstream *NIN* in rhizobial infection, in nodule organogenesis, as well as in mycorrhization, again suggesting common infection mechanisms (Sinharoy et al. 2016).

Harrison and Dixon (1993) reported that roots of the mycorrhizal resistant *M. sativa* mutant (*Myc*[−]) do not become colonized, but hyphae grew attached to the root surface forming appresoria. In the presence of *G. versiforme*, levels of

several flavonoids (diadzein, formononetin, FGM, medicarpin, MGM) increased in the *M. sativa* (Myc⁻) roots in the same way as in colonized root of the wild-type (Myc⁺) *M. sativa* (Harrison and Dixon 1993). Volpin et al. (1994) also found an increase of formononetin in inoculated but still uncolonized roots of *M. sativa* (Myc⁺). Thus, these two researches suggested that these flavonoids play a role in the plant–fungus signaling during precolonization and cell-to-cell stage. Further, Harrison and Dixon (1993) also demonstrated that some flavonoids are newly induced after AM fungal root colonization, where coumestrol and 4',7-dihydroxyflavone were only present in colonized roots of (Myc⁺) *M. sativa* and *M. truncatula*, but were not detected in uncolonized roots of the two plants and in roots of inoculated (Myc⁻) *M. sativa*. This further suggested that these flavonoids play a signaling role during the intraradical phase of the hyphae in the AM symbiosis and might be an indication for its role in the formation of highly ramified arbuscules. Naringenin found in root exudates of bean (Hungria et al. 1991) enhanced in axenic culture of *Gi. margarita*, where the number of vesicle clusters per germinated spore increased. Earlier *in vitro* experiments by Gianinazzi-Pearson et al. (1989) evidenced similar effects with apigenin and hesperetin. Luteolin, found in the seed rinse of alfalfa (Hartwig et al. 1990), although showing no stimulation of hyphal growth, stimulated the production of auxiliary cells in *Gi. margarita* (Bécard et al. 1992). Thus, hyphal differentiation possibly requires a different stimulatory mechanism or induction than do hyphal growth. Some flavonoids may be involved in only one of these mechanisms and not in the other. However, quercetin and myricetin, which exhibit a hyphal growth stimulating effect on different AM fungal genera, also enhanced the formation of auxiliary cells (Bécard et al. 1992), suggesting again a more general role of these two compounds compared to other flavonoids. Sometimes similar changes could be observed within a plant family. The level of coumestrol and daidzein increased in colonized roots of *Glycine max*, *M. sativa*, and *M. truncatula* (Morandi et al. 1984; Harrison and Dixon 1993), all belonging to the leguminosae family, and the level of blumenin was enhanced in colonized roots of several members of the Gramineae (Maier et al. 1995). Change of flavonoids level also depends on the AM fungal species, i.e., different AM fungi have different requirements for their development and thus induce different levels of these compounds or that the plant recognizes the two fungi differently. Ponce et al. (2004) reported that 5,6,7,8,9-hydroxy chalcone (NM7), 3,7-hydroxy-4'-methoxy flavone, 5,6,7,8-hydroxy-4'-methoxy flavones (RR4), and 3,5,6,7,4'-hydroxy flavones (RR4-2) could be detected only in non-mycorrhizal roots of white clover, whereas the flavonoids acacetin, quercetin, and rhamnetin were only present in roots inoculated with *G. intraradices*.

Arbuscules act as the epicenter of mutualism as they represent an extreme form of intimacy and compatibility and are site of nutrient transfer from the fungus to the host plant (Hughes et al. 2008; Denison and Kiers 2011; Mohammadi et al. 2011). In return, host-derived carbon is transferred to the fungi and stored in energy-rich vesicles to support vegetative growth or spore formation (Genre and Bonfante 2010). Whereas a high frequency of arbuscules usually indicates cost-effective nutrient exchange in both directions, high vesicular colonization is a potential

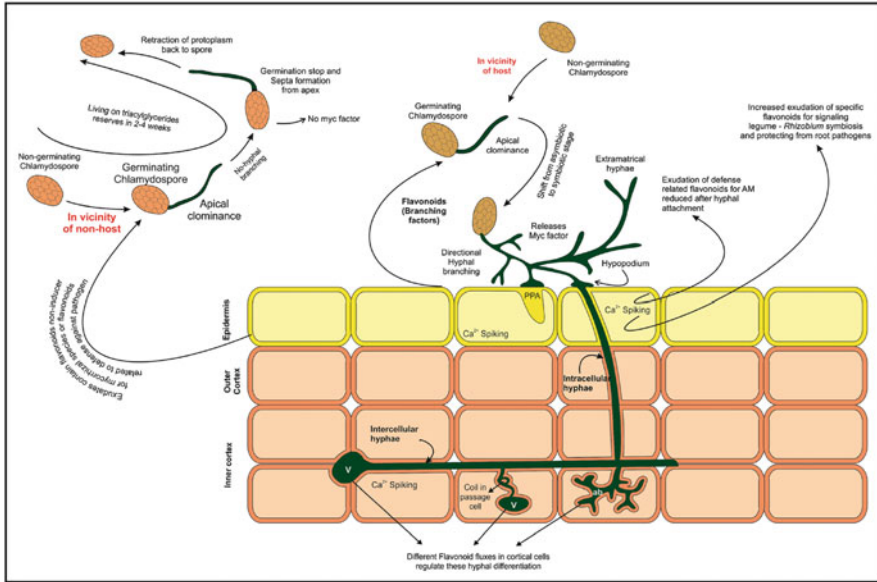


Fig. 8.3 Role of flavonoids in Arbuscular mycorrhizal symbiosis. Abbreviations: *ab* arbuscule; Ca^{2+} Calcium ion; *PPA* prepenetration apparatus; *v* vesicle

indicator of fungal resource hoarding prominently under high external nutrient conditions, when hosts are less dependent on fungal partners (Johnson 2010; Nijjer et al. 2010). Further, host molecules like lysophosphatidylcholine (LPC) potentially allow them to evaluate the amount of P delivered via the mycorrhizal pathway (Bucher et al. 2009). Host will decrease C provision or directly digest arbuscules when there is insufficient P being transferred to the host across the colonized cell (Kobae and Hata 2010). Resource exchange is followed by rapid arbuscule collapse in 4–5 days, with structures degenerating within 2.5–5.5 h (Bonfante and Genre 2010), much more rapidly than the decline in N_2 fixation in nodules (Wong and Evans 1971). The fungal life cycle is completed after formation of asexual chlamydo-spores on the external mycelium, which allow them to propagate and survive in the absence of a host, for more than 10 years (Giovannetti et al. 2010) (Fig. 8.3).

8.4.4 Role of Flavonoids in Autoregulation of Mycorrhization

An already mycorrhizal plant develops one mechanism to repulse further colonization by fungi, i.e., to not differentiate between AMF and soilborne pathogenic fungi (Vierheilg and Piché 2002; Mechri et al. 2015). Flavonoids again play a role in autoregulating mycorrhization, as depending on the flavonoids and their

concentration, they can exhibit an inhibitory and/or a stimulatory effect on fungi (Vierheilig et al. 1998; Subramanian et al. 2007; reviewed by Lira et al. 2015). Larose et al. (2002) found that after an application of a mixture of spores and extraradical hyphae of *G. intraradices* to *M. sativa* roots, coumestrol, ononin, and daidzein levels were increased, whereas formononetin was detected at lower levels, indicating that at this stage of symbiosis, i.e., during appressoria formation, fungal partner, with signals released by its mycelium, is actually asking for a change of the concentration of certain flavonoids, which exhibit stimulatory effects on fungi. The accumulation of medicarpin (defense phytoalexin) only at the stage of appressoria formation, when the fungus has formed its first structures in the root, indicates that once the fungal partner has formed its appressoria it was perceived in a similar way as a pathogen. Later the perception of fungi by the plant changed and at a stage of intense AM root colonization, no altered medicarpin accumulation was observed, while coumestrol accumulation further increased. Interestingly, formononetin/daidzein levels were linked inversely, as at the beginning of root colonization formononetin was reduced in mycorrhizal roots whereas daidzein increased and at the end of the experiment, when formononetin increased daidzein levels seemed to decrease. Besides coumestrol, medicarpin again accumulated at the end of colonization, thereby pointing toward the role of coumestrol at any stage of root colonization; medicarpin probably plays a role at the beginning of root colonization and at a later stage. There was a positive link between the accumulation of coumestrol, medicarpin, and/or formononetin at the later stages and the autoregulation of mycorrhization (Vierheilig and Piché 2002). Some species-specific quantitative changes were observed, as ononin was highly accumulated in roots colonized by *G. mosseae*, but only weakly accumulated in roots colonized by *G. intraradices* while coumestrol was high in roots colonized by *G. intraradices* but lower in roots colonized by *G. mosseae*. The only compound that accumulated to similar levels in all AMF-colonized roots was genistein, and the similar accumulation pattern of genistein in mycorrhizal roots independent of the root-colonizing AMF indicated a general, non-species- or non-genus-specific regulatory role of this compound during mycorrhization (Larose et al. 2002). Later on, Scervino et al. (2005a) reported that acacetin and rhamnetin inhibited the formation of AM penetration structures as well as colonization and pointed toward a possible involvement of these two flavonoids in the autoregulation process of the AM symbiosis. Even, flavonoid NM7 inhibited the number of entry points and the percentage of root length colonization of the test plant by *Gigaspora* and *Glomus* species. The presence of 5,6,7,8,9-hydroxy chalcone in non-AM colonized clover roots and the disappearance of this flavonoid in mycorrhizal clover roots indicated that the AM fungus *G. intraradices* induced a mechanism, which reduces this inhibitory molecule in the mycorrhizal root. Quercetin, 5,6,7,8-hydroxy-4'-methoxy flavones, and 3,5,6,7,4'-hydroxy flavone increased the number of entry points and the AM colonization of tomato roots by *Gigaspora* (not *Glomus*). Further, Scervino et al. (2005b) linked flavonoids changes in mycorrhizal roots directly to regulatory processes of the AM symbiosis for the first time. Interestingly, RR4 and RR4-2 stimulated most of the presymbiotic stages of *Gigaspora* (but not of the two *Glomus* species), supporting

the implication of Akiyama et al. (2002) that a flavonoid, stimulating root colonization by AM fungi, was only present in non-mycorrhizal but not in mycorrhizal roots. They further suggested that the compound might be involved in one stage of the fungal regulation, but not in another, as RR4 increased the percentage of spore germination of *Gi. margarita*, however, inhibited the hyphal growth, and showed no effect on hyphal branching and the cluster formation of auxiliary cells. NM7 was detectable only in non-mycorrhizal white clover roots and inhibited the presymbiotic development of all AM fungi tested. There was stimulatory effect of newly synthesized quercetin on spore germination and/or hyphal growth of *Gigaspora* species. However, acacetin and rhamnetin exhibited an inhibitory effect on nearly all fungal parameters of both AM genera (*Gigaspora* and *Glomus*) tested, pointing toward an implication in the autoregulation of mycorrhization (Scervino et al. 2005b). Scervino et al. (2009) reported that 2 μM concentration of 3-methoxy-5,6,7,8-hydroxy-4'-hydroxy flavone (NMHTV) isolated from shoots of non-arbuscular mycorrhizal (AM) inoculated clover did not affect the percentage of germination of spores but significantly increased the formation of entry points and symbiotic stage of *Gi. margarita* spores on colonization of tomato (*Lycopersicon esculentum*), while at higher concentration of 8 μM concentration inhibited the hyphal length of *Gi. rosea*. The absence of stimulation of the AM presymbiotic and symbiotic stages in tomato by exogenous application of the newly synthesized flavonoids 5,6,7,8-hydroxy-3-methoxy flavone (MH-1), 5,6,7,8-hydroxy-4'-hydroxy flavone (MH-2), and 5,7-hydroxy-3,4'-methoxy flavone (MH-3), isolated from AM clover (*T. repens*) shoots, indicated that the autoregulation of the AM symbiosis can be, at least partially, due to the disappearance of flavonoids in AM-colonized plants that stimulated the AM symbiosis.

Working with split-root systems of barley, alfalfa, and soybean, it was reported that root colonization of one side of a split-root system strongly suppressed mycorrhization of "autoregulated roots" on the other side (Vierheilig 2004b; Meixner et al. 2005). Meixner et al. (2005) reported that the soybean supernodulating mutant *nts1007* [mutated in the receptor kinase gene *GmNARK* (Searle et al. 2003)], which lacks the autoregulatory mechanism to control nodulation, did not autoregulate AMF root colonization. Thus, these reports indicated a similar mechanism of autoregulation in both symbioses. Alike *Rhizobium*-legume symbiosis, flavonoids have also been suggested to be involved in the regulation of mycorrhization as roots treated with certain flavonoids exhibited increased AM fungal root colonization (Vierheilig and Piché 2002; Vierheilig 2004a; Scervino et al. 2005a, b). Catford et al. (2006) studied split-root systems (SRS) of alfalfa plants, where one side of SRS was inoculated with *S. meliloti* or *Glomus mosseae* or was treated with Nod factor. All the three applications resulted in accumulation of daidzein but reduced levels of formononetin, ononin, and medicarpin. These treatments given on one side altered isoflavonoids of the medicarpin synthesis pathway on the other side, thereby suggesting that levels of formononetin and its glycoside ononin were not only locally reduced but also systemically downregulated in parts of the root system that were not in contact with the invading symbionts. Thus,

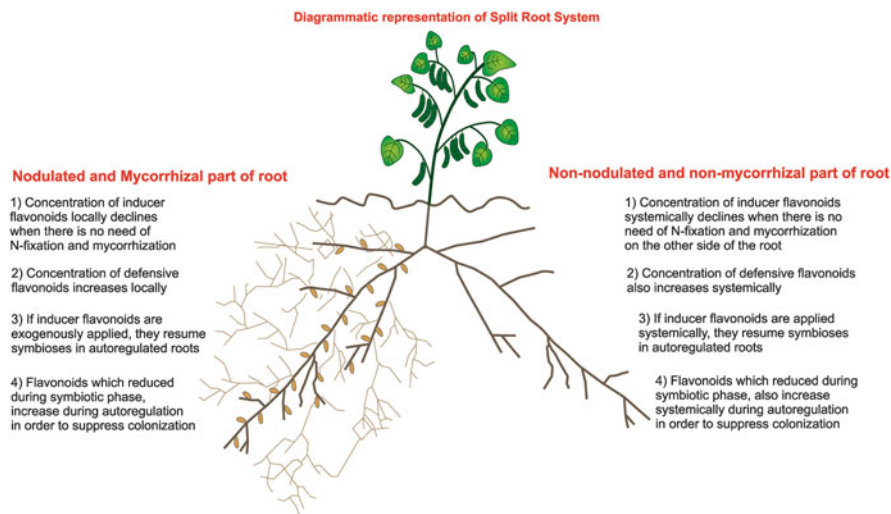


Fig. 8.4 Role of flavonoids in autoregulation of nodulation and mycorrhization

Catford et al. (2006) suggested that systemic downregulation of formononetin and ononin levels in non-treated root parts is a metabolic response to the putative autoregulation signals that mediate suppression of symbioses. Exogenous application of isoflavonoids to roots inactivated or compensated the symbiosis-suppressing effects induced by long-distance autoregulation signals and promoted nodule formation and stimulated establishment of the AM fungal symbiosis. Ononin (but not formononetin) promoted AMF root colonization, whereas formononetin stimulated nodulation in autoregulated parts of the root system. Hence, although autoregulation of nodulation and mycorrhization seem to share some common signaling events (Vierheilig 2004a; Meixner et al. 2005; Vierheilig et al. 2008), the effects of systemically regulated isoflavonoids on establishment of symbiosis were different (Fig. 8.4).

Many legume plants benefit from the tripartite symbiosis of arbuscular mycorrhizal fungi and rhizobia. Siqueira et al. (1991b) reported that in isoflavonoid formononetin and biochanin A (considered “anti-nod inducers”) treated plants, *Rhizobium* nodulation was enhanced in the presence of AM fungi which might have resulted from improved mycorrhizal colonization and plant growth. Soil applications of these compounds had also shown to stimulate mycorrhizal formation and growth of non-nodulated plants in the presence of AM fungi (Siqueira et al. 1991a), thus indicating that isoflavonoid growth stimulation was not nodulation mediated. However, Xie et al. (1995) found that the inoculation of *G. max* with *B. japonicum* increased the colonization by *G. mosseae*, and an increased flavonoid concentration in root exudates was suggested to be responsible for this effect. Xie et al. (1997) reported the increase in mycorrhizal colonization (from <30 to 65%) and sporocarp formation of *G. mosseae* (Nicol. & Gerd.) Gerdemann & Trappin in roots of *Lablab purpureus* (L.) Sweet treated with Nod factors purified from

Rhizobium sp. NGR234. Antunes et al. (2006) found that the concentration of daidzein was at least four times greater in soybean root than in the seed, whereas coumestrol, which was absent in the seed, was newly synthesized. The significant increase in diadzein and coumestrol appeared to result mainly from the development of AM, which might have helped in bradyrhizobial symbiosis (diadzein and coumestrol are involved in bradyrhizobial symbiosis) and thus played a key role in the early stages of the tripartite symbiosis. Morandi et al. (2009) reported a new *M. truncatula* mutant B9, defective for nodulation but was hypermycorrhizal. It represented a new tool for the study of plant metabolites differentially regulating mycorrhiza and nodulation symbioses, in particular those related to autoregulation mechanisms. In contrast to wild A17, mutant was characterized by considerably higher root concentrations of the phytoestrogen coumestrol (*nod* gene inhibitor for *S. meliloti* and hyphal growth inducer of *Gi. margarita*) and coumestrol conjugate malonyl glycoside, thus leading to low and late nodulation but high mycorrhizal colonization in B9 mutant. Fokom et al. (2010) pointed out significant interaction between arbuscular mycorrhizal fungi and 3-*o*-glucoside kaempferol in enhancing P nutrition, N₂ fixation, as well as phenolic metabolism in *Vigna unguiculata* (L.) Walp growing in low-P soil of southern Cameroon. Many flavonoids like quercetin, luteolin, and other substituted flavones and flavanones are released by germinating seeds and living roots of legume crops over time and their interactions with fungal pathogens suggest that while stimulating mycorrhizal fungi they may be equally important for plant protection against soilborne pathogens (Hassan and Mathesius 2012). Cordeiro et al. (2015) evidenced from field experiment that formononetin can improve mycorrhizal colonization, nodules number, and reduce the negative effects of fungicides Carbendazim + Thiram in soybean production. Thus, utilizing flavonoids can be an excellent opportunity to utilize and manipulate *Rhizobium* and AM fungi in order to enhance crop productivity in a cost-effective manner and with reduced agricultural chemical inputs. Currently, seed exudates, which are mixture of flavonoids, are being used commercially to promote *Rhizobium*–legume symbiosis and N₂ fixation in agricultural practices (Skorupska et al. 2010). A commercial product “SoyaSignal” (mixture of genistein and daidzein) in Northern America is being applied either directly to the seed or in furrows in soils that contain adequate populations of *Bradyrhizobium* (Smith and Zhang 1999). SoyaSignal significantly improved nodulation and nitrogen fixation which resulted in an average 7% increase in grain yield (Leibovitch et al. 2001; Broughton et al. 2003). This study linked the function of different flavonoids to the establishment of the tripartite symbiosis and suggested that these compounds are produced and released into the rhizosphere as a function of the colonization process.

8.5 Conclusion

Thus, this chapter highlighted the significance of flavonoids as common architects in the establishment of two agriculturally important root symbioses—nodulation and mycorrhizal interactions. Information attained from these studies illustrated that besides stimulating microbial swarming motility toward host and activating symbiosis-related microbial genes, multifaceted flavonoids contribute in various stages of symbiosome developmental program. Their differential endogenous accumulation regulates modulation of plant innate defense reactions, infection thread/hyphal growth, intraradical hyphal differentiation, cortical cell morphogenesis for providing optimal niche to the microbes, and prevention of metabolically inefficient symbioses. Deciphering the complexity of flavonoids induced responses and their interplays with other major regulators of symbioses such as hormones make flavonoids a vital player in plant–microbe symbioses. However, gaps still remain in the knowledge base and further researches involving the endogenous regulation of flavonoid biosynthesis during these mutualistic associations as well as use of flavonoids as potential soil amendments are needed to optimize their implementation in improving rhizobial and mycorrhizal symbioses.

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