Chapter 1
Journey to Nodule Formation: From Molecular Dialogue to Nitrogen Fixation

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1.1 Introduction

1.1.1 Nitrogen Fixation and Legumes

The use of legumes in agricultural rotations was documented by Pliny the Elder in 147 BC (Crawford et al. 2000). The Leguminosae family is taxonomically categorized into three subfamilies which encompass nodulating plants, the Caesalpinioideae, Mimosoideae, and Papilionoideae (Doyle and Luckow 2003), although most nodulation occurs in the Papilionoideae. They are the third largest family of angiosperms consisting of more than 650 genera and over 18,000 species (Lewis et al. 2005) and are second only to Gramineae in their importance as human food accounting for 27 % of the world's crop production and contributing 33 % of the dietary protein nitrogen needs of humans (Graham and Vance 2003). Legumes include a large number of domesticated species harvested as crops for human and animal consumption as well as for oils, fiber, fuel, fertilizers, timber, medicinals, chemicals, and horticultural varieties (Lewis et al. 2005). One of the reasons legumes are so popular is their ability to satisfy their nutritional needs by communicating and establishing symbiotic relationships with microbes in the rhizosphere. Like 80 % of the terrestrial plant species, legumes form arbuscular mycorrhizal (AM) associations, where the fungus colonizes the cortical cells to access carbon supplied by the plant and the fungus helps the plant in the transfer of mineral nutrients, particularly phosphorus, from the soil (Smith and Read 2008). AM is a very ancient symbiosis more than 400 million years old. In contrast, the endosymbiosis of plants with nitrogen-fixing bacteria is limited to only a few plant families and is more recent evolutionarily, approximately 60 million years old (Godfroy et al. 2006). Around 88 % of legumes examined to date form nodules in association

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with rhizobia, and important agricultural legumes alone contribute about 40–60 million metric tons of fixed N_2 annually while another 3–5 million metric tons are fixed by legumes in natural ecosystems (de Faria et al. 1989; Smil 1999).

The legume–rhizobia symbiosis has been investigated using *Pisum sativum* (pea), *Vicia sativa* (vetch), *Medicago sativa* (alfalfa), *Medicago truncatula* (barrel medic), *Glycine max* (soybean), *Phaseolus vulgaris* (bean), *Sesbania rostrata* (sesbania), and *Lotus japonicus* (lotus). The large genome size and low efficiency of transformation of many crop legumes combined with the advent of genomic research resulted in a concentration on two symbiotic models, *M. truncatula* and *L. japonicus* (Oldroyd and Geurts 2001; Udvardi and Scheible 2005). These systems provide an opportunity for researchers to study both bacterial and fungal symbioses not supported by the well-studied model plant, *Arabidopsis thaliana*, at the molecular level. Both species have all the tools for a model system such as a small diploid genome, self-fertility, ease of transformation, short life cycle, high level of natural diversity, and a wealth of genomic resources (Handberg and Stougaard 1992; Cook 1999). Both belong to the Papilionoideae subfamily, and based on the nature of the nodule they develop, they are classified as determinate (*L. japonicus*) and indeterminate (*M. truncatula*) nodulators. Determinate nodules are characterized by a nonpersistent meristem which ceases development at early stage. This results in round nodules with a homogeneous central fixation zone composed of infected rhizobia-filled cells interspersed with some uninfected cells. In contrast, indeterminate nodules are cylindrical and consist of a gradient of developmental zones with a persistent apical meristem that supports indeterminate growth, an infection zone, a fixation zone, and zone of senescence (Fig. 1.1). The two model plants represent the two nodule development strategies, and most findings discussed in this chapter come from these systems.

1.1.2 Early Steps in Legume–Rhizobia Symbiosis

1.1.2.1 Overview

Soil bacteria belonging to the genera Rhizobium, Bradyrhizobium, Sinorhizobium, Allorhizobium, and Mesorhizobium establish a unique beneficial interaction with most legumes and a few nonleguminous plants in the family of Ulmaceae (*Parasponia* sp.). The interaction between rhizobia and the host plant results in the formation of N_2-fixing nodules. Within these nodules, bacteria are provided with a carefully regulated oxygen and carbon supply which makes it possible for the bacteria to reduce nitrogen efficiently for the plant. The early steps of the symbiosis begin with the exchange of discrete signals, a molecular dialogue, between the bacteria and the plants (Shaw and Long 2003). Plants produce and release chemicals, mainly flavonoids and isoflavonoids, into the rhizosphere. These molecular signals initiate root nodulation by the induction of *nod* genes in rhizobia, promoting bacterial movement towards the plant and enhancing the growth of the

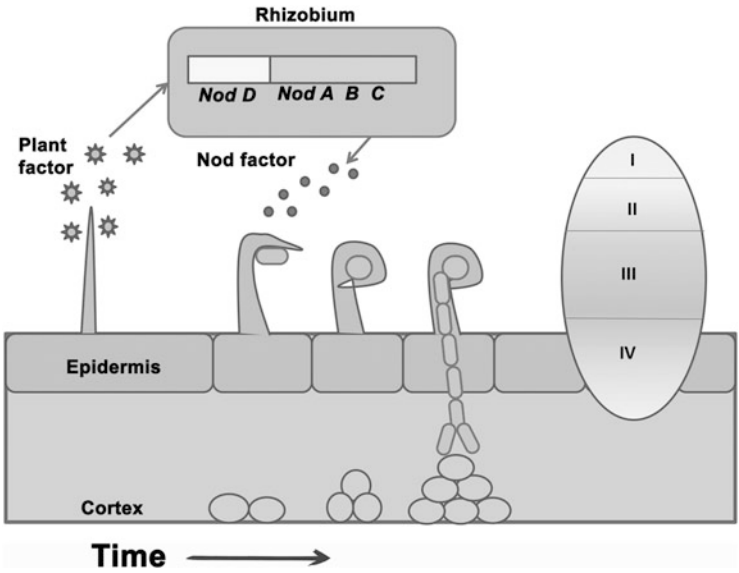

Fig. 1.1 Initial phases in the legume–rhizobium symbiosis. The interaction between rhizobia and legume microsymbionts is determined by two specific steps in the mutual signal exchange. First, bacterial nodulation (*nod*) genes are activated in response to plant-secreted signal molecules (plant factors), especially flavonoids, resulting in biosynthesis and secretion of Nod factors by rhizobia bacteria. In the second step, Nod factors elicit two simultaneous processes in the host plant roots, triggering the infection process and nodule formation (cortical cell division). The infection process includes curling of the root hair around the attached bacteria, infection thread formation, and release of rhizobia from the infection thread into the dividing cortical cells while nodule formation includes mitotic activation of the inner cortical cells, division, and establishment of a meristem zone (I) and infection zone (II), a nitrogen fixation zone (III), and in a senescence zone (IV)

bacterial cells (Phillips and Tsai 1992). The plant factors are recognized by rhizobial NodD proteins, transcriptional regulators that bind directly to a signaling molecule, and are able to activate downstream *nod* genes (Mulligan and Long 1985). Rhizobial *nod* genes are responsible for the production and secretion of species-specific Nod factors, lipochitooligosaccharidic signaling molecules (Zhu et al. 2006). Upon exposure to Nod factors, the plant root hair cells growth is altered, a periodic calcium spiking is induced, a preinfection thread structure is formed, gene expression is altered, and inner cortical cells in the root are mitotically activated, which together leads to the formation of nodule primordia (Ane et al. 2004; Kuppusamy et al. 2004; Mitra et al. 2004; Geurts et al. 2005; Middleton et al. 2007). The infection thread housing the bacteria advances through this actively dividing zone of cells to the nodule primordia. The subsequent release of the bacteria into individual cortical cells by endocytosis results in the enclosure of the bacteria within a plant membrane called the peribacteroid or symbiosome membrane. The peribacteroid membrane effectively isolates the bacteria from the host cell cytoplasm while controlling transport of selected metabolites in both

directions (Puppo et al. 2005). The bacteria inside the symbiosome membrane differentiate into bacteroids that produce nitrogenase for nitrogen fixation (Lodwig et al. 2003). However, for effective nitrogen fixation, nitrogenase needs a low oxygen environment, while at the same time rapid transport of oxygen to the sites of respiration must be ensured. These conflicting demands are met by the presence of millimolar concentrations of the oxygen-binding protein leghemoglobin within the cytoplasm of nodule cells (Ott et al. 2005). Recent work using leghemoglobin RNA interference lines in *L. japonicus* showed altered bacterial and plant cell differentiations, decreased amino acid levels in nodules, and a defect in nitrogen fixation (Ott et al. 2009). The resulting physiological and morphological changes in the host plant lead to the formation of nodules, a suitable environment for bacterial nitrogen fixation (Fig. 1.1). The fixed nitrogen obtained by the plant is not without cost, as the plants in return must contribute a significant amount of energy in the form of carbon skeletons to the bacteria.

1.1.2.2 Plant-Derived Signals

The plant starts the molecular dialogue by releasing flavonoid and isoflavonoid compounds to the rhizosphere (Redmond et al. 1986; Kosslak et al. 1987). Flavonoids have multiple roles in rhizobia–legume symbiosis. These compounds serve as chemoattractants for the rhizobial symbiont and trigger the biosynthesis and release of Nod factors from the bacteria. They do so by acting as a signaling molecule, binding to the bacterial transcription factor *NodD*. *NodD* in turn activates the expression of rhizobial *nod* genes, which are responsible for the production of Nod factors (lipochitin oligosaccharides). The perception of Nod factors by a receptor in the legume host triggers a sequence of events, including curling of root hairs around the invading rhizobia, the entry of the rhizobia into the plant through infection threads, and the induction of cell division in the root cortex that marks formation of the nodule primordium. The recognition of specific flavonoids secreted by the root by compatible rhizobia is the earliest step in determining host specificity.

Flavonoids are also involved in the initiation of the nodule through their action on the plant hormone auxin and could thus play a developmental role in addition to their action as *Nod* gene inducers (Hirsch 1992). RNA interference (RNAi) in *M. truncatula* used to silence the enzyme that catalyzes the first committed step of the flavonoid pathway, chalcone synthase (CHS), reduced the level of flavonoids, and the silenced roots were unable to initiate nodules, even though normal root hair curling was observed (Wasson et al. 2006). In addition, Wasson et al. (2006) rescued nodule formation and flavonoid accumulation by supplementing plants with the precursor flavonoids naringenin and liquiritigenin. Subramanian et al. (2006) used a similar RNAi-mediated approach to silence isoflavone synthase (the entry point enzyme for isoflavone biosynthesis in soybean). Isoflavonoid levels in these plants were below detection, and a major decrease in nodulation was observed suggesting that endogenous isoflavones are essential for the establishment

of symbiosis between soybean and *Bradyrhizobium japonicum*. In *M. truncatula*, RNA interference-mediated suppression of two flavone synthase II (MtFNSII) genes, the key enzymes responsible for flavone biosynthesis, resulted in flavone depleted roots and significantly reduced nodulation providing genetic evidence that flavones are important for nodulation in *M. truncatula* as well (Zhang et al. 2007). Combined, this genetic evidence reinforces the importance of flavonoids in nodule initiation and establishment.

Even though flavonoids are the most potent *Nod* gene inducers, other non-flavonoid compounds such as jasmonates, aldonic acid, betaines, and xanthones can also induce the expression of *nod* genes in rhizobia but only at high concentrations (Phillips et al. 1992; Gagnon and Ibrahim 1998; Mabood and Smith 2005). In addition to their role in defense response against pathogens, both jasmonic acid and methyl jasmonate strongly induced the expression of *nod* genes in *B. japonicum* (Mabood and Smith 2005). *B. japonicum* inoculants preincubated with jasmonic acid and methyl jasmonate can accelerate nodulation, nitrogen fixation, and plant growth of soybean under controlled environment conditions (Mabood and Smith 2005). While *Lupinus albus* secretes diverse compounds into the rhizosphere, the majority are other non-flavonoid compounds, aldonic acids. The family of aldonic, erythronic, and tetronic acids (4-C sugar acids) induced the expression of *nod* genes in several bacteria (such as *Rhizobium lupini*, *Mesorhizobium loti*, and *Sinorhizobium meliloti*) and led to low but significant increases in β-galactosidase activities (Gagnon and Ibrahim 1998). In addition to the flavonoids, alfalfa (*M. sativa*) releases two betaines, trigonelline and stachydrine, that induce *nod* genes in *Rhizobium meliloti* (Phillips et al. 1994). These compounds are secreted in large quantities by germinating alfalfa seeds. Another plant-derived signal important for bacterial attachment to the plant root is plant lectin. Lectins are glycoproteins secreted from the tip of root hairs which mediate specific recognition of the bacterial surface carbohydrate molecules. Several experiments have shown the host specificity of plant lectin-mediated bacterial attachment by expressing plant lectin genes from one legume species in another and cross-inoculated with noncompatible rhizobia (Diaz et al. 1989; van Rhijn et al. 1998). Diaz et al. (1995) also reported the sugar binding activity of pea lectin in white clover and the localization on the external surface of elongating epidermal cells and tips of emerging root hairs, similar to the result observed in pea.

1.1.2.3 Bacterial-Derived Signals

Rhizobia establish the nodulation symbiosis in different legume plants by exchanging chemical signals with their legume partners. The molecular communication begins on the bacterial side with the recognition of the flavonoids by rhizobial NodD proteins (NodD1, D2, and D3). These proteins are transcriptional regulators which bind directly to a signaling molecule and activate downstream *nod* genes (Oldroyd and Downie 2004; Mandal et al. 2010). Upon activation of the *nod* genes by the plant signal, the bacteria release species-specific Nod factors to the

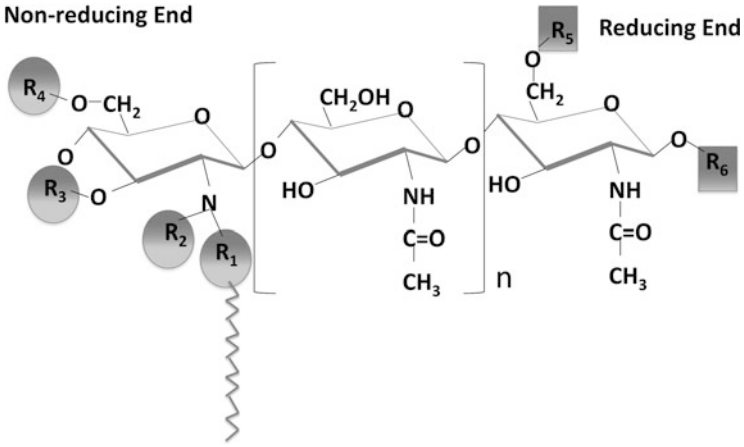

Fig. 1.2 Generalized structure of Nod factors. Species-specific Nod factors have a variety of substitutions at the positions noted by shape. At the nonreducing end (*circles*), R1 is an acyl group with 16–20 carbons in a chain with various levels of unsaturation. R2 is a hydrogen or a methyl group, R3 a hydrogen or carbonyl, and R4 a hydrogen, carbonyl, or acetyl group. At the reducing end (*squares*), R5 can be a hydrogen, sulfate, acetate, fucosyl, sulfo-methylfucosyl, acetyl-methylfucosyl, or arabinose, while R6 is a hydrogen or glycerol. The degree of oligomerization (*n*) can vary from 1 to 4

rhizosphere. Nod factor molecules are lipochitooligosaccharides consisting of three to five β-1, 4 linked *N*-acetyl-glucosamine residues that are acylated with a fatty acid of 16–20 C-atoms in length on the amino group of the nonreducing glucosamine (Fig. 1.2 and Price and Carlson 1995). The common *nod* genes *nodABC* are structural *nod* genes important for the biosynthesis of the core backbone of all the Nod factors and have a pivotal role in infection and nodulation process (Spaink et al. 1993). The *nod*ABC operons are structurally conserved and functionally interchangeable among different rhizobia without altering the host range (Martinez et al. 1990). This common core structure may, however, be modified by a number of species-specific substituents on the distal or reducing terminal residues which make each bacterial factor unique for each host plant. The substituents include acetate, sulfate, carbamoyl, glycosyl, methyl, arabinose, fucose, and mannose groups. Therefore, the host-specific *nod* genes (*nodHPQGEFL*) are important to specify the different substitution present on the backbone of Nod factors, allowing nodulation of a specific host plant (Brelles-Marino and Ane 2008). Mutation of these particular genes leads to an extended or altered host range (Djordjevic et al. 1985). In general, a correct chemical structure is required for induction of a particular plant response and Nod factor-induced signal transduction cascade.

Nod factors act as signal molecules to simultaneously initiate the nodule formation process programmed in the host plant as well as to trigger the infection process (Kouchi et al. 2010). But several other bacterial molecules are important in the legume–rhizobial interaction. For example, rhizobial extracellular polysaccharides (EPS) are host plant-specific molecules involved in signaling or in root hair

attachment. Extracellular polysaccharides are species- or strain-specific polysaccharide molecules with a large diversity in structure and are secreted into the environment or retained at the bacterial surface as a capsular polysaccharide (Laus et al. 2005). EPS-deficient mutants are impaired in efficient induction of root hair curling and especially in infection thread formation which finally leads to the formation of ineffective nodules (Pellock et al. 2000; van Workum et al. 1998). K-antigen polysaccharides are among the most studied acidic polysaccharides involved in nodulation (Becker et al. 2005). The mutation on both *rkpJ* and *rkpU* genes of *S. fredii* HH103 which are vital for production of K-antigen polysaccharides led to reduced nodulation and starvation for nitrogen; their expression was unaffected by flavonoids (Hidalgo et al. 2010). Hence, other bacterial molecules besides Nod factors play a critical role in the progression of the rhizobia–legume interaction.

1.1.3 Nod Factor Signal Transduction Pathway

Genetic studies in the model legumes *M. truncatula* and *L. japonicus* led to the identification of plant genes involved in the early steps in nodulation (Limpens and Bisseling 2003; Levy et al. 2004). A series of mutant screens identified a number of key regulators essential for Nod factor (NF) signaling. Similar set of genes have been found for the two model systems and are described below in spatial/temporal order from the surface of the root hair, based on their mutant phenotypes and diagrammed in Fig. 1.3.

At the cell surface are the LysM-RK receptor kinases (LYK3 and NFP in *M. truncatula* and their counterparts NFR1 and NFR5 in *L. japonicus*) which perceive Nod factors and trigger the signal transduction cascade essential for all early symbiotic events (Limpens et al. 2005; Smit et al. 2007; Broghammer et al. 2012). *MtNFP* is orthologous to *LjNFR5*, and a knockout mutation in this gene causes complete loss of Nod factor-inducible responses (Amor et al. 2003). *M. truncatula* LYK3, a putative high-stringency receptor that mediates bacterial infection, has been localized in a punctate distribution at the cell periphery, consistent with plasma membrane localization and upon inoculation co-localizes with FLOTILLIN4 (FLOT4) tagged with mCherry, another punctate plasma membrane-associated protein required for infection (Haney et al. 2011). Catoira et al. (2001) reported that the *hair curling* (*hcl*) mutants in *M. truncatula* altered the formation of signaling centers that normally provide positional information for the reorganization of the microtubular cytoskeleton in epidermal and cortical cells. Genetic analysis of calcium spiking in *hcl* mutants showed wild type calcium spiking in response to NF suggesting *HCL* acting downstream of earlier NF signaling events (Wais et al. 2000). Using a weak *hcl* allele, *hcl-4*, Smit et al. (2007) found that *hcl* mutants were defective in *LYK3* (LysM receptor kinase) and act as Nod factor entry receptor important for both root hair curling and infection thread formation. Since both *MtNFP* and *MtLYK3* encode transmembrane

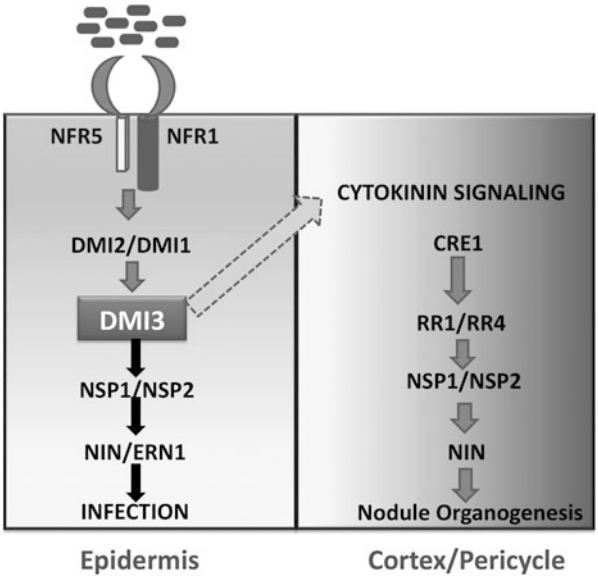

Fig. 1.3 Nod factor signaling cascade in legume–rhizobia symbiosis. The Nod factor receptors NFR5 and NFR1, consisting of extracellular LysM domains and intracellular kinase domain, are positioned at the surface of the root cells to perceive the Nod factor signal from the bacteria and trigger the downstream events. Downstream genes common to both mycorrhizal and bacterial symbiosis (DMI1, DMI2, DMI3) and the mostly rhizobial symbiosis-specific downstream transcription factors (GRAS protein, NSP1 and NSP2, and NIN) are activated upon Nod factor perception. The two processes simultaneously triggered by Nod factor are the infection process in epidermal cells and nodule organogenesis in cortical cells opposite to the xylem poles

receptors containing LysM domains, they were proposed to be good candidates for binding the chitin backbone of NF (Limpens et al. 2003; Arrighi et al. 2006). Recently, two groups reported that the Lotus orthologues of NFP (NFR5) and LYK3 (NFR1) make a heterodimer and perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding (Madsen et al. 2011; Broghammer et al. 2012). A Rho-like small GTPase (*Lt*ROP6) from *L. japonicus* was also identified as an NFR5-interacting protein both in vitro and in planta but did not interact with NFR1 (Ke et al. 2012).

Subsequently, *d*oes not *m*ake *i*nfections genes (*DMI1*, *DMI2*, and *DMI3*) and the *GRAS*-type transcription regulators *n*odulation-*s*ignaling *p*athway genes (*NSP1* and *NSP2*) in *M. truncatula* (Geurts et al. 2005) as well as the *SYMRK, CASTOR, POLLUX, Nup85, Nup133*, and *CCaMK* genes of *L. japonicus* are involved (Ane et al. 2004; Paszkowski 2006; Zhu et al. 2006; Murakami et al. 2007). Plants carrying a single mutation in one of these genes are defective for most of the early responses of Nod factor signaling as well as mycorrhization (Paszkowski 2006; Zhu et al. 2006) with the exception of NSP1 which is nodulation specific (Maillet et al. 2011), indicating that both mycorrhizal and rhizobial symbiosis rely on partially overlapping genetic programs that regulate both signaling pathways.

Since the arbuscular mycorrhizal symbiosis is a very ancient association while the legume/rhizobia symbiosis is relatively more recent (Godfroy et al. 2006), the existence of common genes led to a hypothesis that ancestral legumes may have co-opted part of the signaling machinery of this ancient symbiosis to facilitate the more recent symbiosis with nitrogen-fixing rhizobia (Udvardi and Scheible 2005; Zhu et al. 2006).

As reported by Geurts et al. (2005) and Udvardi and Scheible (2005), among the seven key genes identified in *M. truncatula*, the *DMI1*, *DMI2*, and *DMI3* downstream genes are shared between both rhizobial and fungal symbiosis in the *DMI* dependent signaling pathways. The *dmi1, dmi2*, and *dmi3* mutants do not show root hair deformation, gene expression, or mitotic induction of cortical cells but do show swelling at the tip of the root hairs in response to Nod factor and are blocked early in the establishment of mycorrhizal association (Wais et al. 2000; Oldroyd and Long 2003; Mitra et al. 2004). The DMI1 and DMI3 proteins are highly conserved in most land plants, in contrast to the less conserved DMI2 protein. The *DMI2* gene encodes a receptor-like kinase with extracellular leucine-rich repeats, a transmembrane domain, and intracellular kinase domain (Endre et al. 2002; Levy et al. 2004). DMI2 is called NORK for *n*odule *r*eceptor *k*inase in *M. sativa* and the corresponding orthologue in *L. japonicus* is called *SYMRK* (*sym*biosis *r*eceptor *k*inase) (Endre et al. 2002; Stracke et al. 2002). The only interacting partner for DMI2 reported thus far is 3-hydroxy-3-methyl-glutaryl-CoA reductase (*Mt*HMGR1) (Kevei et al. 2007). Mutagenesis and deletion analysis showed that the interaction requires the cytosolic active kinase domain of DMI2 and the cytosolic catalytic domain of *Mt*HMGR1 (Kevei et al. 2007). Several interacting partners for SYMRK have been reported. These include *S*ymRK-*i*nteracting *p*roteins SIP1 and SIP2, which have essential roles in the early symbiosis signaling and nodule organogenesis (Zhu et al. 2008; Chen et al. 2012), and a *S*ymRK-*i*nteracting *E*3 ubiquitin ligase (SIE3) shown to bind and ubiquitinate SymRK in vitro and in planta (Yuan et al. 2012). The *DMI1* gene encodes an ion channel-like protein which mediates the early ion fluxes observed in root hairs responding to Nod factors (Ane et al. 2004; Zhu et al. 2006). DMI1 and its orthologues are important either to trigger the opening of calcium release channels or compensate for the charge release during the calcium efflux as counter ion channels. DMI1 is required for the generation of Nod factor-induced, nucleus-associated Ca^{2+} spikes that are critical for nodule initiation, and protein localization to the nuclear envelope of *M. truncatula* root hair cells correlates with the nuclear association of Ca^{2+} spiking (Peiter et al. 2007). Both DMI1 and DMI2 are upstream of calcium spiking, and plants with mutations in these genes are blocked for calcium spiking and downstream nodulation events (Shaw and Long 2003). The DMI1 orthologues CASTOR and POLLUX were initially reported to localize in the plastids of pea root cells and onion epidermal cells (Imaizumi-Anraku et al. 2005). However, a functional DMI1::GFP fusion protein localized to the nuclear envelope in *M. truncatula* roots when expressed both under a constitutive 35S promoter and a native *DMI1* promoter (Riely et al. 2007). Recently, immunogold labeling localized the endogenous CASTOR protein to the nuclear envelope of *L. japonicus* root cells,

consistent with a role of CASTOR and POLLUX in modulating the nuclear envelope membrane potential (Charpentier et al. 2008).

Calcium spiking is a central component of the common symbiotic pathway. Recent work using calcium chameleon reporters in *M. truncatula* roots suggests that tightly regulated Ca^{2+}-mediated signal transduction is key to reprogramming root cell development at the critical stage of commitment to endosymbiotic infection (Sieberer et al. 2012). Two nucleoporin genes (*NUP133* and *NUP85)* have been identified at an equivalent position in the Nod factor signaling pathway with the DMI1 protein and are required for the common symbiotic pathways and for calcium spiking responses (Kanamori et al. 2007; Saito et al. 2007). *NUP133* encodes a protein that has sequence similarity to human nucleoporin Nup133 and localizes in the nuclear envelope, indicating that both NUP133 and NUP85 are members of the nuclear pore complex in legumes (Kanamori et al. 2007). Both genes are required for the calcium spiking that is induced in response to Nod factors, but further research is required to clarify the roles of *NUP133* and *NUP85* in leguminous plants.

DMI3 acts immediately downstream of calcium spiking in the nodulation-signaling pathway and is required for both nodulation and mycorrhizal infection (Levy et al. 2004). In contrast to the other mutants mentioned, calcium spiking and root hair swelling in response to Nod factor are wild type in a *dmi3* mutant background whereas symbiotic gene expression or cell divisions for nodule formation are defective (Mitra et al. 2004). The *DMI3* gene encodes a Ca^{2+} and calmodulin-dependent protein kinase (CCaMK) that responds to the Ca^{2+} signal (Mitra et al. 2004; Geurts et al. 2005). These protein families are multifunctional, with a kinase domain, a calmodulin (CaM)-binding domain and a Ca^{2+}-binding domain with three EF hands (Oldroyd and Downie 2004). The CCaMKs have the capacity to bind calcium in two ways, either by direct binding to the three EF hands or by forming a complex with calmodulin to regulate the kinase activity. The interaction of the Ca^{2+} with the C-terminal EF hands results in autophosphorylation of the CCaMK and allows CaM binding, which leads to substrate phosphorylation (Cook 2004; Levy et al. 2004). In general, CCaMK perceives the calcium spiking signature and transduces this to induction of the downstream genes involved in mycorrhizal or rhizobial symbiosis. Split yellow fluorescent protein complementation and yeast-2-hybrid systems demonstrated that the highly conserved nuclear protein IPD3 is an interacting partner of DMI3 and that the interaction is through a C-terminal coiled-coil domain (Messinese et al. 2007). In separate report, characterization of three independent retrotransposon Tos17 insertion lines of rice *OsIPD3* upon AM fungus *Glomus intraradices* inoculation revealed that the *Osipd3* mutants were unable to establish a symbiotic association with *G. intraradices* confirming the role of this CCaMK in root symbiosis with AM fungi (Chen et al. 2008).

Beyond this point, in the common symbiotic pathway transcriptional regulators of the *NIN, GRAS (NSP1, NSP2*), and *ERF* families are required for upregulation of nodulation-expressed genes and initiation of nodulation (Madsen et al. 2010). Both *NSP1* and *NSP2* encode putative transcriptional regulators of the GRAS protein

family (Smit et al. 2005; Kalo et al. 2005). The NSP1 protein has been localized in the nucleus similar to the upstream gene DMI3 (Smit et al. 2005). NSP2, however, migrates from its original location in the nuclear envelope and endoplasmic reticulum into the nucleus where it regulates the transcription of early nodulin genes after Nod factor elicitation (Kalo et al. 2005). Since both NSP1 and NSP2 form a complex (Hirsch et al. 2009) and are genetically downstream of DMI3, at least one of these genes is the target of DMI3 action. Cross species complementation studies also showed *NSP1* and *NSP2* functions are conserved in nonlegumes. *OsNSP1* and *OsNSP2* from rice were able to fully rescue the root nodule symbiosis-defective phenotypes of the mutants of corresponding genes in the model legume, *L. japonicus* (Yokota et al. 2010). Recently, Liu et al. (2011) reported that NSP1 and NSP2 are also a vital component of strigolactone biosynthesis in *M. truncatula* and rice. Mutations in both genes reduced expression of DWARF27, a gene essential for strigolactone biosynthesis (Liu et al. 2011). Downstream of NSP1 and NSP2, another putative transcription factor, NIN, first identified in *L. japonicus*, is essential for coordinating nodule organogenesis and bacterial entry (Marsh et al. 2007). *NIN* encodes a transmembrane transcriptional regulator with homology to Notch of Drosophila (Schauser et al. 1999). Early NF-induced gene expression using an *ENOD11:GUS* reporter fusion in the *Mtnin-1* mutant showed that *MtNIN* is not essential for early Nod factor signaling but may function downstream of the early NF signaling pathway to coordinate and regulate temporal and spatial formation of root nodules (Marsh et al. 2007). The perception of Nod factors also leads to the activation of another transcription factor with DNA-binding capability, ERN1, an AP2-like transcription factor in the ERF subfamily, which is necessary for nodulation and functions in early Nod factor signaling (Middleton et al. 2007). Mutations in *ERN* block the initiation and development of infection threads and thus block nodule invasion by the bacteria. *ERN1* is induced rapidly after *S. meliloti* inoculation and is necessary for Nod factor-induced gene expression. Unlike wild type plants, *ern1* mutants do not form spontaneous nodules when transformed with activated calcium- and calmodulin-dependent protein kinase, and Nod factor application does not induce *ENOD11:GUS* expression (Middleton et al. 2007). A second ERF transcription factor, EFD (for *e*thylene response *f*actor required for nodule *d*ifferentiation), is required for the differentiation of functional Fix+nodules and may participate in an ethylene-independent feedback inhibition of the nodulation process as well as regulating the expression of the primary cytokinin response regulator MtRR4 (Vernie et al. 2008).

The formation of nodule primordia involves dedifferentiation and reactivation of cortical root cells to establish the nodule primordium, a mass of rapidly proliferating undifferentiated cells, opposite to the protoxylem poles (Timmers et al. 1999; Penmetsa and Cook 1997). Both gain-of-function and loss-of-function mutants have shown that cytokinin signaling through the cytokinin receptor kinase (LHK1) is important for reactivation of cortical cells (Tirichine et al. 2007; Murray et al. 2007). Plet et al. (2011) also reported cytokinin signaling in *M. truncatula* integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in

an *MtCRE1* dependent manner. Simultaneous with the formation of the primordia is the endocytic-like entry of the bacteria to the plant root cells, associated with the plant driven infection thread formation, bacterial cell division within the infection thread, progression of the infection threads towards the dividing nodule primordia, and finally the invasion of the developing nodule. Several genes important to Nod factor recognition have been reported to affect infection thread initiation and growth. The *M. truncatula* Hair Curling (*HCL*) gene encodes the LYK3 receptor-like kinase a specific function of which is to initiate the infection thread on Nod factor recognition (Limpens et al. 2003; Smit et al. 2007). Besides its role in nodule initiation, the receptor kinase DMI2 plays a key role in symbiosome formation and is expressed both on the host cell plasma membrane and the membrane surrounding the infection thread (Limpens et al. 2005). Also required for both infection thread growth in root hair cells and the further development of nodule primordia are the orthologous *LIN*/*CERBERUS* genes in *M. truncatula* and *L. japonicus*, which encode predicted E3 ubiquitin ligases containing a highly conserved U-box and WD40 repeat domains functions and function at an early stage of the rhizobial symbiotic process (Kiss et al. 2009 Yano et al. 2009). The Vapyrin (*VPY*) gene is essential for the establishment of the arbuscular mycorrhizal symbiosis and is also important for rhizobial colonization, and nodulation. *VPY* acts downstream of the common signaling pathway (Murray et al. 2011). In addition, flotillin (FLOT2 and FLOT4) and remorin (*Mt*SYMREM1) proteins, which promote trafficking and aggregation of membrane proteins, are required for infection by rhizobia, possibly by acting as scaffolds for recruitment of membrane proteins involved in nodulation signaling [for review, see Oldroyd et al. (2011)].

1.1.4 Nodule Autoregulation

The symbiosis between leguminous plants and rhizobia under conditions of nitrogen limitation leads to the development of new plant organs, the N_2-fixing nodules that are usually formed on roots but also on stems in a few plants. The bacteria require energy and a suitable environment for nitrogenase, the enzyme important for nitrogen fixation (Crawford et al. 2000). Hence, the nodulation process is energy intensive, and the plants need to maintain a balance between cost and benefit by limiting the number of nodules that form. Plants use local and long-distance or systemic signaling to coordinate and adjust the number of nodulation events (Kosslak and Bohlool 1984; Caetano-Anolles and Gresshoff 1991). This negative feedback inhibition, in which the earlier nodulation events suppress the subsequent development of nodules in young tissues, is called autoregulation of nodulation (AON) (Pierce and Bauer 1983; Searle et al. 2003; Oka-kira and Kawaguchi 2006). AON employs root-derived and shoot-derived long-distance signals. The root-derived signal is generated in roots in response to rhizobial infection and then translocated to the shoot, while the shoot-derived signal is generated in shoot and then translocated back to the root to restrict further nodulation. AON is activated

upon the perception of Nod factor in the elongation zone of the root with emerging root hairs where rhizobial infection occurs being most affected (Bhuvaneswari et al. 1981).

AON is under both environmental and developmental controls and appears to be universally used by legumes to control the extent of nodulation. Mutations affecting AON lead to supernodulation or hypernodulation, associated with root developmental defects. Genetic analysis of AON began with the isolation of supernodulating mutants, which have lost their ability to autoregulate nodule numbers. AON mutants are characterized by forming an excessive number of nodules and a short root compared to their wild type counterparts. For instance, *Glycine max nts* (Carroll et al. 1985); *Lotus japonicus har1* (Krusell et al. 2002), *tml* (Magori and Kawaguchi 2009), and *klavier* (Oka-Kira et al. 2005); *Pisum sativum sym29* (Krusell et al. 2002) and *nod3* (Sidorova and Shumnyi 2003); *Medicago truncatula sunn* (Penmetsa et al. 2003; Schnabel et al. 2005), *lss* (Schnabel et al. 2010), and *rdn1* (Schnabel et al. 2011) mutants are defective in autoregulation and thus form an excessive number of nodules. The *NTS*/*NARK, HAR1*, *SYM29*, and *SUNN* genes encode a leucine-rich repeat receptor kinase with homology to Arabidopsis *CLAVATA1* (Searle et al. 2003; Krusell et al. 2002; Schnabel et al. 2005). *KLAVIER* also encodes a different LRR receptor kinase (Miyazawa et al. 2010) while *NOD3* and *RDN1* encode proteins of unknown function (Schnabel et al. 2011). Since autoregulation is mediated through a long-distance signaling involving shoot and root, shoot to root reciprocal grafting studies using wild type and autoregulation defective mutants revealed that there are both shoot controlled as well as root controlled supernodulators. It is believed that the shoot-controlled supernodulators are impaired in either in the perception of the root-derived infection signal or in the transmission of the shoot-derived autoregulation signal. On the other hand, the root controlled mutants are thought to be impaired in either the transmission of the root-derived infection signal or in the perception of the shoot-derived autoregulatory signal.

Nodule initiation and development are also determined by physiological conditions and phytohormones. Successful nodule formation and subsequent nitrogen fixation occur normally only under nitrogen-limiting conditions (Schultze and Kondorosi 1998). However, the mutants defective in AON are partially nitrate tolerant (Caba et al. 1998; Carroll et al. 1985). This suggests that at some stage, the autoregulation signal and the nitrate signal talk to each other to inhibit nodule formation. In addition, the gaseous phytohormone, ethylene, is also a negative regulator of nodule organogenesis. The *M. truncatula* mutant, *skl*, encoding an EIN2 orthologue (Penmetsa et al. 2008), is insensitive to ethylene and shows a tenfold increases in nodule number relative to the wild type (Penmetsa and Cook 1997). Rhizobial inoculation and exogenous ACC induce ethylene synthesis and thereby lead to suppressed nodule and root development in *sunn* mutants (Penmetsa et al. 2003). Similarly, the addition of the ethylene inhibitors like L-α-aminoethoxyvinylglycine enhanced nodule development in common bean and pea (Guinel and Sloetjes 2000; Tamimi and Timko 2003). Another phytohormone, auxin, is mostly produced in younger plant shoots

and moves long distance to the root tip following an auxin concentration gradient to trigger root, nodules, and other plant organ development (Pacios-Bras et al. 2003; Schnabel and Frugoli 2004). In fact, IAA produced by the rhizobia is reported to increase nodule formation (Pii et al. 2007). In uninoculated roots of *sunn* mutant plants, auxin transport from shoot to root is approximately three times higher than the wild type (van Noorden et al. 2006), and auxin transport inhibitors such as NPA significantly reduce nodulation in wild type plants and *sunn* mutants but not *skl* mutants (van Noorden et al. 2006; Prayitno et al. 2006), suggesting a role for auxin in regulating nodule number as well.

The plant hormone, cytokinin, is also implicated in nodulation. Exogenous application of cytokinins to legume roots induced responses similar to rhizobial Nod factors, including cortical cell division, amyloplast deposition, and induction of early nodulin gene expression (Bauer et al. 1996). Gonzalez-Rizzo et al. (2006) identified a *M. truncatula* homologue of Arabidopsis Cytokinin Response1 (CRE1), a cytokinin receptor histidine kinase. Using RNA interference to downregulate *MtCRE1*, they demonstrated that MtCRE1 acts as a negative regulator of lateral root formation and as a positive regulator of nodulation. Expression analysis of genes downstream in cytokinin signaling, *MtRR1* and *MtRR4*, and the early nodulin *MtNIN1* in *M. truncatula* suggests that these three genes are involved in crosstalk between Nod factor and cytokinin signaling pathways depending on MtCRE1 (Gonzalez-Rizzo et al. 2006). Cytokinin activation of MtCLE13, a short peptide involved in nodulation (Mortier et al. 2010), depends on CRE1 and NIN but not on NSP2 and ERN1, suggesting two parallel pathways triggered by cytokinin in the root cortex, activation of cortical cell division and activation of MtCLE13 to inhibit further nodulation (Mortier et al. 2012). In addition, CLE genes (12–13 amino acid long secreted peptides) which are involved in both shoot and root meristem homeostasis, vascular differentiation, and nodulation comprise a gene family of up to 40 members and play a role in either activation of the root-derived AON signal or have the potential to interact with leucine-rich repeat receptor kinases such as SUNN (Mortier et al. 2010). Identification of these AON genes in combination with phytohormones and other growth regulators and the intensive study of the nodulation-signaling cascade will aid understanding of the fascinating and complex events leading to legume nodule formation and regulation and plant development in general.

1.1.5 Nodule Senescence

Functional nodules are not maintained throughout the life cycle of the host plant. The peak nitrogen fixation period in both determinate and indeterminate nodules is restricted to between 3 and 5 weeks after infection (Lawn and Burn 1974; Puppo et al. 2005). During the vegetative growth stage and flowering, nodules are the major carbon sink in legumes. In the course of pod filling, however, the seed is the strongest sink for photosynthate, and nodules start to gradually senesce. The first

symptoms of senescence are the deterioration of leghemoglobin, resulting in a pink to green color change in the nodule, and loss of turgidity in old nodules (Perez Guerra et al. 2010). In mature indeterminate nodules, the senescence zone starts in zone IV (Fig. 1.1). Upon aging, this senescence zone gradually moves in a proximal–distal direction until it reaches the apical part and leads to nodule degeneration (Puppo et al. 2005; Van de Velde et al. 2006). On the other hand, in determinate nodules it expands radially from the center to the periphery. The primary targets for nodule senescence are symbiosomes, in the same manner as chloroplasts in leaf senescence, with several common senescence associated genes both up- and downregulated in leaf and nodule senescence suggesting a shared pathway (Van de Velde et al. 2006). Since symbiosomes are derived from the uptake of prokaryotic cells that fix nitrogen, and chloroplasts are postulated to have originated from the uptake of cells that fix carbon, this common pathway is not surprising. Using transmission electron microscopy, two consecutive stages were distinguished during nodule senescence: a first stage, characterized by bacteroid degradation with a few dying plant cells and a more advanced stage of nodule senescence, during which cells had completely resorbed their symbiosomes and started to decay and collapse (Van de Velde et al. 2006). Hence, the final fate of the bacteria and the plant cells that form the nitrogen-fixing organelle is death. Plant cysteine proteinases are important in controlling nodule senescence. An Asnodf32 protein which encodes a nodule-specific cysteine proteinase in *Astragalus sinicus* was reported to play an important role in the regulation of root nodule senescence. In Asnodf32-silenced hairy roots, the period of bacteroid active nitrogen fixation was significantly extended and enlarged nodules were also observed (Li et al. 2008). Recently, an *M. truncatula* transcription factor, MtNAC969, was also reported to participate in nodule senescence. MtNAC969 is induced by nitrate treatment and, similar to senescence markers, was antagonistically affected by salt in roots and nodules; MtNAC969 RNAi silenced nodules accumulated amyloplasts in the nitrogen-fixing zone and were prematurely senescent (de Zelicourt et al. 2012). Nodule senescence is an active process programmed in development; thus, reactive oxygen species, antioxidants, hormones, and proteinases also play a key role (Puppo et al. 2005).

1.1.6 Concluding Remarks

Both the plant and the bacteria have coevolved a complex series of signals and responses to establish the symbiosis. Understanding of the plant side of the symbiosis has increased rapidly in recent years and continues to accelerate as genomic and molecular tools are brought to bear on the problem. Interestingly, the majority of signal transduction molecules on the plant side of the symbiosis are not exclusive to legumes, suggesting that the bacteria have co-opted existing plant pathways to establish the symbiosis and regulate nodulation. This suggests that in the future, it may be possible to establish nitrogen-fixing symbiosis in plants that currently do not nodulate.

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