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Invited Expert Review

Molecular Analysis of Legume Nodule Development and Autoregulation

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Abstract

Legumes are highly important food, feed and biofuel crops. With few exceptions, they can enter into an intricate symbiotic relationship with specific soil bacteria called rhizobia. This interaction results in the formation of a new root organ called the nodule in which the rhizobia convert atmospheric nitrogen gas into forms of nitrogen that are useable by the plant. The plant tightly controls the number of nodules it forms, via a complex root-to-shoot-to-root signaling loop called autoregulation of nodulation (AON). This regulatory process involves peptide hormones, receptor kinases and small metabolites. Using modern genetic and genomic techniques, many of the components required for nodule formation and AON have now been isolated. This review addresses these recent findings, presents detailed models of the nodulation and AON processes, and identifies gaps in our understanding of these process that have yet to be fully explained.

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Introduction

Nitrogen is arguably the most important nutrient required by plants, being an essential component of all amino and nucleic acids. However, nitrogen availability is limited in many soils, and although the earth's atmosphere consists of 78.1% nitrogen gas (N_2), plants are unable to use this form of nitrogen. To compensate, modern agriculture has been highly reliant on industrial nitrogen fertilizers to achieve maximum crop productivity. However, a great deal of fossil fuel is required for the production and delivery of nitrogen fertilizer. Indeed, industrial nitrogen fixation alone accounts for about 50% of fossil fuel usage in agriculture. This can be exceedingly expensive. In recent years the price of chemical nitrogen fertilizers has increased dramatically due to rising fossil fuel costs. Moreover, carbon dioxide (CO_2) which is released during fossil fuel combustion contributes to the greenhouse effect, as does the decomposition of nitrogen fertilizer, which releases nitrous oxides (NOx), itself about 292 times more active as a greenhouse gas than carbon dioxide (Crutzen et al. 2007). In addition, applying chemical fertilizers is a largely inefficient process as 30–50% of applied nitrogen fertilizer is lost to leaching, resulting in significant environmental problems, such as the eutrophication of waterways (Graham and Vance 2003). Thus, there is a strong need to reduce our reliance on chemical nitrogen fertilizers and instead optimize alternative nitrogen inputs.

Legumes and Nitrogen Fixation

Biological nitrogen fixation is one alternative to nitrogen fertilizer. It is carried out by prokaryotes using an enzyme complex termed nitrogenase and results in atmospheric N_2 being reduced into forms of nitrogen the plant is able to use, such as ammonia. One family of plants, the *Leguminosae*, has evolved a symbiotic relationship with specific soil bacteria, called rhizobia (including the genera *Azorhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*). Once the symbiosis is established, the rhizobia fix atmospheric nitrogen and provide it to their legume host plant. Because nitrogen is a key limiting factor for plant growth and development, the ability of legumes to enter into a symbiosis with nitrogen-fixing rhizobia provides them with a distinct advantage over other plant species.

Legumes include major food and feed crop species, such as soybean, pea, clover, chickpea, alfalfa and mungbean. They represent the third largest group of angiosperms and are the second largest group of food and feed crops grown globally. Indeed, they are cultivated on 12–15% of available arable land and are responsible for more than 25% of the world's primary crop production with 247 million tons of grain legumes produced annually (European Association for Grain Legume Research 2007). In addition to food and feed crops, legumes such as soybean and *Pongamia pinnata* (also called *Millettia pinnata*) have garnered a great deal of attention as future sustainable biofuel sources because of their high seed oil content (Scott et al. 2008).

The rhizobia invade the roots of compatible legume plants, leading to the development of specialized root structures called nodules. In the nodule, the bacteria differentiate into bacteroids and catalyze the reduction of N_2 into ammonia using the nitrogenase enzyme complex, a process commonly referred to as "symbiotic nitrogen fixation". The legume-rhizobia symbiosis is the most important symbiotic association in terms of biological nitrogen fixation, producing roughly 200 million tons of nitrogen annually (Graham and Vance 2003; Peoples et al. 2009). A common farming practice is to rotate crop species, with one typically being a legume such as clover or alfalfa. Thus, these species are often referred to as "green manure". Often, the entire plant is ploughed back into the field, thus dramatically improving the organic content and volume of the soil.

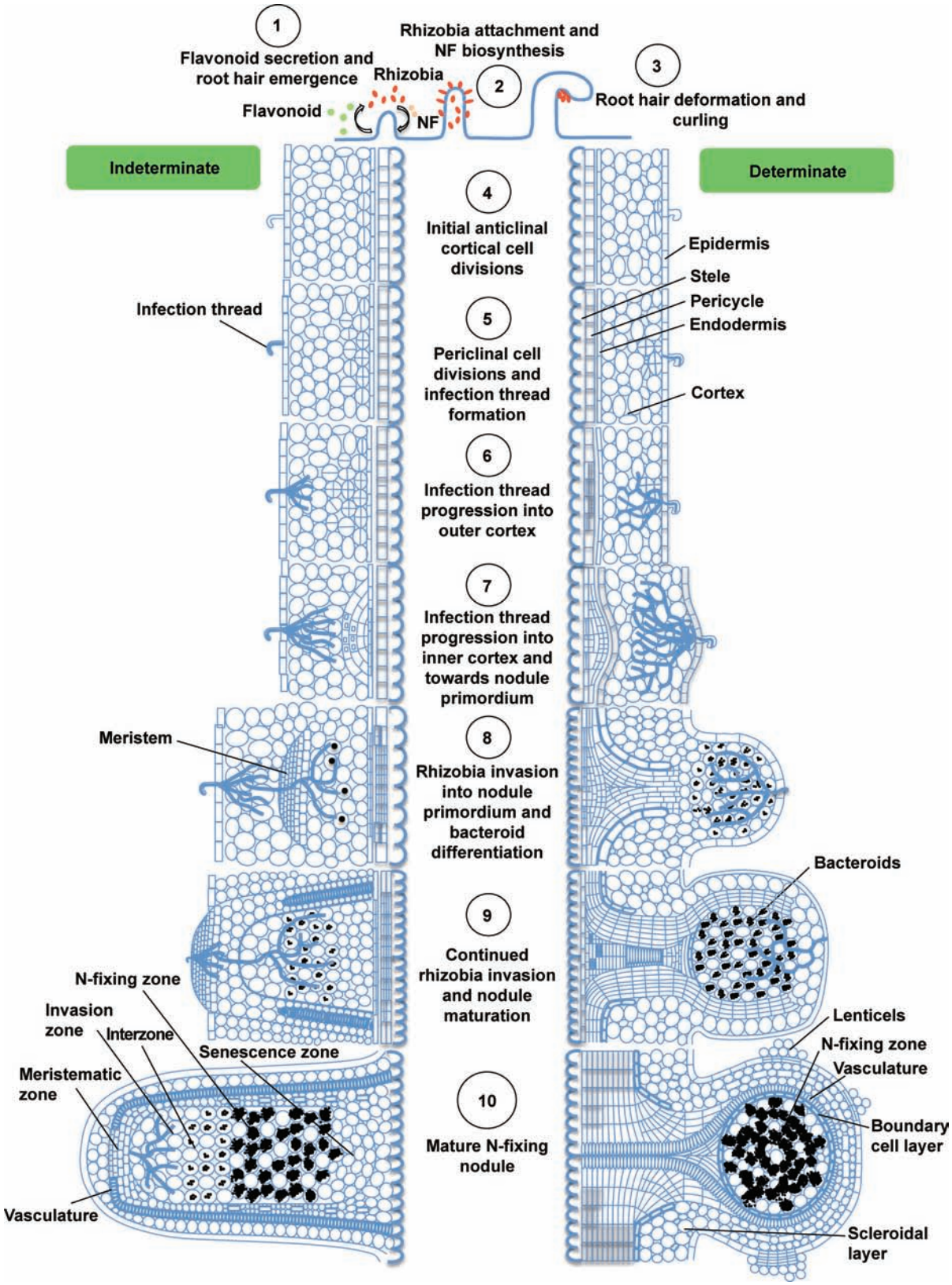
Nodule Organogenesis

Nodule formation is initiated by the host plant roots exuding phenolic flavonoid compounds into the rhizosphere (Redmond et al. 1986) (**Figure 1**; step 1). The exudate partly determines the specificity of the symbiotic relationship as each rhizobia species responds to specific flavonoids. Most rhizobia species interact with only a select few legumes, but some have been shown to have a broad host range (Pueppke and Broughton 1999).

Flavonoid perception attracts the bacteria to the root and activates rhizobia *nod* (nodulation) gene expression, leading to the production and secretion of strain-specific lipo-chito-oligosaccharides, also known as nod factors (NF) (Caetano-Anollés and Gresshoff 1991; Dénarié et al. 1996; Spaink 2000) (**Figure 1**; step 2). The exceptions are some recently identified photosynthetic *Bradyrhizobium* strains that can induce nodule development despite not having the critical *nodABC* genes required for NF biosynthesis (Giraud et al. 2007). NFs have an oligosaccharide backbone of *N*-acetyl-D-glucosamine units with a fatty acyl group attached to the non-reducing sugar. A major determinant of host-symbiont specificity is attributed to the different NF substituents attached to the oligosaccharide backbone (Lerougé et al. 1990; Dénarié et al. 1996).

The presence of compatible rhizobia species and their corresponding NF is generally sufficient to trigger nodule development. The tip of emerging root hairs is the primary target for infection by rhizobia, probably because their thinner and less cross-linked cell walls allow for the re-arrangement of underlying microtubules, changing vesicle trafficking to the growing tip and thus better enabling subsequent penetration by rhizobia. Attachment of rhizobia to root hairs stimulates root hair deformation within 6–8 h (Yao and Vincent 1969; Bhuvaneswari et al. 1981; Bhuvaneswari and Solheim 1985) and also promotes cortical cell divisions (Calvert et al. 1984; Mathews et al. 1989) (**Figure 1**; step 3).

Rhizobia have two main ways of entering the plant root: via the root hair or through cracks in root epidermal tissue (reviewed in Oldroyd and Downie 2008). Root hair infection is the most common and involves the formation of infection threads, which are tubular structures composed of plant cell wall components that act as a passage for the bacteria into the cortical cells of the plant (reviewed by Gage 2004). The rhizobia enter through the deformed root hair tip, which encapsulates a small proportion of the dividing bacteria (Callaham and Torrey 1981; Turgeon and Bauer 1985) (**Figure 1**; steps 4 and 5). The enclosed microcolony presumably has an enriched NF concentration as well as cell wall degrading enzymes. Penetration of the host cell wall, but not its plasma membrane, is followed by resynthesis and re-digestion. This re-occurring cycle coupled with viscous extracellular matrix embedding of the microcolony and continued bacterial growth produces a 'forward' pressure that is needed to 'push' against the root hair turgor pressure. The dynamics of this process results in the formation of the plant-cell wall derived infection thread (**Figure 1**; steps 6 and 7) filled with proliferating bacteria embedded in the ever-hardening extracellular matrix (Gage 2004). In soybean,


1
Flavonoid secretion and root hair emergence
Rhizobia attachment and NF biosynthesis
Rhizobia
2
3
Root hair deformation and curling
Flavonoid
NF
Indeterminate
Determinate
4
Initial anticlinal cortical cell divisions
Epidermis
Stele
Pericycle
Endodermis
Cortex
Infection thread
5
Periclinal cell divisions and infection thread formation
6
Infection thread progression into outer cortex
7
Infection thread progression into inner cortex and towards nodule primordium
Meristem
8
Rhizobia invasion into nodule primordium and bacteroid differentiation
Bacteroids
9
Continued rhizobia invasion and nodule maturation
N-fixing zone
Invasion zone
Interzone
Senescence zone
Meristematic zone
Lenticels
N-fixing zone
Vasculature
Boundary cell layer
10
Mature N-fixing nodule
Vasculature
Scleroidal layer

root hair infection takes place up to 12 h after contact with rhizobia (Turgeon and Bauer 1982, 1985).

It is possible that invading rhizobia, still capable of NF production as evidenced by *NodC::LacZ* fusion expression, stimulate ever-increasing NF levels that lead to mitotic activation of cortical cells in the root. This eventually results in the development of the nodule primordium (**Figure 1**; step 8). The radial position of the cell divisions, and thus the primordium, is controlled by positional gradients for hormones such as ethylene (Heidstra et al. 1997; Lohar et al. 2009). Accordingly, most nodules develop close to the xylem radial cells, away from the phloem. The infection thread grows through the root hair into the root cortex and the newly induced dividing cells. Bacteria are released from near the growing tip of the infection thread into an infection droplet in the host cell cytoplasm. Through a process resembling endocytosis, the bacteria are surrounded by a plant-derived membrane, called the peribacteroid membrane, which forms what is known as the symbiosome (Udvardi and Day 1997).

The membrane-enveloped bacteria continue to divide within the host cells before they differentiate into bacteroids and start to fix nitrogen (Roth and Stacey 1989a,b). Atmospheric N_2 is converted into ammonia by bacteroids and is subsequently assimilated into the plant following its conversion to glutamine by glutamine synthase. Glutamine is further converted to glutamate by glutamte synthase. The rapid conversion of ammonia generates a differential gradient which is thought to primarily drive its export from the bacteroids (Udvardi and Day 1997). Vascular tissues, as well as central tissues composed of invaded and non-invaded cells, are contained in the cortex (Newcomb et al. 1979; Calvert et al. 1984) (**Figure 1**; steps 9 and 10). Between the nodule interior and the neighboring plant cells the plant and bacteroids exchange essential nutrients. Passive transport driven by membrane potential across the peribacteroid membrane facilitates nutrient uptake into the symbiosomes (Udvardi and Day 1997). These mechanisms allow assimilation of photosynthates (as dicarboxylic acids; i.e. malate) into the nodule for the bacteroids, and the export of various compounds, including fixed nitrogen (i.e. glutamine), into the root.

Determinant and Indeterminant Nodule Structures

Two major morphological types of nodules exist in legumes: determinate and indeterminate (**Table 1** and **Figure 1**). The type of nodule is determined by the host plant. 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 (Newcomb et al. 1979; Gresshoff and Delves 1986; Rolf and Gresshoff 1988). For indeterminate nodules, the first cell division events occur anticlinally in the inner cortex, followed by periclinal divisions in the endodermis and pericycle (**Figure 1**; steps 4 and 5). Collectively, these divisions lead to the formation of the nodule primordia. Indeterminate nodules have a more persistent meristem, which results in nodules of cylindrical shape, as exemplified by nodules of alfalfa (*Medicago sativa*), clover (*Trifolium repens*), pea (*Pisum sativum*) and *Medicago truncatula* (Bond 1948; Libbenga and Harkes 1973; Newcomb 1976; Newcomb et al. 1979). The apical meristem continuously produces new cells that become infected with bacteria. At maturity, indeterminate nodules contain a heterogenous population of nitrogen-fixing bacteroids due to continued cell division activity, giving rise to a gradient of developmental states as the nodule continues to elongate (**Figure 1**). These nodules also have a different, less branched vascular system than determinate nodules.

Determinate nodules, on the other hand, are usually spherical, lack a persistent meristem, and do not display an obvious developmental gradient (**Table 1** and **Figure 1**) (Newcomb et al. 1979; Turgeon and Bauer 1982; Calvert et al. 1984; Mathews et al. 1989). The first cell division events of a determinate nodule typically occur sub-epidermally in the outer cortex. Exceptions exist, such as the nodules of *Lotus japonicus*, which do not exhibit the initial sub-epidermal cell divisions (Wopereis

Figure 1. Developmental stages of indeterminate and determinate legume nodules.

Illustrated are the developmental stages of pea (indeterminate; left) and soybean (determinate; right) nodules. Emerging root hairs exude flavonoid compounds, which attract compatible rhizobia and stimulate them to produce nod factors (NF). The root hair deforms and forms a pocket, in which the rhizobia become entrapped. Infection thread structures initiate in the pocket enabling the rhizobia to enter the plant. Cell divisions are first observed in the inner cortex for indeterminate nodules or the sub-epidermal cell layer for determinate nodules. Additional cell layers later divide leading to the formation of the nodule primordium. The infection threads progress towards this primordium and release the rhizobia into infection droplets, in which they differentiate into nitrogen-fixing bacteroids. At the top of the primordium of indeterminate nodules, a meristem develops that continually gives rise to new cells. As these new cells mature, many subsequently become infected, leading to successive zones of rhizobia invasion and differentiation within the nodule. In contrast, determinate nodules do not develop a persistent meristem and hence their invaded cells are all at a similar developmental phase. The various developmental stages, tissue types and nodulation zones are labeled.

Table 1. Major differences between indeterminate and determinate nodule types

	Indeterminate	Determinate
Site of initial cell divisions	Inner root cortex next to the xylem pole	Outer, or middle, cortex next to the xylem pole
Meristem type	Persistent meristem	No persistent meristem
Normal nodule form	Cylindrical/branched	Spherical
Infection thread	Broad	Narrow
Infected cells	Highly vacuolated	Minimal vacuolation
Major bacteroid form	Enlarged, branched, low viability; one per symbiosome	Normal rod size, high viability; multiples per symbiosome
Geographic region of plant origin	Temperate regions	Subtropical and tropical
Examples	*Medicago*, clovers and pea	Soybean, bean, *Pongamia pinnata*, and *Lotus*

et al. 2000). At maturity, determinate nodules contain a relatively homogenous population of nitrogen-fixing bacteroids, as differentiation of the infected cells occurs synchronously, followed by senescence. These nodules have a life-span of a few weeks. When old nodules senesce, new nodules are formed on recently developed portions of the root (Rolfe and Gresshoff 1988).

It will be interesting to identify the role of the *cochleata* gene in meristem-less determinate nodules, as it has a role in meristem identity in indeterminate nodules, causing a homeotic phenotype and root-nodule hybrid structures in pea (Ferguson and Reid 2005). Determinate nodules also form lenticels, which are structures that act to enhance gas exchange (**Figure 1**; step 10). Legumes that form determinate nodules are predominately tropical and subtropical species, including soybean (*Glycine max*), pongamia (*Pongamia pinnata*) and bean (*Phaseolus vulgaris*), but also include other more temperate species such as *L. japonicus*.

Nod Factor Perception

A predominately genetic approach has been used to unravel the mechanisms underlying NF perception. The current model predicts two receptor-like kinases (RLK) located on epidermal cells that are involved in nod factor binding: in *L. japonicus* LjNFR1 and LjNFR5, in *P. sativum* PsSYM2A and PsSYM10, in *M. truncatula* MtLYK3/MtLYK4 and MtNFP, and in soybean GmNFR1α/β and GmNFR5α/β (**Figure 2**; Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003; Arrighi et al. 2006; Indrasumunar 2007; Indrasumunar et al. 2009). These NF receptors consist of an intracellular kinase domain, a transmembrane domain and an extracellular portion having LysM domains. LysM domains are common in bacterial cell wall-degrading enzymes and are thought to bind to peptidoglycans which, similarly to NFs, contain *N*-acetylglucosamine residues (Steen et al. 2003). Although they do exist in eukaryotes, they are not very common. The presence of LysM domains in conjunction with transmembrane and kinase domains is exclusive to plants (Gough 2003). Interestingly, LjNFR1/PsSYM2A/MtLYK3/MtLYK4/GmNFR1α/β has a typical serine/threonine kinase domain, while LjNFR5/PsSYM10/MtNFP/GmNFR5α/β lacks the activation loop (Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003; Indrasumunar 2007; Indrasumunar et al. 2009) where the site of phosphorylation is usually located in most eukaryotic protein kinases (Huse and Kuriyan 2002). 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 domain functioning in downstream signal transduction (Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003). However, interactions between these two RLKs and other signal transduction components remain to be elucidated.

Another RLK involved in NF signaling has leucine rich repeat (LRR) and serine/threonine kinase domains and is encoded by *M. sativa NORK*/*PsSYM19*/*LjSYMRK*/*MtDMI2*/*GmNORK* (**Figure 2**; Endre et al. 2002; Stracke et al. 2002; Mitra et al. 2004; Capoean et al. 2005; Indrasumunar 2007). It is located on the plasma membrane and on the infection thread membrane (Limpens et al. 2005), and is predicted to function in both NF perception and downstream signal transduction since it is required for the earliest detectable root hair responses (Endre et al. 2002; Stracke et al. 2002). Activation of the LysM RLKs is seen as a prerequisite for the activation of this LRR RLK. Indeed, both receptors might be involved in the perception of microbial signal molecules, but it is not yet clear how the LRR RLK integrates fungal and bacterial signals. Whether this occurs directly through the formation of heterocomplexes, or indirectly via secondary signals, remains to be elucidated. Based on downstream responses, the LysM RLKs may have a specific role in the NF signaling cascade (described below), whereas the LRR RLK may function more in initiating bacterial infection events (**Figure 2**).

The Nod Factor Signaling Cascade

Nod factor perception initiates a downstream signal transduction cascade (**Figure 2**). This involves potassium

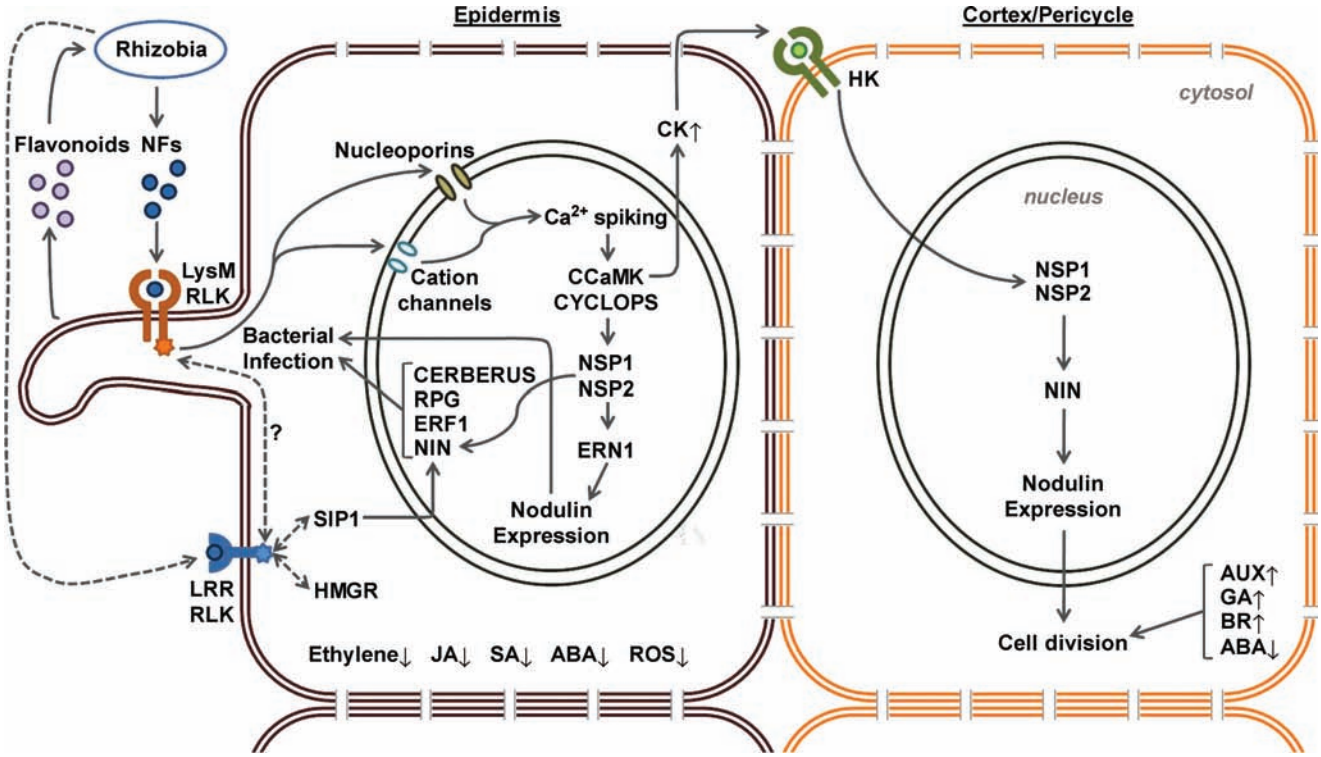


Figure 2. Molecular events associated with the early stages of nodulation.

Legume roots initiate nodulation by exuding flavonoids into the rhizosphere. This attracts compatible rhizobia to the root and stimulates them to produce nod factors (NF). NF is reported to be perceived at the epidermis by a leucine-rich repeat receptor-like kinase (LRR RLK) which triggers a number of downstream events involved in bacterial infection. Concomitantly, NF is also reported to be perceived by two LysM RLKs, which leads to the NF signaling cascade, cortical/pericycle cell divisions, and bacterial infection events. A mobile signal (possibly the phytohormone cytokinin) is presumed to relay NF perception from the epidermis to the cortex where it initiates cell divisions that give rise to the nodule primordia. The levels of many other hormones are also tightly controlled throughout nodule organogenesis and have roles in regulating nodule initiation and development.

ion-channel proteins localized in the nuclear membrane encoded by *MtDMI1, LjCASTOR* and *LjPOLLUX* (Ané et al. 2004; Imaizumi-Anraku et al. 2005; Riely et al. 2007), two nucleoporins encoded by *LjNup133* and *LjNup85* (Kanamori et al. 2006; Saito et al. 2007), and a calcium and calmodulin-dependent protein kinase (CCaMK) encoded by *MtDMI3*/*PsSYM9* (Levy et al. 2004; Mitra et al. 2004).

As quickly as 1 min after NF application, Ca^{2+} fluxes denoted by a rapid influx of Ca^{2+} ions, followed by the membrane depolarization efflux of Cl^- and K^+ occur in root hairs (Felle et al. 1999). Oscillation in cytosolic Ca^{2+} concentrations, known as Ca^{2+} spiking, are subsequently induced in the same cells some minutes after the induction of the Ca^{2+} fluxes (approximately 10 min after NF application; Wais et al. 2000; Walker et al. 2000). The ion-channel proteins and the nucleoporins are required for these Ca^{2+} spiking events and structural studies have indicated that CCaMK may act to perceive the Ca^{2+} spiking signals (reviewed in Oldroyd and Downie 2004). Similar fluctuations and spiking events have previously been shown to transduce signaling events subsequent to ligand binding (Dolmetsch et al. 1998; Li et al. 1998; Allen et al. 2001), indicating a similar effect may be occurring following NF perception. NF perception also leads to root hair deformation and to changes in the root hair actin cytoskeleton that are required for root hair curling and invasion (Cardenas et al. 1998; de Ruijter et al. 1998).

Mutation in genes coding for the NF LRR RLK, 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 (Ané et al. 2004; Imaizumi-Anraku et al. 2005; Kanamori et al. 2006; Miwa et al. 2006; Saito et al. 2007). In contrast, mutations in CCaMK do not affect Ca^{2+} fluxes and Ca^{2+} spiking events yet still 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 (**Figure 2**).

Several transcription factors are activated downstream of CCaMK, including nodulation signaling pathway 1 (NSP1; Smit et al. 2005), NSP2; (Kalo et al. 2005), Ets2 repressor factor (ERF) required for nodultion (ERN; Middleton et al. 2007) and nodule inception (NIN; Schauser et al. 1999; Borisov et al. 2003). *nsp1* and *nsp2* mutants exhibit normal Ca^{2+} responses when treated with NFs; however, they are unable to initiate transcription of the early nodulation (*ENOD*) genes in the epidermis (Catoira et al. 2000; Oldroyd and Long 2003). In the epidermal cells, NSP1 and NSP2 are thought to co-localize with CCaMK in the nucleus (Smit et al. 2005; Oldroyd and Downie 2008). This indicates that *NSP1* and *NSP2* are likely activated after Ca^{2+} spiking, possibly directly downstream of CCaMK. In addition, ERN1 and NSP1 have been shown to bind to the promoter of *ENOD11*; a well characterized *ENOD* expressed in epidermal cells, where the binding of the NSP1 to the *ENOD* promoter requires NSP2 (Andriankaja et al. 2007; Hirsch et al. 2009). In addition to binding to the *ENOD11* promoter, studies by Hirsch et al. (2009) have shown that NSP1 binding to the promoters of *ERN1* and *NIN* is essential for their expression. This suggests that *NSP1, NSP2, ERN1* and *NIN* all work in combination to regulate the expression of *ENOD*s in the epidermis (**Figure 2**).

Genetic and protein–protein interaction studies also identified protein components that interact with CCaMK and are required for NF signaling and nodule development, namely interacting protein of DMI3 (MtIPD3) and LjCYCLOPS (Messinese et al. 2007; Yano et al. 2008). These proteins are predicted to interact through a C terminal coiled-coil domain and are proposed to transduce the calcium spiking signal (Mitra et al. 2004) and regulate *NSP1* expression (Smit et al. 2005).

Operating in parallel with the NF signaling cascade are signaling components required for bacterial infection events that are triggered by the activation of the NF LRR RLK (**Figure 2**). One such component is 3-hydroxy-3-methylglutaryl CoA reductase 1 in *M. truncatula* (MtHMGR), which may be involved in the biosynthesis of isoprenoid-derived phytohormones, such as cytokinins and brassinosteroids (Kevei et al. 2007). However, the precise role of HMGR in nodule development is yet to be determined. SymRK-interacting protein of *L. japonicus* (LjSIP1) and *Rhizobium*-directed polar growth of *M. truncatula* (MtRPG) have also been shown to interact with the LRR RLK. LjSIP1 is a transcription factor that can bind to the promoter of *NIN* to regulate bacterial infection events (Zhu et al. 2008). MtRPG is a coiled-coil protein that has been shown to localize in the nucleus and is also reported to be required for bacterial infection, having a role in directing the polar tip growth of infection threads (Arrighi et al. 2009). However, like MtHMGR there is still much to learn about how LjSIP1 and MtRPG function *in planta*.

Other factors having a role in nodule development include LjCERBERUS, ethylene response factor 1 (LjERF1) and ethylene response factor required for nodule differentiation (MtEFD) (Asamizu et al. 2008; Vernie et al. 2008; Yano et al. 2009). LjERF1 and MtEFD are transcription factors, whereas LjCERBERUS is a U-box protein. They are all localized in the nucleus and have a role in bacterial infection events (**Figure 2**). However, like MtHMGR, LjSIP1 and MtRPG mentioned above, their precise role in nodule organogenesis is yet to be fully understood.

Epidermal and Cortical Responses during Early Stage of Nodulation

Multiple cell types and layers must synchronize their development in order to achieve nodule organogenesis. Indeed, following NF perception in the epidermis, rapid responses are detected in the inner root. 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 of white clover within just 24 h of rhizobia inoculation (Mathesius et al. 2000). To achieve such rapid responses in the inner root after exposing the outer root to rhizobia/NF, some form of signaling communication seems imperative.

A cytokinin receptor functions in the root cortex and is required for cell division events (**Figure 2**). This receptor has a histidine kinase domain and is encoded by *MtCRE1*/*LjLHK1* (Gonzalez-Rizzo et al. 2006; Tirichine et al. 2007). Gain-of-function mutations in *Ljlhk1* result in a spontaneous nodulation phenotype due to controlled cell divisions occurring in the root cortex. Additional studies have shown that downregulation, or loss-of-function, of this cytokinin receptor results in a dramatic decrease in nodule numbers caused by the plant's inability to form nodule primordia (Gonzalez-Rizzo et al. 2006; Murray et al. 2007). Rhizobia infections still take place, but the infection threads lose their directionality and spread laterally rather than growing towards the root cortex (Murray et al. 2007). This suggests that initial bacterial infection events do not require nodule primordia formation or the cytokinin receptor, but that both are subsequently required to guide infection thread growth.

As previously mentioned, the loss-of-function *Mtdmi3* mutants exhibit a non-nodulation phenotype due to the need for CCaMK activity in the epidermis. However, like the cytokinin receptor, gain-of-function mutants of CCaMK result in spontaneous nodulation due to controlled cell divisions occurring in the cortex (Gleason et al. 2006; Tirichine et al. 2006). This mutation also induces the epidermal expression of *ENOD11* (Journet et al. 2001) in a pattern similar to that observed

in plants inoculated with compatible rhizobia (Gleason et al. 2006). Therefore, CCaMK appears to be required for events occurring in both the epidermis and the cortex, yet involving entirely different pathways (**Figure 2**).

NSP1 and NSP2, which act downstream of CCaMK in the epidermis as part of the NF signaling pathway, are also required for cell division events in the root cortex (**Figure 2**; Heckmann et al. 2006). Studies using gain-of-function mutants of CCaMK and the cytokinin receptor have shown that their spontaneous nodulation phenotypes are abolished in the absence of functional copies of *NSP1* or *NSP2* (Gleason et al. 2006; Tirichine et al. 2007). Thus, not only do NSP1 and NSP2 act downstream of CCaMK in the epidermis, but they also act downstream of CCaMK and the cytokinin receptor in the cortex.

Another transcription factor, NIN, also appears to have a role in both epidermal and cortical cells (Schauser et al. 1999; Borisov et al. 2003; Marsh et al. 2007). Mutant *nin* plants exhibit excessive root hair curling and blocked rhizobia infection events in the epidermis (Schauser et al. 1999). In the cortex, *nin* mutant plants are unable to initiate cell divisions and subsequent nodule primordium formation (Schauser et al. 1999; Borisov et al. 2003). Moreover, the action of NIN is essential for nodule development to occur in CCaMK and cytokinin receptor spontaneous nodulation mutants and it has been suggested that NIN is activated following the activation of CCaMK and the cytokinin receptor (Tirichine et al. 2006, 2007; Marsh et al. 2007). These features are similar to what is observed in the *nsp* mutants. However, unlike the *nsp* mutants, *nin* mutants show excessive *ENOD11* expression along the root epidermis, suggesting that NIN is not essential for NF-induced *ENOD11* expression. NIN may therefore act as a negative regulator of NF signaling to regulate the spatial expression of *ENOD11* in the root epidermis (Marsh et al. 2007). Furthermore, the expression of *NIN* is induced by cytokinin or NF application (Gonzalez-Rizzo et al. 2006; Murray et al. 2007), further supporting the idea that NIN positively regulates cortical cell divisions. However, the precise function of NIN at the molecular level remains to be fully established.

That a cytokinin receptor is critical for nodule development highlights the fact that the plant hormone, cytokinin, is a key component of nodule organogenesis. Indeed, it seems possible that cytokinin could be the mobile signal that communicates epidermal perception of NF to the inner root (**Figure 2**). Another plant hormone, abscisic acid (ABA), has also been proposed as a candidate for the mobile signal (Ding and Oldroyd 2009). ABA is typically considered a negative regulator of nodule development and appears to have a role in both the epidermis and cortex (Ding et al. 2008; Biswas et al. 2009; reviewed by Ding and Oldroyd 2009). Other plant hormones have also been reported to have roles in nodule development, including positive regulators such as auxin, brassinosteroids and gibberellins and negative regulators such as reactive oxygen species (ROS), jasmonic acid (JA) and ethylene (Ferguson and Mathesius 2003; Ferguson et al. 2005a; Sun et al. 2006; Kinkema and Gresshoff 2008; Mathesius 2008). However, for many of these signals, direct roles in nodulation have yet to be fully demonstrated and their function may lie more in indirect processes such as cell division, differentiation and maintenance.

The Process of Autoregulation of Nodulation

There are a number of additional external and internal factors that act as negative regulators of nodulation. Mutants unable to synthesize or perceive these factors exhibit increased nodule numbers. Many of these factors function in the autoregulation of nodulation (AON) pathway involving long-distance root-shoot signaling (**Figure 3**). AON is initiated during nodule development by the synthesis of a root-derived signal named 'Q'. Recent work has indicated that Q is likely a CLAVATA3/ESR related (CLE) peptide (Okamoto et al. 2009; D Reid, B Ferguson and P Gresshoff, unpubl. data, 2009). Grafting experiments (Delves et al. 1986) have shown that Q travels to the shoot after inoculation with rhizobia where it, or a product of its action, is perceived by an AON LRR RLK having a serine/threonine kinase domain called *GmNARK*/*LjHAR1*/*MtSUNN* (Krusell et al. 2002; Nishimura et al. 2002a; Searle et al. 2003; Schnabel et al. 2005). This AON LRR RLK is similar to many other protein receptor kinases found in plants and animals and is expressed specifically in the phloem (Nontachaiyapoom et al. 2007). It is not yet known whether the AON LRR RLK functions independently, or as part of a homodimer, or heterodimer, complex with another receptor-kinase or receptor-like protein, to perceive the Q signal (**Figure 4A**).

Recent work in soybean has identified novel components that may interact directly with the AON LRR RLK or function downstream of its activity to regulate AON (**Figure 3**). These include genes coding for kinase-associated protein phosphatases, *GmKAPP1* and *GmKAPP2* (Miyahara et al. 2008). These genes may act to directly translate GmNARK activity as part of the GmNARK signal transduction pathway. They can be transphosphorylated by the GmNARK kinase domain and in turn can dephosphorylate the GmNARK receptor (**Figure 4A**). The signal resulting from this interaction is suggested to be relayed from GmNARK to several unknown downstream effectors.

The perception of Q by the AON LRR RLK in the leaf results in the production of a novel shoot-derived inhibitor, named 'SDI'. The inhibitor appears to enter the phloem and travels down to the root where it acts to inhibit further nodulation events (Gresshoff and Delves 1986; Lin et al. 2009) (**Figure 3**). Recent work using soybean has established that SDI is small (<1 kDa), heat stable, NF-dependent, requires

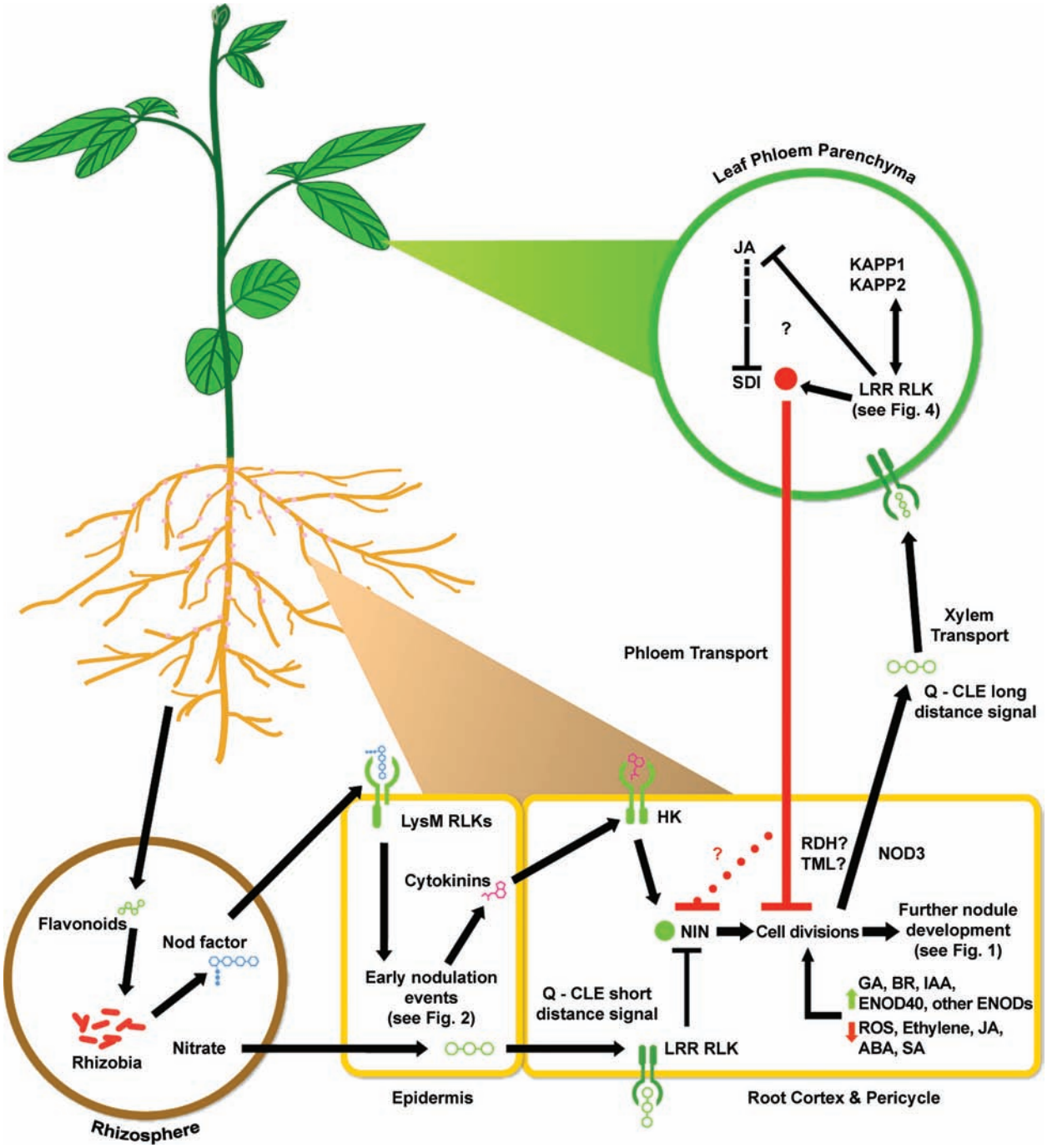


Figure 3. Rhizobia- and nitrate-regulation of nodulation via autoregulation of nodulation (AON).

The plant has inbuilt regulatory mechanisms to control nodule numbers. Rhizobia systemically regulate nodulation by triggering the production of an AON elicitor signal, called Q, at some stage during cell division. Similarly, nitrate also induces the production of a Q signal molecule that can elicit AON activity. Recent evidence indicates that the Q signal molecules are highly similar CLAVATA3/ESR related (CLE) peptides. The rhizobia-induced Q is transported long-distance to the leaf, whereas the nitrate-induced Q acts locally in the root. Interestingly, it appears that the same leucine-rich repeat receptor-like kinase (LRR RLK, encoded by *GmNARK*/*LjHAR1*/*MtSUNN*) is involved in perceiving the rhizobia-induced Q in the leaf and the nitrate-induced Q in the root. Perception of either Q molecule leads to the production of an inhibiting factor that suppresses further nodulation events in the root.

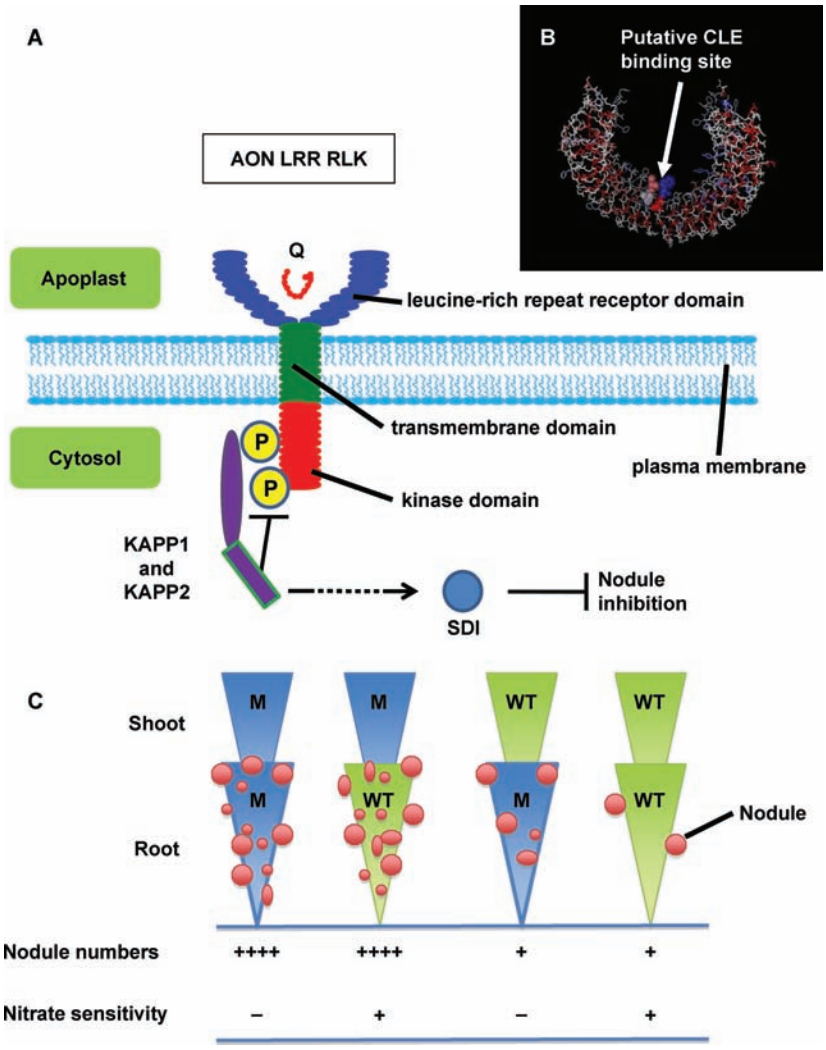


Figure 4. The leucine-rich repeat receptor-like kinase (LRR RLK) involved in regulating legume nodule numbers.

(**A**) Proposed molecular mechanism of autoregulation of nodulation (AON) signal transduction. Elicitor compounds proposed to be CLAVATA3/ESR related (CLE) peptides are synthesized in the root following rhizobia inoculation or nitrate treatment. The LRR RLK perceives the elicitor ligand in the apoplast, triggering downstream signaling events in the cytosol. Perception of the ligand allows for the phosphorylation of the kinase domain of the LRR RLK. KAPP1 and KAPP2 are subsequently transphosporylated, and in turn dephosporylate the LRR RLK kinase domain. Resulting signal(s) are relayed to several unknown downstream effectors. Activation of the LRR RLK also triggers the production of a shoot-derived factor that inhibits further nodulation events.
(**B**) Putative protein structure of the LRR domain of the soybean LRR RLK, *GmNARK*, showing the putative CLE binding domain.
(**C**) Classical grafting studies using wild type and supernodulating mutant plants have shown that the LRR RLK functions in the shoot to control root nodule numbers. More recently, an additional, yet less obvious, role for the LRR RLK was identified in the root by treating grafted plants with high levels of nitrogen. M, mutant genotype; WT, wild type genotype.

GmNARK-activity for its biosynthesis, and is unlikely to be an RNA or a protein (Lin et al. 2009).

Recently, GeneChip and real time polymerase chain reaction (PCR) analyses of leaves from rhizobia-inoculated or -uninoculated soybean plants differing in *GmNARK* function revealed a novel regulation of members of the octodecanoid pathway (Kinkema and Gresshoff 2008). This suggests the involvement of jasmonic acid, a novel plant hormone (related to prostagladinins in humans), in AON (**Figure 3**). Moreover, these genes represent candidates as downstream effectors of GmNARK activity. Functional reverse genetics tools, such as virus-induced gene silencing (VIGS), will be useful in verifying

whether these factors, and the abovementioned GmKAPPs, are indeed critical components of the AON signaling circuit.

Root-specific genes have been identified in pea (*PsNOD3*; Postma et al. 1988) and *L. japonicus* (*LjRDH1*, Ishikawa et al. 2008; *LjTML*, Magori et al. 2009) that may be involved in Q biosynthesis or translocation, or in SDI perception, in the root. Recent work using approach-grafting techniques has elegantly indicated that *PsNOD3* likely functions in the root before the activation of the AON LRR RLK in the leaf. Therefore, *PsNOD3* may have a role in the production or transport of Q in the root (Li et al. 2009).

A number of other genes have also been identified as regulators of nodule numbers. Grafting studies have shown that *LjKLAVIER* has a shoot-specific role in regulating nodule numbers (Oka-Kira et al. 2005). However, the identity of this gene remains unknown. Loss of function of the ERF transcription factor, *MtEFD*, also results in increased nodule numbers, possibly by altering cytokinin signaling (Vernié et al. 2008). Interestingly, *LjASTRAY*, which encodes a bZIP transcription factor with a RING-finger motif, regulates light and photomorphogenic signaling, but also regulates nodulation, as loss-of-function mutants exhibit increased nodule numbers (Nishimura et al. 2002b). Whether these genes function directly or indirectly with AON remains to be determined.

Other factors that reduce nodule numbers include ethylene and nitrate (Carroll et al. 1985a,b; Guinel and Geil 2002; Ferguson and Mathesius 2003; Ferguson et al. 2005b; Gresshoff et al. 2009; Lohar et al. 2009). Ethylene is strongly induced by stress and it seems possible that a mechanism has evolved to prevent precious photoassimilates from being used for nodule development while the plant is under duress. Similarly, because nitrogen is the main component the plant acquires in the legume-rhizobia symbiosis, it seems highly plausible that a mechanism has evolved to prevent the plant from forming nodules when nitrogen levels in the rhizosphere are already sufficient.

Mutations that disrupt the plant's ability to perceive either ethylene or nitrogen alleviate the inhibitory nature of these factors, resulting in increased nodule numbers. This includes genes required for ethylene sensitivity and response, such as *LjETR1* and *LjEIN2*/*MtEIN2* (Penmetsa et al. 2008; Lohar et al. 2009). In addition, *nitrate-tolerant symbiosis* (*nts*) mutants that form many nodules when grown under inhibitory nitrate levels have been isolated in soybean and pea (Carroll et al. 1985a,b; Delves et al. 1986), but *nts* genes not involved in AON remain to be cloned.

Interestingly, recent work has indicated that nitrate inhibition of nodulation may function via an upregulation in the expression of a nitrate-induced CLE peptide in the root (Okamoto et al. 2009; D Reid, B Ferguson and P Gresshoff, unpubl. data, 2009; Gresshoff et al. 2009) (**Figure 3** and **Figure 4C**). This nitrate-induced CLE peptide is highly similar to the rhizobia-induced Q CLE peptide. Both CLEs appear to be perceived by the same AON LRR RLK encoded by *GmNARK*/*LjHAR1*/*MtSUNN* (**Figure 4**), only the nitrate-induced CLE exhibits little-to-no mobility and is perceived in the root, whereas the rhizobia-induced CLE undergoes long distance transport and is perceived in the shoot. The fact that the same receptor is required to perceive both of the Q peptides may demonstrate why all soybean and pea *nts* mutants are both nitrate-tolerant and AON defective.

Conclusions and Perspectives

The environmental and agricultural benefits of legumes have been recognized for centuries. Over the last 10 years, our understanding of the nodulation process that is largely responsible for these benefits has grown immensely. This can be attributed to advances in the available tools and technologies, coupled with the use of model legume and mutagenesis programs, enabling the identification of many key nodulation genes. However, a number of critical questions remain: What is the mobile signal coordinating the developmental programs of the epidermis and the cortex? Why are there three different NF receptors, how do they function to perceive NF and do they interact? Does the LRR RLK required for AON in the shoot indeed have a dual role for nitrogen regulation of nodulation in the root?

With next generation sequencing technologies and relatively-complete genome sequences now available, a new wave of novel genes required for nodule organogenesis, including miRNAs, will undoubtedly be revealed. The subsequent use of cutting-edge techniques, such as RNAi and VIGS, will help confirm the functionality of these genes without the need to generate stable mutant lines. Moreover, the ever increasing sensitivity of analytical instruments should ensure continued advances in nodulation biochemistry. Collectively, although gaps still remain in the knowledge base, they are being filled at an unprecedented rate, and on a global scale, never before experienced in the field of legume nodulation.

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