Integrated Nod Factor Signaling in Plants

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Abstract Many legumes enter symbiotic relationships with rhizobial bacteria to acquire the macronutrient nitrogen. Bacteria reduce nitrogen to ammonia, a form accessible for the plant, in plant-derived organs, called "nodules." The symbiotic interaction is initiated by the release of the bacterial signal Nod factor into the rhizosphere and its recognition by plant roots. The perception of Nod factor in the plasma membrane induces a signaling pathway that uses calcium as a secondary messenger. Genetic analysis in legume species such as *Medicago truncatula* and *Lotus japonicus* revealed many components which are essential for the Nod factor induced signaling pathway. We describe the structural specificity of Nod factor recognition and the genes involved in the activation and perception of calcium oscillations during Nod factor signaling. In addition, the processes that lead to the initiation of nodule organogenesis following Nod factor signaling are briefly discussed.

1 Introduction

One of the most important examples of a beneficial symbiosis in the biosphere is the symbiotic interaction between nitrogen-fixing soil bacteria and their plant hosts. Nitrogen fixation takes place in unique organs predominantly associated with roots, called "nodules." The formation of nodules provides an oxygen-limited environment that is suitable for the activity of the oxygen-inhibited bacterial enzyme nitrogenase. Nitrogen-fixing bacteria convert atmospheric nitrogen into ammonia, which is absorbed by the plant to meet its nutritional needs. As nitrogen is usually lacking from many soils around the world, biological nitrogen fixation has a significant impact on global agriculture. Indeed, early agricultural systems invariantly used co-cultivation or rotation of a legume with a cereal crop to both enhance soil fertility and provide a balance of protein-rich and carbohydrate-rich foods (e.g., soybean/rice in Asia, bean/maize in the Americas, and lentil, chickpea/wheat, barley in Mesopotamia).

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F. Baluška and S. Mancuso (eds.), *Signaling in Plants,* Signaling and Communication in Plants, 71
DOI: 10.1007/978-3-540-89228-1_4,

Nitrogen-fixing root nodule symbioses occur in two major associations: the legume–*Rhizobium* association and the actinorhiza–*Frankia* association. The actinorhiza –*Frankia* symbiosis differs from the interaction of legumes with rhizobia in several morphological and cytological aspects (Pawlowski and Bisseling 1996). However, both legume and actinorhizal symbioses are initiated by an exchange of signals, a process, that has been well studied for the legume–*Rhizobium* interaction but is poorly understood for the actinorhizal symbiosis. The legume–*Rhizobium* association is initiated by the release of plant-made compounds known as flavonoids from the plant root into the rhizosphere and their recognition by rhizobia. Upon recognition of flavonoids, *nod* genes of rhizobia are induced and these produce lipochitooligosaccharide signal molecules called "Nod factors" (Denarie et al. 1996; Long 1996; Spaink 2000). Rhizobial infection of the host plant and formation of the nodule primordia depend on the appropriate recognition of Nod factor by the host legume.

Bacterial infection can occur by both intracellular and intercellular mechanisms. The intracellular mechanism is used in most root nodule symbioses and generally starts with the attachment of rhizobia to the root hairs of legumes. This results in the induction of root hair curling and in the entrapment of the bacteria within a curled root hair, a structure also known as an infection pocket. The bacteria induce the hydrolysis of the plant cell wall and this coupled with plasma membrane invagination forms a tubular structure, the infection thread. Bacteria enter the plant root through the infection threads which grow towards the root cortex. Concomitant with this infection process, Nod factor triggers the formation of the nodule primordia by inducing cell divisions in the root cortex. After the infection thread reaches the nodule primordia, rhizobia are released inside the plant cell and enclosed by a plant-derived membrane. Within this structure the rhizobial bacteria differentiate into bacteroids, and these coupled with the surrounding plant membrane are referred to as "symbiosomes," the cellular structures capable of nitrogen fixation (Newcomb 1981). Intracellular invasion occurs in the majority of legume hosts and in the model legumes *Medicago truncatula* and *Lotus japonicus*. During intercellular infection, which is observed in many tropical legumes, the bacteria enter via cracks in the epidermis that can form where lateral or adventitious roots emerge (Chandler et al. 1982; Goormachtig et al. 2004; James et al. 1992). Although the two infection processes differ in many ways, they both depend on Nod factor and its signal transduction pathway in the plant host (Cullimore et al. 2001; D'Haeze et al. 2000; Oldroyd 2001).

Nod factor is a key player in the coordination of nodulation and therefore we will focus our attention on Nod factor, its perception, and the induction of the Nod factor signaling cascade in legumes.

2 Nod Factor Production in Bacteria

To establish a functional root nodule symbiosis, rhizobial bacteria have to recognize and respond to the presence of the legume host. This is realized by the chemical interplay of flavonoid compounds released from the legume root and bacterially derived Nod factor, a potent signal to the legume host.

2.1 *Flavonoids and* nod *Gene Induction*

Flavonoids are plant secondary metabolites that are synthesized via the phenylpropanoid pathway. They are diverse molecules with a wide range of structures and function. For decades flavones and isoflavones, subclasses of flavonoids, have been known to play a role in nodulation (Peters et al. 1986). Approximately 30 flavonoids which act as inducers of *nod* genes in rhizobia have been isolated (Begum et al. 2001; Cooper 2007; Hungria et al. 1992; Smit et al. 1992). *nod* genes, which are predominantly required for Nod factor synthesis, are mediated by NodD transcriptional regulators. NodDs associate with promoter elements of *nod* genes to activate their transcription (Fisher and Long 1993). It has long been discussed whether flavonoids form a complex with NodD at the DNA, but no evidence for a direct interaction between the two molecules has yet been found. However, it has been observed that flavonoids stimulate an increase in DNA binding of NodD1 to *nod* gene promoters (Peck et al. 2006). Recent work indicates the further importance of flavonoids for nodulation once the bacteria have entered the plant root and exogenous flavonoids are no longer available (Subramanian et al. 2007). In *M. truncatula* and soybean it has been shown that the suppression of endogenous flavonoid production abolishes nodulation (Subramanian et al. 2006; Wasson et al. 2006).

Besides the induction of *nod* gene expression, flavonoids elicit strong chemotactic and growth responses to concentrate rhizobia at the root surface (Rolfe 1988; Stougaard 2000). In addition, the activation of genes expressing type III secretion proteins and rhamnose-rich lipopolysaccharides requires flavonoids (Kobayashi et al. 2004). Furthermore, flavonoids have been proposed to have a role much later in nodule development by regulating auxin transport during the initiation of the nodule primordium (Wasson et al. 2006). Thus, flavonoids may play multiple roles during the legume–*Rhizobium* symbiosis with the induction of Nod factor signal production as a key function.

2.2 *Nod Factor Synthesis, Structure, and Specificity*

Nod factors are lipochitooligosaccharides comprising a backbone of β-1–4-linked *N*-acetyl-D-glucosamine residues with N-linked acyl groups attached to the nonreducing end (Fig. 1). Nod factors show a wide variation in their structure; they differ in the length of the backbone, the saturation of the fatty acid residue, and the number and types of substituent groups (Denarie et al. 1996; Spaink 2000). The additional modifications to the basic molecule can include sulfuryl, methyl, mannosyl, carbamoyl, acetyl, fucosyl, and arabinosyl moieties. The structure of the Nod factor molecule varies between different rhizobial strains. This diversity in Nod factor structure is often the basis for the specificity of interaction between certain bacterial strains and legume species.

As outlined earlier, Nod factor production is a function of the rhizobial *nod* genes. Each rhizobial strain has a characteristic array of *nod* genes. The *nodABC* genes are required for the production of the Nod factor backbone (the N-acylated

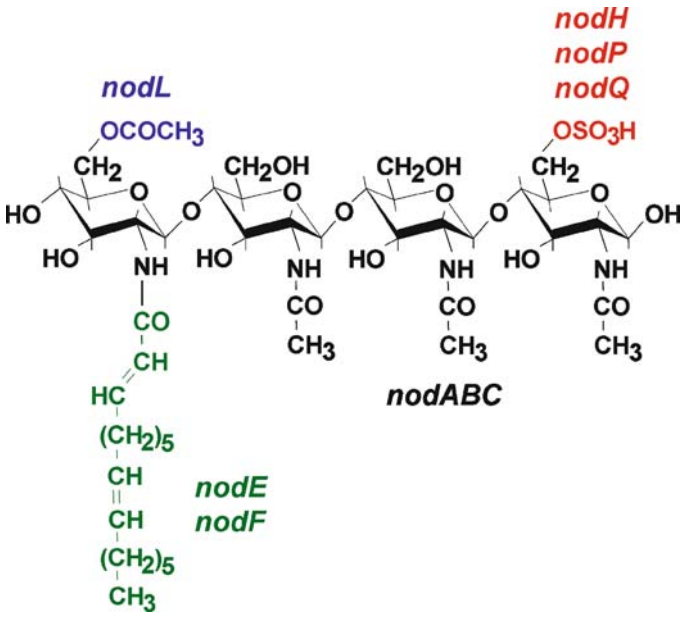

Fig. 1 Structure of Nod factor from *Sinorhizobium meliloti*. The Nod factor backbone of β-1–4-linked *N*-acetyl-D-glucosamine residues, which requires *nodABC*, and its linked *N*-acyl group is common for all rhizobial strains, while the number and types of substituent groups can vary. Nod factor from *S. meliloti* carries an *O*-sulfate group at the reducing end which requires *nodHPQ*. The appropriate attachment of the *N*-acyl group is *nodEF*-dependent and the attachment of the *O*-acetyl group at the nonreducing end depends on *nodL*

chitin) (Fig. 1). It was shown that *nodABC* can be functionally exchanged between rhizobial strains (Denarie et al. 1996), indicating that those genes are not involved in defining the bacterial host range. For the *Medicago* symbiont, *Sinorhizobium meliloti*, *nodE*, *nodF*, *nodL*, *nodH*, *nodP*, and *nodQ* are host-specific *nod* genes. *S. meliloti* Nod factor carries an *O*-sulfate group at the reducing glucosamine residue, which is a combined function of *nodH*, *nodP*, and *nodQ* (Fig. 1) (Roche et al. 1991; Schwedock and Long 1990). *S. meliloti* Nod factor can only induce nodulation in its *Medicago* hosts when this sulfate is present. The lack of the host-specific *nod* genes can alter the host range of *S. meliloti* (Denarie et al. 1996). For example, *nodH* mutants, which lack the *O*-sulfate group, lose their ability to nodulate *Medicago sativa*, but gain the activity on *Vicia sativa* (Faucher et al. 1989).

S. meliloti nodE and *nodF* regulate the attachment of the appropriate $C_{16:2}$*N*-acyl group, while *nodL* is required for the *O*-acetyl modification (Fig. 1) (Demont et al. 1993; Spaink et al. 1991). Mutations in *nodE*, *nodF*, and *nodL* lead to delayed and reduced nodulation in *S. meliloti* hosts *Medicago* spp., but still elicit morphological responses associated with nodulation (Ardourel et al. 1994; Debelle et al. 1986; Swanson et al. 1987). *nodF*/*nodL* double mutants showed a more severe phenotype: they were unable to penetrate into legume hosts and to form infection threads (Ardourel et al. 1994). However, these mutants were able to activate cortical cell

division, indicating that the Nod factor structural requirements are more stringent for bacteria1 entry than for the induction of the nodule primordium (Ardourel et al. 1994). This work and work from other laboratories uncoupled bacterial infection from the activation of earlier Nod factor responses, suggesting that two specificities for Nod factor recognition might exist: low-stringency recognition that is necessary for earlier stages of the symbiosis and high-stringency recognition necessary for bacterial infection (Firmin et al. 1993; Geurts et al. 1997; Oldroyd and Downie 2004; Spaink et al. 1991; Walker and Downie 2000).

3 Nod Factor Recognition in the Plant

Nod factor released from rhizobial bacteria is required to activate several plant processes associated with nodulation (Downie and Walker 1999). Genetic approaches have been used to identify genes involved in the induction of these plant responses.

3.1 Receptor-Like Kinases as Candidates for the Nod Factor Receptor

To identify receptors in legumes responsible for the perception of Nod factor, mutant screens have been performed. Legume mutants lacking all Nod factor induced plant responses were identified in *L. japonicus, M. truncatula*, and *Pisum sativum*. In *L. japonicus* two genes, *LjNFR1* and *LjNFR5*, were found to be essential for Nod factor perception (Fig. 2) (Madsen et al. 2003; Radutoiu et al. 2003). Both genes encode transmembrane receptor-like kinases with two to three extracellular lysine motifs (LysM) and an intracellular serine/threonine protein kinase domain. While LjNFR1 contains an apparently functional serine/threonine kinase domain (Huse and Kuriyan 2002; Schenk and Snaar-Jagalska 1999), *LjNFR5* encodes a kinase lacking the activation loop that usually regulates kinase activity (Madsen et al. 2003). This leads to the hypothesis that LjNFR5 forms a receptor complex with LjNFR1 which contributes the kinase activation domain (Radutoiu et al. 2003). LysM motifs present in the extracellular domains of both receptor-like kinases have previously been identified to bind peptidoglycans (Bateman and Bycroft 2000). The binding occurs to the *N*-acetylglucosamine-*N*-acetylmureine backbone, which is chemically similar to the Nod factor backbone (Steen et al. 2003); but the direct binding of Nod factor to LjNFR1 and LjNFR5 LysM domains remains to be demonstrated. However, recent work revealed that the specificity of Nod factor recognition is mediated by LjNFR1 and LjNFR5 (Radutoiu et al. 2007). The expression of both receptor-like kinases from *L. japonicus* in *M. truncatula* and *Lotus filicaulis* extends their host range to include the *L. japonicus* symbiont *Mesorhizobium loti*. Furthermore, domain swaps and amino acid substitutions demonstrated the importance of the LysM domains, especially the LysM2 domain of LjNFR5 in Nod factor specificity (Radutoiu et al. 2007).

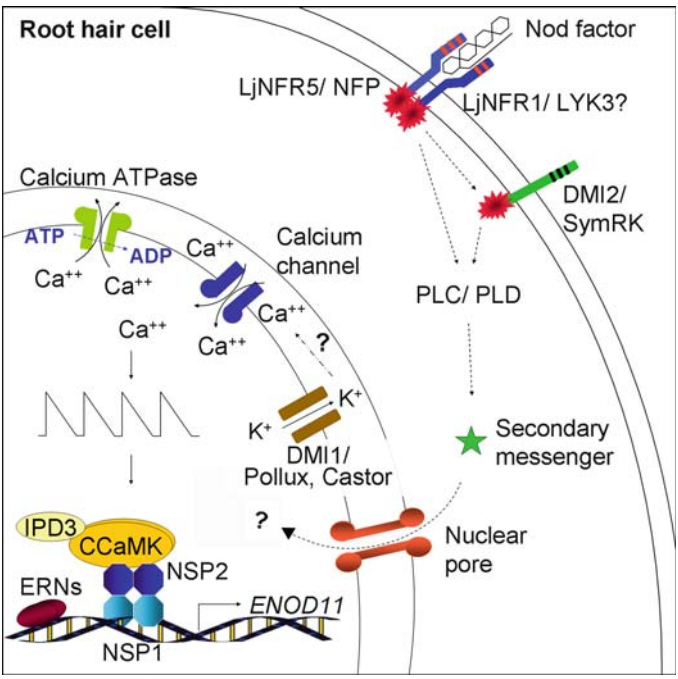

Fig. 2 Nod factor signaling in the legume-*Rhizobium* symbiosis. Nod factor recognition requires two receptor-like kinases, LjNFR1 (LYK3?) and LjNFR5/NFP, consisting of extracellular LysM domains and an intracellular kinase domain. A second type of receptor-like kinase is involved in Nod factor signaling: DMI2/SymRK, which contains extracellular leucine-rich repeat motifs. Phospholipase C, phospholipase D, and a secondary messenger are proposed to link Nod factor recognition to calcium spiking in the nucleus. The activation of calcium spiking also depends on components of the nuclear pore complex, NUP85 and NUP133, and up to two potassium channels, DMI1/Castor, Pollux, on the nuclear membrane. The calcium signal is presumed to be decoded and transduced by CCaMK, NSP1, NSP2, and ERNs. *PLC* phospholipase C, *PLD* phospholipase D, *CCaMK* calcium/calmodulin-dependent protein kinase, *ENOD11* early nodulation gene

Characterization of *M. truncatula* and *P. sativum* mutants has led to the identification of the *LjNFR5* orthologues *MtNFP* and *PsSYM10*, respectively (Amor et al. 2003; Arrighi et al. 2006; Madsen et al. 2003; Walker et al. 2000). Loss-of-function mutations in *MtNFP* and *PsSYM10* caused the loss of all Nod factor inducible plant responses, similar to *Ljnfr5*. Several homologues of *LjNFR1* exist in *M. truncatula* and mutations or RNA interference of two of these, *LYK3* and *LYK4*, show specific defects in *S. meliloti* infection, but not in the induction of earlier Nod factor responses (Limpens et al. 2003; Smit et al. 2007). Further analysis of a weak *lyk3* allele revealed that LYK3 is involved in the recognition of the *O*-acetyl modification of the *S. meliloti* Nod factor (Fig. 1). This work provides evidence in *M. truncatula* for the previously proposed two-receptor model that was based on genetic work in *S. meliloti* (Ardourel et al. 1994): *MtNFP* provides low-stringency recognition for early Nod factor responses, while *LYK3* provides high-stringency recognition that is required to allow *S. meliloti* infection.

3.2 The Role of Lectin Nucleotide Phosphohydrolase in Nod Factor Recognition

During the past few years much less attention had been paid to a possible alternative or additional candidate for Nod factor perception. In 1999 a lectin was isolated from roots of the legume *Dolichos biflorus* (Quinn and Etzler 1987) that catalyzes the hydrolysis of phosphoanhydride bonds of nucleoside diphosphates and triphosphates (Etzler et al. 1999). It was therefore called "lectin nucleotide phosphohydrolase" (LNP). LNP binds to Nod factors and Nod factor binding induces its phosphohydrolase activity in vitro. An antiserum to LNP of *D. biflorus* and *Glycine max* inhibits root hair deformation and nodule formation, suggesting that this protein has a function in the legume–*Rhizobium* symbiosis (Day et al. 2000; Etzler et al. 1999). Moreover, the expression of an LNP gene in *G. max* is induced by rhizobia, while LNP overexpression enhances nodulation (Day et al. 2000; McAlvin and Stacey 2005). Although these data highlight the relevance of LNP in nodulation, a role for LNP as a Nod factor receptor remains controversial.

4 The Role of Calcium in Nod Factor Signaling

The calcium ion is an important component in a diverse array of signaling pathways in plants. It is a ubiquitous intracellular secondary messenger that has been found to increase upon many physiological stimuli. Oscillations in calcium ion concentrations have been observed in stomatal guard cells (McAinsh et al. 1995) and have been shown to specifically regulate stomatal closure (Allen et al. 2001). Calcium ions also play a pivotal role in Nod factor signaling (Fig. 2). Application of Nod factor induces two separable calcium responses: a rapid influx of calcium ions and oscillations in cytosolic calcium around the nucleus.

With use of ion-specific microelectrodes an influx of calcium ions into *M. sativa* root hair cells could be shown after Nod factor addition (Felle et al. 1999a, b). The calcium ion influx is followed by the efflux of chloride ions within a few seconds, leading to Nod factor induced membrane depolarization (Felle et al. 1998). To rebalance the membrane charge, cation channels are activated, allowing the efflux of potassium ions and the repolarization of the membrane (Felle et al. 1998). Calcium ion influx associated with the root hair tip has also been observed in *Phaseolus vulgaris*, *P. sativum*, and *M. truncatula* root hair cells (Cardenas et al. 1999; Shaw and Long 2003; Walker et al. 2000), suggesting that it is a common feature in the legume–*Rhizobium* symbiosis. Calcium ion influx occurs within 1 min after the application of Nod factor at a minimum concentration of 1–10 nM (Felle et al. 1998; Shaw and Long 2003).

In addition to the calcium flux response, Nod factor induces oscillations in cytosolic calcium ion concentrations associated with the nucleus, called "calcium spiking" (Fig. 2). This was first shown in *M. sativa* root hair cells using calcium-sensitive reporter dyes (Ehrhardt et al. 1996). Since then calcium spiking has been

observed in *P. vulgaris, L. japonicus*, *M. truncatula*, and *P. sativum* root hair cells following the addition of Nod factor (Cardenas et al. 1999; Harris et al. 2003; Oldroyd et al. 2001; Wais et al. 2000; Walker et al. 2000). A calcium spike always shows a characteristic shape: a sudden increase in calcium levels followed by a more gradual decline (Meyer and Stryer 1988). In contrast to the Nod factor induced calcium flux, calcium spiking starts with a lag of approximately 10 min after Nod factor addition. The reason for this lag between Nod factor addition and calcium spiking induction has yet to be explained. Interestingly, calcium spiking appears at a minimum Nod factor concentration of 1–10 pM, a roughly 1,000-fold lower concentration than that required for induction of the calcium flux (Oldroyd et al. 2001; Shaw and Long 2003). In addition, calcium spiking is activated by Nod-factor-like chitin oligomers without the activation of the calcium flux (Walker et al. 2000). These discoveries indicate that for the induction of calcium spiking a relatively low stringency of Nod factor perception is required, while a much higher concentration and specific Nod factor structures are needed to elicit the calcium flux. This correlates with previous work indicating a high stringency of Nod factor recognition required for bacterial infection, and a model has been proposed which suggests a role of the calcium flux in the activation of infection thread formation (Miwa et al. 2006). This model predicts that upon the first contact between the legume root and the rhizobia the perception of a low Nod factor concentration initiates calcium spiking and subsequently promotes the accumulation of bacteria inside the infection foci. Once rhizobia are trapped in the root hair curl, the Nod factors should accumulate to high concentrations and therefore activate calcium flux, which could then lead to the initiation of the infection thread. In contrast to the observed lag phase using calcium-sensitive dyes, in this model the calcium flux occurs later than the calcium spiking response. Indeed, it has been shown that root hair cells initially induced to spike were able to show a calcium ion influx when Nod factor levels were secondarily raised (Shaw and Long 2003).

Pharmacological and genetic research indicate a function for calcium spiking in the activation of early Nod factor induced responses such as gene expression (Charron et al. 2004; Wais et al. 2000; Walker et al. 2000). It has been widely suggested that the nature of the calcium oscillations encodes information to define the outcome of the downstream response (Allen et al. 2001; Evans et al. 2001; Oldroyd and Downie 2006).

5 Genes Involved in the Activation of Calcium Spiking

Calcium spiking plays a crucial role in the Nod factor induced signaling pathway; it probably acts as a secondary messenger to transduce the Nod factor signal. Mutant screens have revealed several components required for the Nod factor dependent activation of calcium spiking. In addition to the LysM receptor-like kinases (see Sect. 3.1), a leucine-rich repeat (LRR) receptor-like kinase (Endre et al. 2002; Stracke et al. 2002), up to two putative cation channels (Ane et al. 2004; Edwards et al. 2007; Imaizumi-Anraku et al. 2005; Riely et al. 2007), and two nucleoporins

(Kanamori et al. 2006; Saito et al. 2007) are necessary for the induction of calcium spiking (Wais et al. 2000). Biochemical and pharmacological studies further indicate that multiple phospholipid signaling pathways are linked to Nod factor signaling (Charron et al. 2004; den Hartog et al. 2001; Engstrom et al. 2002; Sun et al. 2007).

5.1 LRR Receptor-Like Kinase

In addition to the LysM receptor-like kinases, the Nod factor signal transduction pathway requires a second type of receptor-like kinase that contains three LRR motifs in the extracellular domain (Fig. 2). The importance of this LRR receptor-like kinase for nodulation has been characterized in *Sesbania rostrata* (*SrSymRK*), *M. sativa* (*MsNORK*), *P. sativum* (*PsSym19*), *M. truncatula* (*DMI2*), and *L. japonicus* (*LjSymRK*) (Capoen et al. 2005; Endre et al. 2002; Stracke et al. 2002). In addition, the orthologues of this receptor-like kinase have been shown to be critical for nodulation in the actinorhizal plants *Casuarina glauca* (*CgSymRK*) and *Datisca glomerata* (*DgSymRK*), indicating genetic mechanisms shared between the two types of root nodule symbioses (Gherbi et al. 2008; Markmann et al. 2008). This LRR receptor-like kinase is essential for the activation of calcium spiking and early nodulin gene expression, but is not required for the activation of the calcium flux (Catoira et al. 2000; Miwa et al. 2006; Shaw and Long 2003; Wais et al. 2000). In contrast, the LysM receptor-like kinases are necessary for all Nod factor induced responses including the calcium flux, indicating a bifurcation in the Nod factor signaling pathway immediately downstream of the LysM receptor kinases, with one branch leading to calcium spiking and one branch leading to the calcium flux. In addition, partial suppression of *DMI2* and *SrSymRK* using RNA interference revealed a function for these genes at later stages of the symbiosis, during bacterial release and symbiosome formation (Capoen et al. 2005; Limpens et al. 2005). Consistent with these observations, the receptor-like kinase is expressed in roots prior to infection and is induced in the preinfection zone of *M. truncatula* nodules (Bersoult et al. 2005; Limpens et al. 2005).

5.2 The Role of Ion Channels in Nodulation Signaling

Calcium spiking is associated with the nucleus and it has been proposed that the nuclear envelope and the endoplasmic reticulum may act as the internal stores for this calcium response. A number of genes have been identified that are necessary for Nod factor induced calcium spiking and potentially function in regulating the release of calcium from these stores (Miwa et al. 2006; Shaw and Long 2003; Wais et al. 2000). In *L. japonicus* there are two highly homologous genes, *CASTOR* and *POLLUX*, encoding for predicted integral membrane proteins (Imaizumi-Anraku et al. 2005). In *M. truncatula* and *P. sativum* only one such gene, *DMI1* and *SYM8,*

respectively, was identified (Fig. 2) (Ane et al. 2004; Edwards et al. 2007). *DMI1* and *SYM8* are orthologous and are more closely related to *POLLUX* than they are to *CASTOR*. DMI1 has been reported to localize to the periphery of nuclei of root cells (Riely et al. 2007). DMI1, SYM8, CASTOR, and POLLUX show weak homology to MthK, a calcium-gated potassium channel from *Methanobacterium thermoautotrophicum* (Jiang et al. 2002). Observations using *sym8* and a structural model of the SYM8 channel indicate that SYM8/DMI1 may form a cation channel (but not a calcium channel) which could be opened by the binding of a signaling ligand (Edwards et al. 2007). SYM8/DMI1 may allow cation flow across the nuclear membrane, thus changing the membrane polarization, and this could regulate the opening of an as yet unidentified calcium channel. The hypothesis that SYM8/DMI1 regulates calcium channels is supported by the discovery that DMI1 interferes with calcium release from internal endoplasmic reticulum stores in yeast (Peiter et al. 2007).

5.3 Two Nucleoporins Required for Calcium Spiking

Two additional genes essential for Nod factor activation of calcium spiking have been identified in *L. japonicus*, *NUP133* and *NUP85*, and they both encode nucleoporins (Fig. 2) (Kanamori et al. 2006; Saito et al. 2007). Nucleoporins are components of the nuclear pore that consists of more than 30 proteins and function in the transport of large proteins and RNAs (Suntharalingam and Wente 2003). Nuclear pore complexes are additionally necessary for the appropriate localization of proteins to the inner nuclear membrane (Suntharalingam and Wente 2003). A NUP133-green fluorescent protein fusion localizes to the nuclear rim of root and root hair cells (Kanamori et al. 2006), indicating that NUP133 and NUP85 are members of a nuclear pore complex in legumes. Therefore, we might hypothesize that both nucleoporins are involved in the activation of calcium spiking by the possible transport and localization of calcium or other cation channels, such as SYM8/DMI1. However, further research will be required to clarify the role of NUP133 and NUP85 in early symbiotic signal transduction.

5.4 Phospholipase C and Phospholipase D

During the past few years a function for phospholipids in early Nod factor signaling has been discovered (Fig. 2). Phospholipase C, which is activated by heterotrimeric G-proteins, hydrolyzes plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate, in turn, can lead to the release of calcium from intracellular stores, such as the endoplasmatic reticulum. Mastoparan, a tetradecapeptide, activates heterotrimeric G-proteins and so artificially induces effector enzymes downstream

of G-proteins. It has been shown that mastoparan treatment activates calcium spiking, nodulin gene expression, and root hair deformation, indicating a role of G-proteins in Nod factor signaling (den Hartog et al. 2001; Pingret et al. 1998; Sun et al. 2007). Nodulin gene expression triggered by mastoparan requires the calcium/calmodulin-dependent protein kinase CCaMK (see Sect. 6.1), but does not require DMI1 and DMI2 (Charron et al. 2004; Sun et al. 2007). This suggests that mastoparan activates a component of the Nod factor signaling pathway downstream of DMI1/DMI2 and upstream of calcium spiking and CCaMK. Additionally, phospholipase C and phospholipase D were both found to be activated by Nod factor (den Hartog et al. 2001; den Hartog et al. 2003) and their requirement in signal transduction has been revealed by pharmacological studies. Root hair deformation and nodulin expression were inhibited by application of neomycin, an inhibitor of phospholipase C (Charron et al. 2004; den Hartog et al. 2001; Pingret et al. 1998). Cyclopiazonic acid and U-73122, inhibitors of type IIA calcium pumps and phospholipase C, abolish Nod factor induced calcium spiking (Engstrom et al. 2002). *n*-Butanol and cyclopiazonic acid inhibit both nodulin gene expression and mastoparan-induced calcium spiking (Charron et al. 2004; Sun et al. 2007). These pharmacological studies support the requirement of phospholipase C, phospholipase D, and calcium pumps as components in Nod factor-induced calcium signaling.

6 Genes Involved in the Perception of Calcium Spiking

Recent discoveries have deciphered components in the Nod factor signaling pathway which link Nod factor perception to the pivotal calcium spiking response. In addition, a number of components of the Nod factor signaling pathway have been defined that function in the perception and transduction of the calcium spiking signal. A calcium/calmodulin-dependent protein kinase and at least three transcription factors are likely to link calcium to the activation of nodulation gene expression.

6.1 The Function of a Calcium/Calmodulin-Dependent Protein Kinase

A calcium/calmodulin-dependent protein kinase (CCaMK) required for the legume–*Rhizobium* symbiosis has been identified in *M. truncatula* (*DMI3*), *L. japonicus* (*LjCCaMK*), and *P. sativum* (*PsSYM9*) (Levy et al. 2004; Mitra et al. 2004). Analogous to proteins that function upstream of calcium spiking, CCaMK is necessary for Nod factor induced gene expression, but is not necessary for the activation of calcium spiking (Catoira et al. 2000; Wais et al. 2000). This indicates that CCaMK plays a role in signaling downstream of the calcium oscillations (Fig. 2). The CCaMK protein consists of a serine/threonine kinase domain, followed by a calmodulin binding domain and three calcium binding EF-hand domains. It belongs to a family of

plant-specific proteins, but its calmodulin binding domain is similar to the mammalian CaMKII which has the ability to decode calcium spiking (De Koninck and Schulman 1998). In general, plant chimeric calcium/calmodulin dependent protein kinases are dually regulated: free calcium ions, which bind to the EF-hand domains, induce autophosphorylation and thereby enhance the binding of calcium complexed with calmodulin. The binding of calmodulin in turn promotes substrate phosphorylation (Gleason et al. 2006; Patil et al. 1995; Ramachandiran et al. 1997; Takezawa et al. 1996). An additional domain important for the function of CCaMK is the autoinhibitory domain which negatively regulates kinase activity. Specific removal of this domain leads to gene expression and spontaneous nodule formation in the absence of rhizobia or Nod factor (Gleason et al. 2006). A point mutation in the autophosphorylation site of *LjCCaMK* also resulted in spontaneous nodulation when expressed from its native promoter (Tirichine et al. 2006), implying that CCaMK is a central regulator for nodule organogenesis. A newly isolated protein, IPD3, has been shown to interact directly with *M. truncatula* CCaMK in yeast and in planta (Messinese et al. 2007). IPD3, which lacks homology to functionally characterized proteins, might act downstream of CCaMK in the Nod factor induced signaling pathway.

6.2 A Suite of Transcription Factors Transduce the Signal Downstream of CCaMK

NSP1 and NSP2 are two putative GRAS domain transcriptional regulators which function in Nod factor signaling downstream of calcium spiking (Kalo et al. 2005; Oldroyd and Long 2003; Smit et al. 2005; Wais et al. 2000). Both GRAS domain proteins have been identified in *M. truncatula* and *L. japonicus* (*LjNSP1*, *LjNSP2*) (Heckmann et al. 2006). The GRAS family of putative transcriptional regulators is found throughout the plant kingdom and these proteins have diverse roles in plant development (Bolle 2004). Although NSP1 and NSP2 both encode GRAS family members they are not very similar (17% identity, 32% similarity), indicating different functions in the Nod factor signaling pathway. *M. truncatula nsp1* and *nsp2* show defects in the activation of the nodule primordium, bacterial infection, and Nod factor induced gene expression and show reduced root hair deformation (Catoira et al. 2000; Oldroyd and Long 2003). The constitutive CCaMK construct did not autoactivate gene expression in the *nsp2* mutant, but showed partial activation in the *nsp1* mutant (Gleason et al. 2006). These observations place NSP1 and NSP2 downstream of CCaMK and indicate that the calcium signal is transduced by CCaMK through the two GRAS domain regulators (Fig. 2). Recently, it has been shown that NSP1 and NSP2 homodimerize and heterodimerize with each other and that NSP1 associates with the promoter of the early nodulation gene *ENOD11* (Hirsch et al., unpublished results). The fact that NSP2 also heterodimerizes with the kinase domain of CCaMK indicates a direct activation of the GRAS protein complex by CCaMK and links calcium spiking to the induction of nodulation gene expression through these transcription factors.

Like NSP1 and NSP2, ERN1, an ethylene response factor (ERF) transcription factor, is required for nodulation and acts downstream of CCaMK (Middleton et al. 2007). ERN1 and two close homologues, ERN2 and ERN3, have been identified to associate with the *ENOD11* promoter close to the NSP1 binding site (Fig. 2) (Andriankaja et al. 2007). Although this might indicate a direct heterodimerization between NSP1 and the ERN proteins, no such interaction has yet been described. While ERN1 and ERN2 function as transcriptional activators, ERN3 represses ERN1/ERN2-dependent transcriptional activation of *ENOD11* (Andriankaja et al. 2007). Another protein which negatively regulates *ENOD11* expression is NIN (Marsh et al. 2007; Schauser et al. 1999). *NIN* encodes a protein with transmembrane domains, but also a putative DNA binding domain. NIN negatively regulates the spatial pattern of *ENOD11* expression 24 h after Nod factor treatment (Marsh et al. 2007). Despite this apparent negative regulation of early nodulation gene expression, genetic studies have revealed that NIN is a positive regulator of nodulation and bacterial infection. Thus, NIN is likely to act in the activation of gene expression at later stages of nodule organogenesis.

7 The Arbuscular Mycorrhizal Symbiosis

More than 80% of terrestrial plants form beneficial symbioses with arbuscular mycorrhizal (AM) fungi. The AM symbiosis is thought to have evolved more than 400 million years ago and is therefore one of the oldest symbioses entered into by plants. The AM fungi enhance the uptake of macronutrients such as organic nitrogen and phosphate through the plant root (Harrison 1999; Hodge et al. 2001). Interestingly, nodulation and mycorrhizal signaling pathways share several common features, including the activation of calcium oscillations and the induction of a common set of signaling genes. At least seven genes that play a role in both types of symbioses have been identified so far. The LRR receptor-like kinase (Sect. 5.1) and the putative ion channels (Sect. 5.2) are dually involved in the activation of calcium spiking in nodulation and arbuscular mycorrhization (Catoira et al. 2000; Kosuta et al. 2008). CCaMK and IPD3 (Sect. 6.1), which act downstream of calcium spiking, are also shared signaling components, while NSP1 and NSP2 (Sect. 6.2) are not (Catoira et al. 2000; Messinese et al. 2007). The fact that root nodule and AM symbioses harbor conserved early-signaling genes supports the model that nodulation genes have been recruited from the more ancient AM symbiosis.

8 Cytokinin, a Positive Regulator of Nodulation

Hormones which possess a general role in plant organogenesis have also been shown to be important for nodule formation. Cytokinin, an adenine-derived signaling molecule, is a positive regulator of nodule organogenesis. The exogenous cytokinin

application to legume roots activates Nod-factor-like responses such as cortical cell division and early nodulin gene expression (Cooper and Long 1994; Fang and Hirsch 1998; Mathesius et al. 2000). With use of a cytokinin response gene from *Arabidopsis thaliana* cytokinin levels could be measured in transgenic *L. japonicus* root tissue during plant development and symbiotic interactions with rhizobia. The cytokinin response regulator was expressed in curled root hairs and nodule primordia after rhizobia inoculation (Lohar et al. 2004), indicating that cytokinins play a role in the legume–*Rhizobium* symbiosis. In support of this, suppression of the cytokinin receptor by RNA interference led to cytokinin-insensitive roots, which showed a strong reduction in nodule formation in *M. truncatula* (Gonzalez-Rizzo et al. 2006). A loss-of-function mutation in the cytokinin receptor gene of *L. japonicus*, *LHK1*, also dramatically reduced nodule formation (Murray et al. 2007). *lhk1* mutants showed a block in the activation of nodule primordia, but a hyperinfection phenotype with a large number of infection threads that lost directionality during their growth in the root cortex. Furthermore, a gain-of-function mutation in *LHK1* resulted in spontaneous nodule formation in the absence of rhizobia or Nod factor and hypersensitivity to exogenous cytokinin (Tirichine et al. 2007). These observations highlight the importance of cytokinins in nodule organogenesis, but also indicate that they are dispensable for bacterial infection in the epidermis.

9 Conclusions

Genetic and biochemical studies have defined many components of the Nod factor signaling pathway. It has become clear that calcium oscillations, one of the earliest measurable plant responses, play a central role in nodulation. Two receptor-like kinases with extracellular LysM domains and one receptor-like kinase with an extracellular LRR domain are essential for the activation of calcium oscillations. The necessity of three receptor-like kinases at the plasma membrane suggests that a phoshorylation cascade is involved in the initiation of Nod factor signaling. Two cation channel(s), nucleoporins, and phospholipase C/phospholipase D are also required for the induction of calcium spiking, but their exact functions remain to be defined. Although the requirement of calcium channels and calcium pumps for internal calcium stores is critical for calcium spiking, the proteins fulfilling these functions in Nod factor signaling have not yet been identified. A calcium/calmodulin-dependent protein kinase, CCaMK, which acts downstream of calcium oscillations is an obvious candidate for decoding the calcium signal. Its dual mode of calcium binding might enable the protein to decipher the information encoded in the amplitude and frequency of the calcium spikes. Recent advances indicate that the calcium signal is directly linked to nodulation gene expression through two GRAS domain proteins and a suite of ERF transcription factors acting downstream of CCaMK. Cytokinin is a key regulator of nodule organogenesis and changes in cytokinin levels are likely to be a target for Nod factor signaling.

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