Two Types of Calcium Signalling in Legume-Rhizobia Symbiosis

A thesis submitted to the University of East Anglia
for the degree of Doctor of Philosophy

Giulia Morieri

John Innes Centre

Norwich, September 2010

To my family

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Abstract

The legume-*Rhizobium* symbiosis results in the formation of nitrogen-fixing nodules. Bacterial infection occurs through the formation of a tunnel-like structure called an infection thread. The rhizobially-made Nod factor is essential for the establishment of this symbiosis. When Nod factor is added to legume root hair cells two calcium responses are observed: a rapid influx of calcium in the root hair tip and periodic calcium oscillations in and around the nuclear area. The role of these calcium responses in the infection of legume by rhizobial has been studied in this thesis. The function of the calcium flux was investigated in *Medicago truncatula*. Structurally-modified Nod factors produced by rhizobial mutants that are unable to infect roots successfully via infection threads, were impaired for induction of calcium flux. *M. truncatula* and *L. japonicus* infection mutants were also tested for the calcium flux response. Together the results point towards a key role for the Nod factor–induced calcium flux during infection thread initiation. In order to study whether root hair calcium spiking is essential for cortical nodule organogenesis, Nod factor-induced calcium spiking was tested in the *Lotus japonicus symrk14 har1-1* mutant. Inoculation of this mutant results in the formation of nodules that are not infected by mean of infection threads. The lack of infection thread and the huge reduction of calcium spiking in the *symrk14-har1-1* mutant shows a link between calcium spiking and bacterial infection. Finally, the role of the Lectin–nucleotide phosphohydrolase (LNP) in the Nod factor signalling pathway was investigated. The LNP antisense line blocked for nodule formation was found to be defective for Nod factor-induced calcium spiking, calcium flux and *NIN* gene expression, thus revealing a novel component of the Nod factor signalling pathway.

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LIST OF ABBREVIATIONS

Abbreviations	Definitions
2-APB	2-amino-ethoxydipheylborate
ABA	Abscisic acid
ADP	Adenosine 5’-diphosphate
AM	Arbuscular Mycorrhiza
amu	atomic mass unit
AVG	L-a-(2-aminoethoxyvinyl) glycine
ATP	Adenosine 5’-triphosphate
BHQ	2',5'-di(tert-butyl)-1,4-benzohydroquinone
BNM	Buffered nodulation medium
bv.	Biovar
cDNA	Complementary deoxyribonucleic acid
CaM	calmodulin
CCaMK	Calcium/calmodulin-dependent protein kinase
CCD	Charge-coupled device
CFP	Cyan fluorescent protein
cm	Centimetre
CPA	Cyclopiazonic acid
CT	Threshold cycle
dpi	Days post inoculation
DC	Direct current
Db	Dolicus biflorus
DWA	water agar medium
EPS	Exopolysaccharides
F1	First filial generation
F2	Second filial generation
Fix-	Non-nitrogen fixation minus
FP	Fahraeus Plant (medium)
FRET	Fluorescence resonance energy transfer
g	Gram
GFP	Green fluorescent protein
G-protein	Guanine nucleotide binding protein
GUS	β-glucuronidase
h	hour
HPLC	High Performance Liquid Chromatography
IP_3	inositol-1,4,5-triphosphate
IT	Infection thread
LCO	Lipo-chito-oligosaccharide

Abbreviations	Definitions
LNP	lectin nucleotide phosphohydrolase
LRR	Leucine rich repeat
Lj	*Lotus japonicus*
M	Molar
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
Mt	*Medicago truncatula*
mV	milliVolt
MW	Molecular weight
Myc-	Mycorrhization minus
nm	Nanometre
Nod-	Non-nodulating
NUP	Nucleoporin
PCR	Polymerase chain reaction
PA	phosphatidic acid
PLC	Phospholipase C
PLD	Phospholipase D
Ps	*Pisum sativum*
qPCR	Quantitative Polymerase chain reaction
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
RT-PCR	Reverse-transcriptase polymerase chain reaction
Sec	Seconds
SERCA	sarcoplasmic/endoplasmic reticulum calcium channel ATPases
spp.	species
SYM	Symbiosis
Sr	*Sesbania rostrata*
X-glcA	5-bromo-4-chloro-3-indolyl-beta-D-glucuronide sodium salt
YC	yellow cameleon
YFP	yellow fluorescent protein
WT	Wild type
µg	Microgram
µl	Microlitre
µM	MicroMolar
µm	Micrometre

PUBLICATIONS

During the course of this thesis the following manuscripts have been published (or are being prepared) based on data presented:

CHAPTER 3

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CHAPTER 4

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Yokota K, Fukai E, Madsen LH, Jurkiewicz A, Rueda P, Radutoiu S, Held M, Hossain MS, Szczyglowski K, **Morieri G**, Oldroyd GE, Downie JA, Nielsen MW, Rusek AM, Sato S, Tabata S, James EK, Oyaizu H, Sandal N, Stougaard J (2009). Rearrangement of actin cytoskeleton mediates invasion of *Lotus japonicus* roots by *Mesorhizobium loti*. Plant Cell 21: 267-284**.**

CHAPTER 5

Kosuta S[§], Held M, Hossain MS, **Morieri G**, MacGillivary A, Johansen, Antolín-Llovera M C, Parniske M, Oldroyd GED, Downie JA, Karas B, Szczyglowski K (in revision). Mutations in the conserved GDPC motif of *Lotus japonicus* SYMRK block constitutive ectodomaim cleavage and reveal a predominantly epidermal function of the receptor in symbiosis.

CHAPTER 6

Morieri G, Roberts NJ, Kalsi G, Rose A, Stiller J, Gresshoff PM, Oldroyd GED, Downie JA, Etzler ME (In revision). Rhizobial and mycorrhizal symbioses in *Lotus japonicus* require Lectin nucleotide phosphohydrolase, which acts upstream of calcium signaling.

CHAPTER 1

General introduction

Nitrogen Availability: a Global Problem

The growth of plants is limited by the availability of nutrients, including nitrogen, which is essential for the synthesis of amino and nucleic acids. Legumes are rich in protein and have long been known to increase soil fertility. Because many soils around the world are usually poor in nitrogen, early agricultural systems usually used co-cultivation or rotation of a legume with a cereal crop in order to restore soil fertility and provide a balance diet of protein and carbohydrate (e.g., bean/maize in the Americas, soybean/rice in Asia and lentil, chickpea/wheat, barley in Mesopotamia).

Nowadays, agriculture is highly reliant on industrial nitrogen fertilizer to maximize crop production by enhancing the amount of nitrogen in soils. It is estimated that at least one third of the food that is required to sustain the present world population depends on industrially produced nitrogen fertilizer (Smil, 1997), albeit that several environmental and economical problems are associated with the production and use of nitrogen fertilizer. Up to 50% of fossil fuel consumption in agriculture is used for production, the transport and application of nitrogen fertilizer. In addition, around 50% of the applied nitrogen is lost into the soil-aquatic systems (Mosier, 2004) and this is responsible for increasing plant and algal blooms that are now a global problem. The rising cost of fossil fuels is causing an increase of the cost of nitrogen fertilizers. This, together with the need for improved sustainability, is making alternative sources of nitrogen a future challenge.

Symbiotic Nitrogen Fixation

Despite its abundance in the atmosphere, atmospheric di-nitrogen is not accessible to plants. Nitrogen must first be fixed or combined with oxygen or hydrogen in

order to be used by plants. Some fixation occurs in lightning strikes, but biological nitrogen fixation is accomplished by bacteria. These bacteria contain the nitrogenase enzyme which combines gaseous nitrogen with hydrogen to release ammonia. In the course of evolution, legumes and some other plants have developed a symbiotic relationship with nitrogen-fixing bacteria in order to access nitrogen from the atmosphere. Nitrogen fixation in free-living bacteria is limited by availability of carbon and by the inhibition by oxygen of the nitrogenase enzyme. These symbioses result in the formation of specialized plant organs, called nodules. Inside the plant nodules, bacteria are provided with a perfect environment for nitrogen fixation and this is characterised by carefully regulated oxygen and carbon supply.

Nitrogen-fixing root nodules can be formed by two major plant-bacteria interactions: the *Rhizobium* and the *Frankia* symbioses. These two microsymbionts are considerably different (Pawlowski and Bisseling, 1996). *Frankia* are Gram positive filamentous and branching bacteria. They interact with eight different families of dicotyledonous plants, mostly tree and woody shrubs, collectively called actinorhizal plants (Pawlowski K, 2008). Rhizobia include eleven genera of unicellular Gram negative bacteria such as *Rhizobium, Sinorhizobium, Mesorhizobium* and *Bradyrhizobium*. They interact almost exclusively with legumes and one non-leguminous *Parasponia* sp. The legume family (Leguminosae), with more than 18 000 species and 650 genera, is the third largest angiosperm family (Soltis et al., 1995). It has three subfamilies, the Mimosoideae, Papilionoideae and the Caesalpinoideae. Many important crop species are legumes, examples are: soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), pea (*Pisum sativum)* and lentils (*Lens culinaris*). Phylogenetic analysis suggests that within the legumes, nodulation evolved independently at least on three separate occasions (Doyle, 1998). Despite the differences between *Rhizobium* and *Frankia* symbioses, phylogenetic analysis of these nodulating lineages revealed that they are closely related, indicating a common evolutionary basis of root nodulation symbioses (Soltis et al., 1995).

Certain cyanobacteria can enter specific interactions with members of the Gunneraceae. However, because the cyanobacteria colonize existing glandular tissue of

the plant without creating novel organs, this is usually not considered a real form of bacterial endosymbiosis (Gualtieri and Bisseling, 2000).

Brief Overview of Legume-*Rhizobium* Symbiosis

The legume–*Rhizobium* symbioses start with a molecular dialogue between legumes and rhizobia. Rhizobia perceive plant-made compounds known as flavonoids and this perception induces the bacteria to release lipochitooligosaccharide molecules called "Nod factors" (Spaink et al., 1991; Denarie et al., 1996; Long, 1996). Nod factors are perceived by the plant and act as signalling molecules triggering many of the plant responses necessary for the formation of a nitrogen fixing nodule. In the cortex, Nod factors activate cortical cell division forming a developing nodule, called a nodule primordium. At the same time, in the epidermis of many legumes, root hair cells deform, upon Nod factor perception, in such a way to entrap the bacteria within a curl, thereby forming an infection pocket. From the centre of the curl, bacterial infection starts through the formation of a tunnel-like structure called an infection thread. The infection thread crosses several cell layers until it reaches the cortex where it delivers the bacteria into the developing nodule. Rhizobia are, thus, released from the infection thread to inside the plant cell, where they differentiate into nitrogen-fixing forms, termed bacteroids. Bacteroids enclosed by a plant-derived membrane differentiate to form "symbiosomes", defined as the cellular structures capable of nitrogen fixation.

Early Steps of Legume-*Rhizobium* Symbiosis: a Molecular Communication

Flavonoids

The first step in the molecular dialogue between the plant and the bacteria is the detection by rhizobia of flavonoids and related molecules that are secreted from the legume roots. Several studies have shown in last decades that flavonoids are plant secondary metabolites playing a role in nodulation (Peters et al., 1986). Flavones and

isoflavones, a subclass of flavonoids, are diverse molecules, with a wide range of structures and function, and are synthesized via the phenylpropanoid pathway. Approximately 30 of them have been shown to induce *nod* genes in rhizobia (Hungria et al., 1992; Smit et al., 1992; Begum et al., 2001; Cooper, 2007).

The rhizobial regulatory protein NodD recognises and interacts with the appropriate flavonoids and then induces the transcription of *nod* genes required for Nod factor synthesis (Fisher and Long, 1993). NodD proteins bind to the promoter elements of *nod* genes to trigger their transcription. However, it has long been discussed whether flavonoids form a complex with NodD on the DNA or bind to NodD promoting its interaction with DNA. There is no direct biochemical evidence for flavonoid binding to NodD although it has been observed that flavonoids stimulate an increase in DNA binding of *S. meliloti* NodD1 to *nod* gene promoters (Peck et al., 2006).

In addition to the activation of *nod* gene expression, flavonoids also play a pivotal role concentrating rhizobia at the root surface by inducing strong chemotactic and growth responses (Rolfe and Gresshoff, 1988; Stougaard, 2000). There is increasing evidence that flavonoids contribute to nodulation once the bacteria have entered the plant root and exogenous flavonoids are no longer available (Subramanian et al., 2007). *M. truncatula* and soybean plants suppressed for endogenous flavonoid production abolishes nodulation (Subramanian et al., 2006; Wasson et al., 2006; Zhang et al., 2009). Later in nodule development, flavonoids also regulate auxin transport during the initiation of the nodule primordium (Wasson et al., 2006; Zhang et al., 2009). Indeed, recent work has shown that flavonoid-deficient roots only nodulated when supplemented with two flavonoids: a *nod* gene inducer and an auxin transport inhibitor (Zhang et al., 2009). Hence, flavonoids can play multiple roles at different stages of nodulation during the legume–*Rhizobium* symbiosis with a key function in the induction of Nod factor signal production.

Nod Factor

Nod factor is a highly potent bacterially-derived signalling molecule that can be active at picomolar concentrations (Long, 2001). The structure of Nod factor is characterized by a backbone of four or five β-1,4-linked *N*-acetylglucosamine residues

with an *N*-linked fatty acyl chain attached the non-reducing end (Figure 1.1A) (Lerouge et al., 1990; Denarie et al., 1996; Downie and Walker, 1999). Modifications of this basic structure determine which legume species will be nodulated by a given rhizobial strain (Lopez-Lara et al., 1996; Corvera et al., 1999; Pacios Bras et al., 2000; Perret et al., 2000; Rodpothong et al., 2009). Hence, for example, the length and saturation of the acyl chain is different in the Nod factors made by *Sinorhizobium meliloti* (which nodulates alfalfa) compared from those of *Rhizobium leguminosarum* bv. v*iciae* (which nodulates pea and vetch). *S. meliloti* Nod factors carriy an O-linked sulphate group at the reducing end, whereas *R. leguminosarum* bv. v*iciae* releases a mixture of four Nod factors in which a tetrameric or pentameric sugar backbone carries a $C_{18:1}$ or $C_{18:4}$ N-linked acyl group and an O-acetyl group on the acylated sugar (Spaink et al., 1991). Nod factor released by *Mesorhizobium loti* (which nodulates *Lotus* spp.) carries a lipid C_{18} chain, a methyl and carbamoyl residue at the reducing terminal, and an acetylated fucose at the non-reducing terminal of pentamer of *N*-acetyl-glucosamine (Figure 1.1B). It is this diversity in Nod factor structure that largely defines specificity between symbiotic partners (Perret et al., 2000).

Nod factor production is dependent on functions of the rhizobial *nod* genes. The *nodABC* genes are essential for the production of the Nod factor backbone (the N-acylated chitin) (Figure 1.1A) but are not involved in defining the bacterial host range as they can be functionally exchanged between rhizobial strains (Denarie et al., 1996). In the *Medicago* symbiont, *S. meliloti* , *nodE*, *nodF*, *nodL*, *nodH*, *nodP*, and *nodQ* are host-specific *nod* genes. The *O*-sulphate group at the reducing glucosamine residue require the combined function of *nodH*, *nodP*, and *nodQ* (Figure 1.1) and is essential in determining the specificity between *S. meliloti* and *Medicago* hosts. (Schwedock and Long, 1990; Roche et al., 1991). The absence of the host-specific *nod* genes can modify the host range of *S. meliloti* (Denarie et al., 1996). For example, *nodH* mutants, which produce the Nod factor lacking the *O*-sulfate group, lose their ability to nodulate *Medicago sativa*, but become able to nodulate *Vicia sativa* (Faucher et al., 1989). *S. meliloti nodE* and *nodF* determine the attachment of the appropriate $C_{16:2}$ *N* –acyl group, while *nodL* is required for the *O*-acetyl modification (Figure 1.1) (Demont et al., 1993; Bloemberg et al., 1994). Mutations in these genes delayed and reduced nodulation of *Medicago* spp. by *S.*

meliloti, but the mutants still elicited morphological responses associated with nodulation (Debelle et al., 1986; Swanson et al., 1987; Ardourel et al., 1994). *nodF*/*nodL* double mutants exhibited a more severe phenotype: they are not capable of infecting and penetrating into the legume hosts (Ardourel et al., 1994). Nevertheless, these mutants were still able to induce cortical cell division, revealing that the Nod-factor structural requirements are more stringent for bacterial entry than for the induction of the nodule primordia (Ardourel et al., 1994). Infection threads are normally preceded by the formation of cytoplasmic bridges traversing the central vacuole in outer cortical cells, called pre-infection threads. Van Brussel et al. (1992) and Spaink et al. (1991), showed that in vetch both pre-infection-threads and nodule cell division required $C_{18:4}$ Nod factors. Purified $C_{18:1}$ Nod factors, normally released by *R. leguminosarum* bv. *viciae nodE* mutant, did not induce pre-infection-threads or nodule cell division (Spaink et al., 1991; van Brussel et al., 1992), suggesting that pre-infection-thread formation requires high Nod factor structure specificity as observed for bacterial infection. In *M. loti* the acetylfucose decoration of the Nod factor was found to play a significant role during infection thread initiation and bacterial entry into the root hair cells but dispensable for root hair curling and cortical cell division (Rodpothong et al., 2009). These findings, together with other investigations, uncoupled the earlier Nod factor responses from bacterial infection. Thus, before the Nod factor receptors were actually been cloned, the possibility was raised of two specificities for Nod factor recognition: a low-stringency recognition would be required for earlier stages of the symbiosis, while a high-stringency recognition would be necessary for bacterial infection (Spaink et al., 1991; Firmin et al., 1993; Ardourel et al., 1994; Geurts et al., 1997; Walker and Downie, 2000; Oldroyd and Downie, 2004). The question that still remains open is whether these two phases of Nod factor recognition would be accomplished by two different receptors: a signalling and an entry receptor as defined by Ardurel and associates (1994), or by a single-receptor complex able to induce two or more output responses depending on the structure of the binding ligand (Oldroyd et al., 2001a).

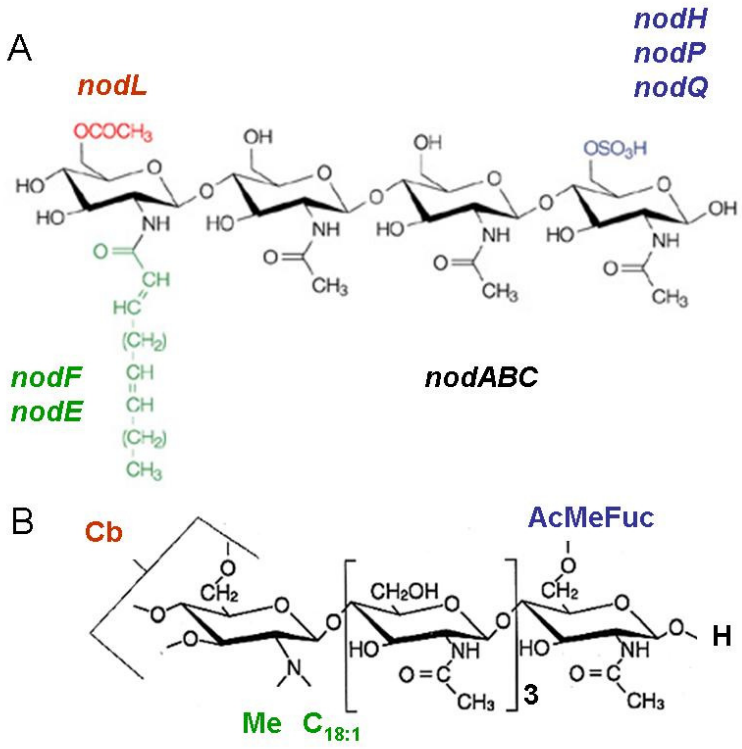


Figure 1.1. Structure of Nod factors from *Sinorhizobium meliloti* (A) and *Mesorhizobium loti* (B).

The Nod factor backbone of β-1-4-linked *N*-acetylglucosamine residues carrying a linked *N*-acyl group is common for all rhizobial strains and requires *nodABC* genes, while the number and types of substituent groups can vary. (A) Nod factor from *S. meliloti* (which nodulates *Medicago* spp.) 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 requires *nodL.* (B) The Nod factor released by *Mesorhizobium loti* (which nodulates *Lotus* spp.) is a pentamer of *N*-acetyl-glucosamin containing a lipid C_{18} chain, a methyl and carbamoyl residue (Cb) at the reducing terminal, and an acetylated fucose (AcMeFuc) at the non-reducing terminal of end (modified from Oldroyd and Downie, 2004).

Nod Factor Recognition in the Plant

Several plant processes associated with nodulation depend on plant perception of Nod factors released from rhizobia. Nod factors can induce responses in the host plant at concentrations as low as picomolar, suggesting that plants must have high-affinity Nod factor receptors. With the use of genetic approaches several genes involved in the induction of these plant responses induced by Nod factor have been identified.

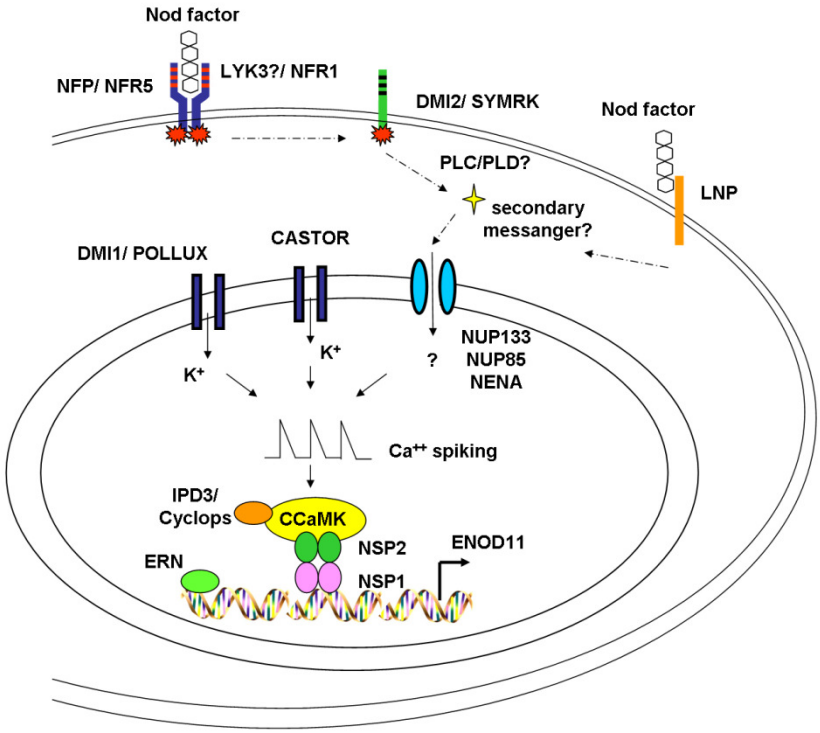


Figure 1.2. Model of Nod factor signalling in root hair cell.

Nod factor is thought to be perceived by two receptor-like kinase *Mt*NFP/*Lj*NFR5 and *Mt*LYK3?/ *Lj*NFR1 formed by extracellular LysM domain and intracellular kinase domain. A second receptor-like kinase found to be involved in Nod factor signalling is *Mt*DMI2/*Lj*SYMRK containing leu-rich repeat regions. Nod factor has been also shown to bind LNP. The signal generated at the plasma membrane is then transduced to the nucleus through an unidentified secondary messenger probably produced by phospholipase C (PLC) and Phospholipase D (PLD). In the nuclear envelope, cation channel *Mt*DMI1/*Lj*POLLUX, *Lj*CASTOR and the nucleoporins *Lj*NUP133, *Lj*NUP85 and NENA are all required for the induction of nuclear calcium spiking. CCaMK, NSP1, NSP2, *Mt*IPD3/*Lj*CYCLOPS and ERN are proposed to decode and transduce the calcium signal into expression of gene required for nodulation (e.g. ENOD11).

Receptor-Like Kinases

Mutant screens with *L. japonicus, M. truncatula*, and *P. sativum* have identified legume mutants lacking all Nod-factor-induced plant responses due to mutations in the receptors responsible for the perception of Nod factor. In *L. japonicus* two genes, *NFR1* and *NFR5*, encoding transmembrane receptor-like kinases with an intracellular serine/threonine protein kinase domain, were found (Figure 1.2) (Madsen et al., 2003; Radutoiu et al., 2003). Both receptor-like kinases contain two or three extracellular lysin motifs (LysM) in the extracellular domains, which are predicted to bind peptidoglycans (Bateman and Bycroft, 2000). Although no direct binding of Nod factor to NFR1 or NFR5 has been reported several lines of evidence suggest that NFR1 and NFR5 are involved in Nod factor perception. First, it is known that the LysM binds to the peptidoglycan *N*-acetylglucosamine- *N*-acetylmureine backbone which is chemically similar to the *N*-acetylglucosamine Nod factor backbone (Steen et al., 2003). Second, it has been demonstrated that NFR1 and NFR5 generate specificity in Nod factor recognition (Radutoiu et al., 2007), because *M. truncatula* and *Lotus filicaulis* expressing *Lj*NFR1 and *Lj*NFR5 gain the ability to be nodulated by *L. japonicus*-specific *M. loti*. Third, domain swaps and amino acid substitutions highlighted the essential role of the LysM domains, particularly the LysM2 domain of NFR5, in determining Nod factor specificity (Radutoiu et al., 2007). The topology of these receptors and the lack of the kinase activation loop of NFR5 suggested that the activation of NFR5 kinase is achieved by forming a complex with NFR1 where NFR1 would supply the kinase activation domain and trigger signalling events (Schenk and Snaar-Jagalska, 1999; Huse and Kuriyan, 2002; Madsen et al., 2003; Radutoiu et al., 2003). Recent work has shown that the presence of the Nod factor receptors NFR1 and NFR5 is not sufficient to determine the infection phenotype of *Lotus* spp. Addition of *LjNFR1* and *LjNFR5* to *L. pedunculatus* roots inoculated with *M. loti* did not restore the infection phenotype. In addition, chimeric receptors containing the extracellular regions of *L. pedunculatus* NFR1 and NFR5 and the corresponding *L. japonicus* kinase domain did not complement the *L. japonicus nfr1-1* or *nfr5-2* mutants or the double mutant *nfr1-1nfr5-2*, suggesting that additional signals independent of Nod factor may be required for the initiation of infection threads (Bek et al., 2010). Recently, the LysM receptor kinase

gene family containing 17 members have been identified in *L. japonicus* (Lohmann et al., 2010).

Similarly, mutational screens in *M. truncatula* and *P. sativum* identified the *LjNFR5* orthologues *MtNFP* and *PsSYM10*, respectively (Walker et al., 2000; Amor et al., 2003; Madsen et al., 2003; Arrighi et al., 2006). As observed in the *Ljnfr5* mutant, the *M. truncatula* mutants *nfp* and the *P. sativum sym10* are completely deficient for all Nod factor-induced responses. In *M. truncatula* there are several homologues of *LjNFR1:* nine new families of LysM-Receptor-like-kinases have been identified revealing at least 17 genes. Most of these are expressed in roots or nodules, suggesting that several of these receptor may be involved in nodulation (Arrighi et al., 2006). Mutations or RNA interference of two of these, *LYK3* and *LYK4,* led to specific defects in *S. meliloti* infection, though the induction of earlier Nod factor responses was unaffected (Limpens et al., 2003; Smit et al., 2007). Work on a weak *lyk3* allele has showed that the recognition of the *O*-acetyl modification of the *S. meliloti* Nod factor is probably mediated by LYK3 (Smit et al., 2007) (Figure 1.2) This evidence is consistent with the previously proposed two-receptor model (Ardourel et al., 1994) in which a low-stringency receptor (*NFP*) induces early Nod factor responses, while bacterial infection requires a high-stringency Nod factor receptor (*LYK3*).

Lectin Nucleotide Phosphohydrolase

Despite several pieces of evidence implicating receptor-like kinases as candidates for Nod Factor perception, little information is available on possible alternative or additional candidates for Nod factor perception. A lectin, isolated originally from roots of the legume *Dolichos biflorus* (Quinn and Etzler, 1987), was found to catalyse the hydrolysis of nucleoside diphosphates and triphosphates (Etzler et al., 1999) and was therefore named “lectin nucleotide phosphohydrolase” (LNP). Using a competitive binding assay, Etzler et al. (1999) showed that LNP binds to Nod factors produced by the rhizobial strain that nodulates *Dolichos biflorus* and that Nod factor binding induced its phosphohydrolase activity in vitro. The observation that antiserum to LNP of *D. biflorus* and *Glycine max* inhibited both root hair deformation and nodule formation revealed that this protein plays a role in nodulation (Etzler et al., 1999; Day et al., 2000). Corroborating

these finding, it was shown that rhizobia induced the expression of an LNP gene in *G. max*, and that the overexpression of LNP enhances nodulation (Day et al., 2000; McAlvin and Stacey, 2005). Recent work has shown that silencing LNP in *Glycine soya* reduced nodulation, and more interestingly that the addition of ADP to silenced roots restored the wild type phenotype (Govindarajulu et al., 2009). Although these data highlight the importance of LNP in nodulation, the role for LNP as a Nod factor receptor remains controversial.

Early Plant Responses to Nod Factors

Purified Nod factors can elicit many of the early responses associated with nodulation (Oldroyd and Downie, 2004), such as membrane depolarization, pH change, calcium influx, calcium spiking and root hair deformation, indicating that Nod factor alone is sufficient for the activation of several events necessary for successful nodulation.

Membrane Depolarization and pH Change

One of the first responses elicited by Nod factors is a rapid and transient depolarization across the plasma membrane of root hair cells and it is associated with a transient extracellular, and sustained intracellular, alkalinization (Ehrhardt et al., 1992; Kurkdjian, 1995; Felle et al., 1996). Voltage clamp studies have shown that *S. meliloti* cell-free filtrates, and nanomolar concentrations of purified Nod factor, triggered a transient depolarization of the membrane potential of 10 to 15 mV in alfalfa root hair cells. This membrane depolarization started approximately 30 to 60 seconds after Nod factor addition, reaching a maximum within 10 min (Ehrhardt et al., 1992). This work also showed that Nod-factor-induced membrane depolarization is specific, as it was not observed in root hairs of the non-legume tomato, and occured in a dose-dependent manner reaching its maximun effect between 10^{-8} and 10^{-7} M Nod factor (Ehrhardt et al., 1992). Kurkdjian et al, (1995) showed that the repolarisation of the plasma membrane involves two steps. During the first fast phase which occurs 30 to 60 seconds after the addition of Nod factor, the membrane potential recovers to about one-half the value of the total depolarization and remains stable for a few minutes. Unlike the first phase, the

second phase is slower lasting about 15 minutes (Kurkdjian, 1995). With improved instrument sensitivity, Felle et al, (1996) reported that the depolarization response was induced only 15 seconds after Nod factor application (Felle et al., 1996). Given that unsulphated Nod molecules and synthetic *N,N′,N″,N‴*-tetraacetylchitotetraose were unable to induce depolarization and root hair deformation, it was found that Nod factor specificity is required for these responses (Kurkdjian, 1995).

Nod factors (10 nM) induced a rapid (within 15 seconds) cytoplasmic alkalinisation of 0.2 to 0.3 pH units in alfalfa root hair cells altering intracellular and extracellular pH (Felle et al., 1996). Curiously, non-sulphated Nod factor, which is unable to induce root hair deformation (Schultze et al., 1992) and membrane depolarization (Felle et al., 1996), could trigger the cytoplasmic alkalinisation as well as the sulphated Nod factors, suggesting independent Nod factor perception systems for the induction of these responses (Felle et al., 1996).

Calcium Responses in Nod factor Signalling

Calcium Signalling in Plant Cells

In eukaryotes, calcium represents one of the most ubiquitous secondary messengers involved in signal transduction. Calcium regulates several cellular activities linking a range of external stimuli to their physiological responses. In plants, many abiotic and biotic external stimuli such as depolarization of root hair-transmembrane potential, touch, cold shock, red light, pathogen perception, treatment of some cells with auxin, cytokines, gibberelic acid and abscisic acid have been shown to induce increases of free cytoplasmic calcium (Sanders et al., 2002). Calcium current (net calcium flux) is required to maintain the growth of expanding cells, such as root hair cells, cells in the elongation zone (White, 1998) and elongation of pollen tubes (Franklin-Tong et al., 1993; Malho et al., 1994; HoldawayClarke et al., 1997). Calcium oscillations have been observed in stomatal guard cells (McAinsh et al., 1995; Allen et al., 2001). When the roots begin to sense a water shortage in the soil, abscisic acid (ABA) is released. ABA binds to receptors in the guard cells plasma membranes, which causes the concentration of free calcium to increase in the cytosol due to influx from outside the cell, and release of calcium from

internal stores such as the endoplasmic reticulum and vacuoles (Kim et al., 2010). ABA-induced calcium transients result in stomatal closure, preventing water loss.

Calcium has also an essential function in Nod factor signalling (Figure 1.2). Addition of Nod factors to root hair cells triggers two different calcium responses: a rapid influx of calcium ions and cytosolic calcium oscillations around the nucleus.

Ion Fluxes

Within one minute of Nod factor addition Felle et al. (1999) observed a rapid calcium influx followed by the efflux of chloride, an efflux of potassium ions and an alkalinisation of the cytoplasm in alfalfa root hairs. The use of stationary ion-selective extracellular electrodes revealed that the chloride efflux was induced in response to elevated cytosolic calcium and was responsible for the membrane depolarization (Kurkdjian, 1995; Felle et al., 1998). Corroborating this observation, pharmacological studies demonstrated that the increase in cytoplasmic calcium is necessary for chloride efflux and membrane depolarization (Felle et al., 1999a). It was also suggested that potassium might operate as a charge balance, in order to stop the depolarization and start a repolarization though a H^+ pump, and that a charge balance could be provided by potassium efflux and re-polarization (Felle et al., 1998).

Rapid increases in cytosolic calcium concentrations have also been observed using calcium-sensitive dyes. Application of 1 nM Nod factor to root segments loaded with the calcium indicators Fura-2 or Fluo-3 induced, within seconds, an increases in intracellular free calcium concentration (Gehring et al., 1997). In *Vicia sativa* root hairs, an apical calcium rise of 6- to 10-fold was measured using acid loading of Indo-1 (de Ruijter et al., 1998). In *Phaseolus vulgaris* root hairs microinjected with dextran-linked Fura-2, a rapid change in cytoplasmic calcium levels from 400 nM to 1500 nM was induced in the apical region, within 5 to 10 minutes after the addition of Nod factors (Cardenas et al., 1999). With the use of an ion-selective intracellular electrode, rapid calcium increase have also been observed in *Medicago sativa* root hairs one to two minutes after the application of Nod factors from *S. meliloti* (Felle et al., 1999a). In *M. sativa,* growing root hairs showed a steep calcium gradient going from approximately 170 nM at the basal region to around 790 nM at tip. Addition of Nod factor decreased cytoplasmic calcium within 10 µm of the

tip and increased calcium behind the tip within 5 minutes in growing root hairs, whereas in non-growing root hairs without a tip-to-base cytosolic calcium gradient, cytoplasmic calcium increased at the root tip as well as at the root hair base (Felle et al., 1999b). The partial dissipation of the calcium gradient observed in growing root hairs was a specific reaction to Nod factors as it was not observed when cells were treated with chitotetraose. It was suggested that response to Nod factors depends on the development stage of the root hairs and that changes in cytosolic calcium could have different functions in Nod-factor signalling: changes of cytosolic calcium concentration in the tip of the root hair could be associated to root hair deformation, whereas the increase in calcium behind the tip might be required for the amplification of the Nod signal to induce downstream events (Felle et al., 1999b).

In root hairs of pea microinjected with Oregon Green, treatment with 10 nM Nod factor from *R. leguminosarum.* bv *viciae* induced an increase in cytoplasmic calcium levels, whereas, chitin oligomers of four or five N-acetylglucosamine residues were inactive even when added at 1 µM (Walker et al., 2000). A different result was obtained in *Glycine max* where addition of both Nod factor and chitooligomers at nanomolar concentration elicted an increase of cytosolic calcium (Muller et al., 2000). These contrasting results could be explained by two major limitations presented in the latter work: first, the investigation was performed on suspension-cultured soybean cells rather than living root hair cells tested with microinjection analysis; second, the calcium imaging system used was based on aequorin, a photoprotein binding calcium which does not allow the detection of calcium changes at the single cell level.

In *M. truncatula,* microinjection of root hair cells with two dyes (Oregon Green, calcium sensitive and Texas Red, calcium insensitive dye) revealed that a Nod factor-elicited calcium flux was located in the tip of root hair and had a biphasic characteristic consisting of a rapid and immediate elevation in cytoplasmic calcium concentration followed by slow decrease in calcium levels. Nod factor analogues, such as sulphated chitotetraose and unsulfated $NodH^-$ Non factor, failed to induce this calcium response. (Shaw and Long, 2003). Calcium flux was also observed in *L. japonicus* root hair cells after addition of 10 nM Nod factor form *M. loti*. The characteristic of this calcium flux in terms of shape and lag phase were found to be variable from cell to cell (Miwa et al., 2006b).

Calcium Spiking

In addition to the calcium flux response, Nod factors trigger oscillations in cytosolic calcium concentrations around the nucleus, termed "calcium spiking" (Figure 1.2). Calcium spiking was originally observed in *M. sativa* root hair cells by Ehrhardt et al., in 1996. These regular oscillations, with a mean period of 60 seconds were induced approximately ten minutes after the root hairs were exposed to Nod factor and lasted up to three hours (Ehrhardt et al., 1996). The observation that a nodulation mutant and non-legumes (tomato) lacked this response suggested that calcium spiking provides a specific signal required for Nod factor signal transduction. Furthermore, chitin oligomers and an unsulphated Nod factor carrying a $C_{18:4}$ fatty acyl chain produced by *R. leguminosarum* failed to induce calcium spiking in alfalfa, suggesting that structural features of the signalling molecules are required to cause calcium oscillations. It was also shown that the calcium elevation was associated with the nuclear area, suggesting that the calcium stores or the channels that mediate calcium release are localized in this region (Ehrhardt et al., 1996). Ratiometric measurements with Fura-2 allowed a more accurate measurement of calcium levels and showed that calcium spiking in the nuclear area consisted of increases of approximately 500 nM calcium (Ehrhardt et al., 1996).

The finding that calcium oscillations are involved in nodulation in alfalfa has spurred, in the last decade, many laboratories to further investigate this response in several legumes. A variety of reports showed that calcium spiking occurs in *P. vulgaris* (Cardenas et al., 1999), pea (Walker et al., 2000), *M. sativa* (Ehrhardt et al., 1996), *M. truncatula* (Wais et al., 2000; Miwa et al., 2006a; Sieberer et al., 2009), *L. japonicus* (Harris et al., 2003; Miwa et al., 2006b) and *S. rostrata* (Capoen et al., 2009) revealing that calcium spiking is common in legumes. In *P. vulgaris*, the repeated cytosolic calcium fluctuations were detected in the perinuclear region after 10-15 min exposure with Nod factors by using fluorescent dye with laser scanning confocal microscopy (Cardenas et al., 1999). In contrast to alfalfa and *M. truncatula* (Shaw and Long, 2003), studies on calcium spiking in pea revealed that chitin oligomers were capable of inducing calcium spiking in wild type but not in nodulation mutant (Walker et al., 2000). The observation that chitin oligomers can induce calcium spiking but fail to trigger root hair deformation (Walker et al., 2000) suggests that these two responses are activated by independent mechanisms.

In addition, it was shown that addition of *S. meliloti* but not *R. leguminosarum,* to *M. truncatula* root hair cells, caused similar calcium spiking to that induced by Nod factor (Wais et al., 2002). Interestingly, when *S. meliloti nod* genes were introduced in *R. leguminosarum* or even in *E. coli*, calcium spiking could be restored (Wais et al., 2002).

Similarly to animal cells (Meyer and Stryer, 1988), a typical "spike" of the Nod-factor-induced calcium oscillation is characterised by a rapid (1-4 seconds) increase in cytosolic calcium, and is followed immediately by a more gradual decline (approximately 30 seconds (Ehrhardt et al., 1996). The first part implicates the opening of calcium channels, while the second phase requires the activity of a calcium ATPase or calcium antiporter to control the movement of calcium against the electrochemical gradient, from the cytoplasm into either an internal store or extracellular space. Once initiated, spikes occur repeatedly for up to three hours with an average frequency of 60-100 seconds, depending on the position of the root hair cells along the root (Miwa et al., 2006a). With the use of a nucleoplasmin-tagged cameleon, recent work has demonstrated that calcium spiking occurs within the nuclear compartment (Sieberer et al., 2009). The initial rapid increase of calcium concentration observed in each spike was associated with the nuclear envelope(Sieberer et al., 2009), consistently with the nuclear envelope location of the cation channels and nucleoporins required for the initiation of calcium spiking (Kanamori et al., 2006; Charpentier et al., 2008; Groth et al., 2010).

Unlike the Nod-factor-induced calcium flux, calcium spiking starts with a lag of approximately 10-20 minutes after Nod factor application. The reason for this lag between Nod factor addition and calcium spiking induction is still an unresolved question. Interestingly, while high concentrations (> 10^{-9} M) of Nod factors induce calcium flux followed by calcium spiking, low concentrations (< 10^{-10} M) trigger calcium spiking but not calcium influx (Shaw and Long, 2003). Furthermore, Nod factor-like chitin oligomers can activate calcium spiking without the induction of the calcium flux (Walker et al., 2000). In *M. truncatula*, Nod factor lacking the reducing end sulphate group, purified from bacteria lacking the *nodH* gene, induced calcium spiking at 10 nM, but failed to induce the calcium flux response (Wais et al., 2002; Shaw and Long, 2003). Thus, in both *P. sativum* and *M. truncatula*, Nod factor-like molecules induce calcium spiking without the induction of calcium flux (Walker et al., 2000; Shaw and Long, 2003). This evidence suggests that while

calcium spiking requires a low stringency of Nod factor perception, a high concentration of specific Nod factor structure is required to elicit the calcium influx. This is consistent with the two receptor model proposed on the bais of genetic analysis in which a low stringency receptor would be required for early Nod factor response while a second receptor, with higher structural stringency, would be involved in the initiation of bacterial infection. Indeed, a model has been proposed in which calcium spiking and other early Nod factor responses, such as root hair deformation, are induced by low Nod factor concentrations released during the first contact between the legume root and rhizobia, whereas calcium flux would be generated by high Nod factor concentrations found in the root hair curl where the rhizobia are trapped and start replicating. The calcium flux induced by such high Nod factor concentrations would then drive the formation of infection threads (Miwa et al., 2006b). Although addition of Nod factor to root hair cells induces calcium flux followed by calcium spiking, this model predicts that calcium flux occurs later than the calcium spiking response. Indeed, it has been shown calcium ion influx can be induced in root hair cells that are already spiking (Walker et al., 2000; Shaw and Long, 2003).

In support for such a model several lines of evidence showed that calcium flux and calcium spiking are two separable responses. The spatial distribution of calcium flux and calcium spiking is different: while calcium spikes originate in the nuclear area and propagate as a wave outward, calcium flux begins at the cell tip and moves towards the nucleus (Ehrhardt et al., 1996; Cardenas et al., 1999; Walker et al., 2000; Shaw and Long, 2003). Additional evidence showing that calcium influx and calcium spiking are separate responses come from genetic analysis. Mutation of the receptor-like kinase gene *DMI2*/*SYMRK/SYM19*, in the cation channel genes *DMI1*/*POLLUX/SYM8, CASTOR* and nucleoporin genes *NUP85* and *NUP133* block calcium spiking but not calcium flux (Walker et al., 2000; Shaw and Long, 2003; Kanamori et al., 2006; Miwa et al., 2006b; Saito et al., 2007). These observations indicate that calcium influx and calcium spiking are independent of one other and, thus, may be involved in activating different (but possibly overlapping) responses.

In order to identify the molecular components involved in the Nod factor calcium signalling pathway, various inhibitors which are known to block calcium signalling in other

eukaryotes have been tested for the effects on nodulation calcium signalling. Calcium ions can enter into the cytoplasm either from outside the cell through the cell membrane via calcium channels, or from some internal calcium stores. To identify the calcium channel and calcium pumps causing calcium spiking, several inhibitors have been tested for their effect on calcium spiking. These studies revealed that Nod-factor-induced calcium spiking is inhibited by 2-amino-ethoxydipheylborate (2-APB), an inhibitor of both IP3-mediated and store depletion-mediated calcium release; by caffeine, an inhibitor of IP3-receptor calcium channels and an agonist of ryanodine receptor calcium channels; by Cyclopiazonic acid (CPA), an inhibitor of type IIA calcium ATPases in plants; by 2,5-Di-t-butyl-1,4-benzohydroquinone (BHQ), an inhibitor of mammalian sarcoplasmic/endoplasmic reticulum calcium channel ATPases (SERCA); by the phospholipase C inhibitor U-73122 and by the phospholipase D inhibitor n-butanol (Miwa Thesis 2006, unpublished data, (Engstrom et al., 2002)). Interestingly, pharmacological antagonists that interfere with intracellular ion channel and calcium pump such as 2-APB, CPA and TMB-8, are also efficient blockers of Nod factor-elicited *ENOD11* gene expression (Charron et al., 2004). Moreover, the addition of the calcium chelator EGTA and the calcium channel blocker La^{3+}, completely inhibited *MtENOD12* induction by Nod factor (Pingret et al., 1998). These observations further demonstrate that Nod-factor-induced calcium spiking is required for the activation of early nodulin genes.

One of the advantages of calcium oscillations is the availability of both amplitude and frequency, which could provide the opportunity for multiple signals to be generated by calcium in the same cells. Indeed, it has been shown that the nature of the calcium oscillations encodes information that can define the outcome of the downstream response (Dolmetsch et al., 1998; Allen et al., 2001; Evans et al., 2001; Oldroyd and Downie, 2006). Recently, different calcium spiking signatures have been shown to correlate with different modes of bacterial infection. In *S. rostrata* root infection by rhizobia can occur intercellularly at cracks caused by lateral root emergence. At lateral root bases cells Nod factor trigger faster and more symmetrical calcium oscillation compared to those induced during root hair invasion. However, when levels of jasmonic acid were raised or when ethylene was decreased calcium spiking slowed down in frequency. Interestingly, these different calcium oscillations were associated with an

activation of intracellular root hair invasion by rhizobia, indicating that intracellular invasion of root hairs is linked with a very specific calcium signature (Capoen et al., 2009).

Genes Involved in the Activation of Calcium Spiking

Calcium spiking plays an essential role in the Nod-factor-induced signalling pathway and it is likely to act as a secondary messenger to transduce the Nod factor signal. Mutant screens have identified a diversity of components necessary for the induction of calcium oscillation. In addition to the LysM receptor-like kinases (see above), a leucine-rich repeat (LRR) receptor-like kinase (Endre et al., 2002; Stracke et al., 2002), two cation channels (Ane et al., 2004; Imaizumi-Anraku et al., 2005; Edwards et al., 2007; Riely et al., 2007; Charpentier et al., 2008), and three nucleoporins (Kanamori et al., 2006; Saito et al., 2007; Groth et al., 2010) are required for the activation of calcium spiking (Wais et al., 2000) (Figure 1.2). Furthermore, biochemical and pharmacological investigations revealed that phospholipid signalling pathways are linked to Nod factor signalling (den Hartog et al., 2001; Engstrom et al., 2002; Charron et al., 2004; Sun et al., 2007).

LRR Receptor-Like Kinase

In addition to the LysM receptor-like kinases, a second type of receptor-like kinase containing three LRR motifs in the extracellular domain, a transmembrane domain and an intracellular protein kinase, is involved in the Nod factor signal transduction pathway (Figure 1.2) (Endre et al., 2002; Stracke et al., 2002). The relevance of this LRR receptor-like kinase in nodulation has been reported in *M. sativa* (*MsNORK*), *P. sativum* (*PsSym19*), *M. truncatula* (*DMI2*), *L. japonicus* (*LjSymRK*) and *S. rostrata* (*SrSymRK*) (Endre et al., 2002; Stracke et al., 2002; Capoen et al., 2005). Moreover, the orthologues of this receptor-like kinase *CgSymRK* and *DgSymRK* have been shown to be essential for nodulation in the actinorhizal plants *Casuarina glauca* and *Datisca glomerata*, suggesting that genetic mechanisms are conserved between the two types of root nodule symbiosis (Gherbi et al., 2008; Markmann et al., 2008). This gene is essential for nodulation and very highly-conserved among legumes, though the function of the protein is not yet fully understood. LRR have been shown to mediate specific protein-protein interaction (Jones

and Jones, 1997) and it has been proposed that SYMRK might interact with an unidentified extracellular protein and mediate the phosphorylation of some components yet to be identified. Indeed, it has been shown that in DMI2, the *M. truncatula* orthologue of SYMRK, interacts with HMGR1, a key enzyme in synthesis of mevalonate and that the interaction requires the cytosolic active domain of DMI2 (Kevei et al., 2007).

LRR receptor-like kinase is not thought to be a Nod-factor receptor because *SYMRK* mutants show some responses to Nod factor, including root hair deformation (Stracke et al., 2002). Unlike the LysM receptor-like kinases, which are known to be required for all Nod-factor-induced responses, this LRR receptor-like kinase is necessary for the activation of calcium spiking and early nodulin gene expression, but is not essential for the induction of calcium flux, suggesting a bifurcation in the Nod factor signalling pathway immediately downstream of the LysM receptor kinases, with one branch leading to calcium spiking and one branch leading to the calcium flux (Catoira et al., 2000; Wais et al., 2000; Shaw and Long, 2003; Miwa et al., 2006b). Furthermore, partial suppression of *DMI2* and *SrSymRK* using RNA interference demonstrated a function for these genes at later stages of the symbiosis, during bacterial release and symbiosome formation (Limpens et al., 2003; Capoen et al., 2005). Corroborating these findings, the receptor-like kinase is expressed in roots prior to infection and is expressed in the preinfection zone of *M. truncatula* nodules (Limpens et al., 2003; Bersoult et al., 2005).

Ion Channels Required for Calcium Spiking

Characterisation of the *M. truncatula* mutant *dmi1*, defective for calcium spiking, expression of nodulin genes and cortical cell division (Catoira et al., 2000; Wais et al., 2000), revealed that the *DMI1* gene encodes a protein with similarity to a ligand-gated cation channel in archea (Ane et al., 2004). Based on predicted structural similarities with MthK, a calcium-activated potassium channel from *Methanobacterium thermoautotrophicuman*, it was suggested that SYM8, the pea orthologous of DMI1, forms a tetrameric channel of a predicted structure similar to the archaebacterial ion channel but containing a different filter region (Edwards et al., 2007). Similarly, in *L. japonicus,* two proteins, POLLUX, the DMI1 orthologoue, and CASTOR, were found to

have structural matches to the calcium-gated potassium channel MthK (Imaizumi-Anraku et al., 2005). In addition, the finding that DMI1 interferes with calcium release from internal endoplasmic reticulum stores in yeast supported the hypothesis that SYM8/DMI1 regulates calcium channels (Figure 1.2)(Peiter et al., 2007).

Using electrophiological studies, recent work showed that CASTOR is a cation channel permeable to potassium (Charpentier et al., 2008). The lack of expression of POLLUX in a cell-free system precluded similar studies with POLLUX, but *POLLUX* was found to complement a yeast mutant deficient for potassium import. In addition, *POLLUX* was also able to complement the nodulation deficiency of the *castor-12* mutant (Charpentier et al., 2008). These observations, together with the finding that CASTOR and POLLUX share identical filter sequences provide support for the hypothesis that POLLUX is also a cation channel (Charpentier et al., 2008). Contradictory studies have been reported in the literature on the localisation of CASTOR and POLLUX. Imainuzi-Anraku et al, (2005) firstly reported that CASTOR and POLLUX were localised in the plastid, whereas the *M. truncatula* POLLUX orthologue, DMI1, was shown to be the nuclear membrane (Riely et al., 2007). In recent work, an extended analysis of the sub-cellular localization of CASTOR reported that this protein is localised in the nuclear envelope of *L. japonicus* cells (Charpentier et al., 2008), consistent with the nuclear-localised calcium spiking. How CASTOR and POLLUX contribute to the generation of calcium oscillations remains unclear. The influx of potassium triggered by the opening of these channels could compensate for the loss of positive charge induced during each spike by the activation of calcium channels. Alternatively, or simultaneously, the movement of potassium ions across the membrane generated by CASTOR and POLLUX could change the nuclear membrane potential, which in turn, could activate voltage-dependent calcium channels on the same membrane, similarly to what has been described in mammals (Stehno-Bittel et al., 1995; Edwards et al., 2007; Charpentier et al., 2008). Although several pieces of evidence have been reported on potassium channels, the molecular identities of the calcium channels required for Nod-factor-induced calcium oscillation remain obscure.

The Role of Three Nucleoporins Proteins Required for Calcium Spiking

Exchange of macromolecules between cytoplasm and nucleus occur thorough nuclear pore complexes, formed by more than 30 nucleoporins (NUP) and other components (Suntharalingam and Wente, 2003). Interestingly, in *L. japonicus,* three genes, *NUP133, NUP85* and *NENA,* encoding three nucleoporins have been found to be required for the generation of calcium spiking (Figure 1.2) (Kanamori et al., 2006; Saito et al., 2007; Groth et al., 2010). Recent work showed that NENA interacts with NUP85 in yeast and that NENA, like NUP133, localises to the nuclear rim of epidermal cells (Kanamori et al., 2006; Groth et al., 2010). The nuclear pore complex allows the movement of small molecules and the selective facilitated transport of large proteins and RNAs (Suntharalingam and Wente, 2003), and it is believed that ions, as calcium, can freely translocate through the nuclear pores (Bootman et al., 2009). Thus, it has been proposed that *NUP133, NUP85* and *NENA* could allow secondary messengers into the nucleoplasm or, alternatively, they might interfere with the localisation of potassium channels and other proteins required for Nod-factor-induced calcium spiking (Oldroyd and Downie, 2008; Groth et al., 2010). Support for this idea comes from evidence showing that nuclear pore complexes mediate the localization of proteins to the inner nuclear membrane (Suntharalingam and Wente, 2003). Further investigations are required to clarify the role of symbiotic NUP proteins in the generation of calcium spiking.

Phospholipid Signalling

In mammalian cells, calcium mobilisation from intracellular stores is predominantly regulated by the secondary messenger, inositol-1,4,5-triphosphate (IP_3), which can cause the release of calcium from intracellular stores by activating IP_3 and Ryanodine-receptors (Berridge et al., 2003). Despite the absence of homologues for animal IP_3 and ryanodine receptor channels in plants (McAinsh and Pittman, 2009), it has been shown that two inhibitors of IP_3-activated channels, TMB-8 and 2-APB, inhibited expression of *ENOD11*, and that 2-APB can also block calcium spiking, suggesting a conserved mechanism of calcium spiking in both mammals and plant systems (Figure 1.2).

Phospholipid signalling in animals involves phospholipase C (PLC), that converts phosphatidylinositol 4,5-biphosphate into diacylglycerol and IP_3 (Oldroyd and Downie,

2004); phospholipase D (PLD), that hydrolyses phosphatidylcholine to form phosphatidic acid (PA), releasing the soluble choline. Studies with pharmaceutical inhibitors indicate that PLC and PLD are involved in Nod-factor-induced calcium spiking and expression of Nod-factor-induced gene expression (Pingret et al., 1998; Engstrom et al., 2002). PLC is activated through heterotrimeric G-proteins induced by G-protein-coupled receptors. Interestingly, the heterotrimeric G-protein agonist mastorparan can induce root hair deformation (den Hartog et al., 2001), DMI3-dependent nodulation gene expression (Pingret et al., 1998) and calcium spiking (Sun et al., 2007). In addition, levels of PLC and PLD were both found to be increased by Nod factor (den Hartog et al., 2001; den Hartog et al., 2003). The calcium oscillations induced by mastoparan are similar to those induced by Nod factor, albeit occurring throughout the cell and showing a slower release of calcium and a higher variability in the lag, and spiking period (Sun et al., 2007).

Remarkably, mastoparan was able to induce calcium spiking and *ENOD11* in the nodulation mutant *nfp*, *dmi1* and *dmi2* carrying mutation in genes required for Nod-factor-induced calcium spiking and gene expression (Charron et al., 2004; Sun et al., 2007). These observations suggested that mastoparan and Nod factor function on different sets of calcium channels or that equivalent calcium channels are differentially triggered by the two molecules (Sun et al., 2007). Therefore, it was proposed that mastorpan could either induce calcium spiking that is mechanistically unrelated to the Nod-factor-induced calcium spiking or could induce components of the Nod factor signalling pathway that are downstream of *NFP*, *DMI1* and *DMI2* (Sun et al., 2007). Although further studies are necessary to discriminate between these possibilities, these results provide further evidence for a function for phospholipids signalling in nodulation.

Genes Involved in the Perception of Calcium Spiking

In order to perceive the signals generated during calcium spiking and to translate the calcium signals into downstream events such as gene expression, a cell must be provided with calcium sensors and calcium effectors. Recent discoveries, in the Nod factor signalling pathway, have found a calcium and calmodulin-dependent protein kinase and at least three transcription factors required for activation of gene expression downstream of calcium spiking.

A Calcium/Calmodulin-Dependent Protein Kinase as a Decoder of Calcium Oscillations

A calcium/calmodulin-dependent protein kinase (CCaMK) essential for nodulation has been identified in *M. truncatula* (*DMI3*), *L. japonicus* (*LjCCaMK*), and *P. sativum* (*PsSYM9*) (Figure 1.2) (Levy et al., 2004; Mitra et al., 2004b). Mutations in this gene block *ENOD* gene expression but not calcium spiking or activation of calcium flux (Catoira et al., 2000; Wais et al., 2000; Shaw and Long, 2003; Miwa et al., 2006b). CCaMK is formed by a serine/threonine kinase domain, an autoinhibitory domain, that overlaps with a calmodulin (CaM)-binding domain, and three EF-hand motifs. Calcium can bind CCaMK either as the free ion to the three EF-hand domains or complexed with CaM. The binding of free calcium ions activates autophosphorylation increasing the binding of CaM, which in turn triggers substrate phosphorylation (Patil et al., 1995; Takezawa et al., 1996; Ramachandiran et al., 1997; Gleason et al., 2006). Removal of the inhibitory domain of *MtCCaMK* or a point mutation in the autophosphorylation site of *LjCCaMK* can induce the autoactivation of the nodulation signalling pathway leading to the formation of spontaneous nodules and early nodulin gene induction in the absence of the symbiotic rhizobia (Gleason et al., 2006; Tirichine et al., 2006b). These results clearly demonstrated the essential role of *CCaMK* in the Nod factor signalling pathway.

Recent work identified two genes proteins: IPD3, in *M. truncatula*, and CYCLOPS, orthologoue in *L. japonicus*, which interact with CCaMK in the nucleus (Messinese et al., 2007; Yano et al., 2008). The lack of homology to functionally characterized proteins did not allow the function of this protein to be predicted. Nevertheless, it has been hypothesised that IPD3/CYCLOPS might act downstream of CCAMK transferring the calcium signals to transcriptional factor or, alternatively it might function as a modulator of CCAMK (Capoen and Oldroyd, 2008).

Transcription Factors Transduce the Signal Downstream of CCAMK

Spontaneous nodulation and nodulin gene expression induced by activated CCaMK depend on the GRAS family transcriptional factors NSP1, NSP2, and the transcriptional factors NIN and ERN (Schauser et al., 1999; Gleason et al., 2006; Heckmann et al., 2006; Marsh et al., 2007; Middleton et al., 2007; Vernie et al., 2008).

This strongly argues that *NIN*, *NSP1, NSP2* and *ERN-1* are on a linear pathway downstream of CCaMK with respect to gene regulation and induction of nodule morphogenesis. Recent work has shown that NSP1 and NSP2 form a complex that is associated with promoters of early nodulin genes, as ENOD11 and NIN, and that NSP2 also interacts with the kinase domain of CCaMK, linking CCaMK and gene induction (Figure 1.2) (Hirsch et al., 2009). In addition, ERN1 and two close homologues, ERN2 and ERN3, have been shown to associate with the *ENOD11* promoter close to the NSP1 binding site (Andriankaja et al., 2007; Middleton et al., 2007). Thus, it has been proposed that NSP1, NSP2, ERN1, ERN2 and ERN3 act together to regulate temporally and spatially the expression of early nodulin genes (Hirsch et al., 2009), although interactions between ERN proteins and NSP1, NSP2 have not yet been described.

Root Hair Deformation

Nod factor has the challenging task to reprogramme the developmental stage of root hairs from cells involved in absorption of water and nutrients, to a suitable host for bacterial infection. This transformation occurs through several stages. Initially, Nod factor induces several cytoplasmic changes in root hair cells which include: changes in rates of cytoplasmic streaming, nuclear movements, changes in the shape of the vacuole, and shifts in the position of the endoplasmic reticulum towards the tip of root hair cells (Allen and Bennett, 1996; Miller et al., 2000). Such alterations of the cytoarchitecture are accompanied by changes of the actin and microtubule cytoskeleton (Cardenas et al., 1998; de Ruijter et al., 1999; Weerasinghe et al., 2003; Vassileva et al., 2005; Weerasinghe et al., 2005). In at least four species, bean (*Phaseolus vulgaris*), alfalfa (*Medicago sativa*), common vetch (*Vicia sativa*) and *L. japonicus*, treatment of root hairs with Nod factor leads to rapid, within three to five minutes, changes in the polymerization pattern of actin (Cardenas et al., 1998; Weerasinghe et al., 2005). In growth-arresting root hairs (those that show root hair deformation in response to Nod factor) endoplasmic microtubules were observed to disappear shortly after Nod factor addition, but they reformed within 20 minutes (Sieberer et al., 2005). Nod factor was shown to trigger fragmentation of long actin bundles extending into the root hair apical tips, and subsequent accumulation of fine bundles of filaments in the apical/subapical

region was also observed in responding root hairs (Cardenas et al., 1998; de Ruijter et al., 1999; Weerasinghe et al., 2005; Yokota et al., 2009).

All these cytoskeleton modification are associated and are probably responsible for the root hair deformation induced by Nod factor, eventually trapping the bacteria within a curl. Thus, the Nod-factor-induced disintegration of the actin cytoskeleton in the apical region of root hairs is associated with the arrest of root hair tip growth occurring within 10 minutes and followed by swellings of root hair tip (Figure 1.3) (Cardenas et al., 1998). A few hours later, Nod factors induce re-initiation of root hair tip growth via the formation of a branch-like outgrowth (Figure 1.3). In order to entrap the rhizobia root hair curling occurs. Local application of Nod factor has been shown to induce root hair curling towards the point of application on root hair cells of *M. truncatula* (Esseling et al., 2003). Thus, it has been proposed that Nod factor-secreting rhizobia lead to the continuous reorientation of the root hair tip until it form a tight curl, usually referred to as a shepherd's crook (Esseling et al., 2003).

Several morphological changes to Nod factor have been widely described in the literature, although the genetic components behind such developmental changes remain to be determined. Pharmacological studies suggested that phospholipid signalling is likely to play a role during root hair deformation. Treatment of root hair cells with mastoparan, a G-protein agonist, was shown to induce root hair deformation (den Hartog et al., 2001). Moreover application of inhibitors of the PLC, inhibited root hair deformation, suggesting that components of the phospholipids signalling, such as PLC might be involved in the induction of the deformation of root hairs (den Hartog et al., 2001).

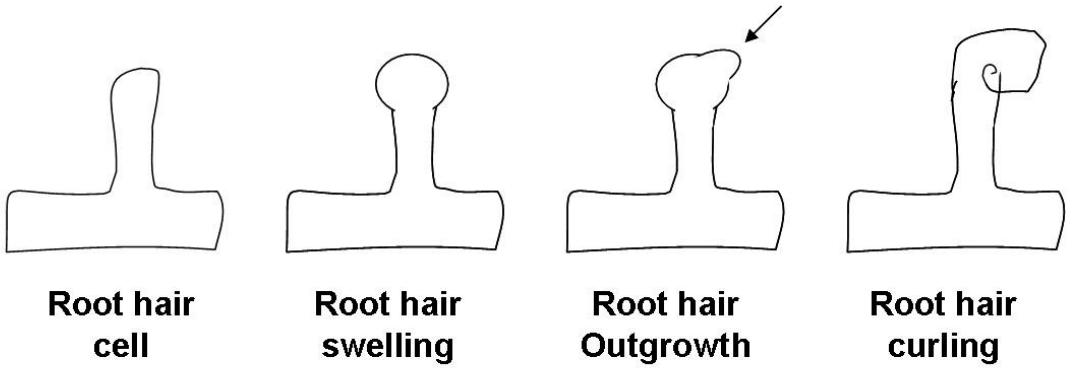


Figure 1.3. Morphology of root hairs responding to Nod factor and rhizobia.

The initial response to Nod factor, observed within one hour, is an arrest of root hair growth and swelling of the root hair tip. Subsequently, Nod factor induce a new outgrowth commonly referred as root hair deformation. In the presence of rhizobium cells, root hairs curl in order to entrap the bacteria within a curl (root hair curling).

Bacterial Infection

Bacterial infection starts when rhizobia growing in the rhizosphere remain entrapped between two cell walls, e.g. within the curl of a root hair cell. In order to facilitate the entry of rhizobia into the plant root, the plant develops a tunnel-like structure, called an infection thread, which permits the bacteria to cross several cell layers and eventually reach the nodule primordium in the inner cortex. It is still an open question how the infection threads start. Rhizobial mutants producing modified Nod factor structures are unable to form infection threads. These observations led to the idea that a high stringency of Nod factor structure is required for the initiation of the infection thread (Ardourel et al., 1994; Walker and Downie, 2000). Nevertheless, it is clear that Nod factor alone is unable to trigger the initiation of infection threads. Recent work has shown that the presence of the Nod factor receptors NFR1 and NFR5 is not sufficient to determine the infection phenotype of *Lotus* spp. Addition of *LjNFR1* and *LjNFR5* to *L. pedunculatus* roots inoculated with *M. loti* did not restore the infection phenotype. In addition, chimeric receptors containing the extracellular regions of *L. pedunculatus* NFR1 and NFR5 and the corresponding *L. japonicus* kinase domain did not complement the *L. japonicus nfr1-1* and *nfr5-2* mutants and the double mutant *nfr1-1nfr5-2*, suggesting that additional bacterial molecules independent of Nod factor are required for the initiation of infection threads (Bek et al., 2010). The presence of rhizobia is, thus, indispensable for the formation of this process and it is possible that a threshold of high Nod factor concentration released by the entrapped microcolony of rhizobial cells is necessary to trigger the initiation of the infection thread (Walker and Downie, 2000; Brewin, 2004). A calcium flux induced by such a high Nod factor concentration have been also hypothesised to drive initiation of the infection thread (Miwa et al., 2006b).

Several decades ago, ultrastructural studies revealed that the infection thread initiates as an invagination of the plant cell wall and this occurs as a consequence of degradation of plant cell walls and subsequent extension of the tubular structure (Jordan et al., 1963; Ridge and Rolfe, 1985). It has been observed that the nucleus of the root hair cells, normally linked to the tip by microtubules, probably in order to direct the deliver of new wall material to the growing apex of uninfected cells (Lloyd et al., 1987), moves toward the base of the root hairs after bacterial infection. This relocation of the nuclei

allows the delivery of new cell wall material to be directed at the tip of the infection thread (Rae et al., 1992). The progression of infection threads through the cortex seems to be guided by the reactivation of inner cortical cells induced by Nod factor, and this involves centralisation of the nuclei and formation of anticlinal transvacuolar cytoplasmic strands, also referred to as pre-infection threads (Rae et al., 1992; van Brussel et al., 1992). These responses are also induced in the outer cortex but in this region the centralisation of nuclei and the formation of cytoplasmic bridges are followed by the activation of cell division which will eventually lead to the formation of the nodule primordia.

Infection threads have a unique and remarkable topology in which bacteria remain topologically exterior to the plant cells (Figure 1.4A). Inside the infection thread, bacterial cells are embebbed in a plant extracellular matrix and are surrounded by the tubular structure defined by a layer of cell wall material and a layer of plasma membrane (Figure 1.4B). Using of fluorescent-tagged (GFP) derivatives of *S. meliloti* it has been shown that bacterial cell divisions occur only in the growing apex of the infection thread filled with one or two column of longitudinally aligned bacteria (Gage et al., 1996). Based on the dimension of rhizobial cells (approximately 1 μm in length) it has been estimated that only 100-150 bacterial cells contribute to the propagation of the infection thread (Gage, 2002). Confocal microscopic analysis has shown that infection thread growth is a discontinuous process with gaps between the file of bacteria. A bacteria free zone was observed at the tip of the growing infection thread suggesting that progression of the infection thread does not require bacterial contact (Fournier et al., 2008). Crossing epidermal and cortical cells infection threads branch and ramify in a tree-like network with a direction of growth that changes as the nodule develops (Monahan-Giovanelli et al., 2006).

Although several infection threads are generated at the epidermis, only 1-5% of them reach the nodule primordia (Vasse et al., 1993). In addition, if the bacterial symbiotic partner does not provide the plant with the correct signals, growth of the infection thread stops. For example, infection threads aborts within the root hair cells after inoculation with exopolysaccharide deficient *S. meliloti* mutants (Gonzalez et al., 1996; Pellock et al., 2000). Microarray analysis of *M. truncatula* plants inoculated with

succinoglycan deficient *S. meliloti* revealed that exopolysaccharide provides a signal that enables the host to be successfully infected by increasing its translation capacity, by altering its metabolic activity and by preparing it for invasion (Jones et al., 2008; Jones and Walker, 2008). These findings indicate that that the infection thread process is carefully regulated by both the plant and bacterial symbiotic partners.

A different type of infection observed in some legume is the intercellular infection thread, where bacteria gain access to the interior of the plant root by cracks resulting form lateral root protrusion. *Sesbania rostrata* is a tropical legume that can grow in both dry and waterlogged condition. In dry conditions the rhizobia infect the plant thorough root hairs with a similar mechanism as that observed for vetch, *Lotus,* and *Medicago* species. In contrast, in waterlogged conditions infection of *S. rostrata* occurs via crack entry and, leads to the formation of cortical infection pockets by eliciting local cell death, (D'Haeze et al., 2003). Interestingly, intercellular infection threads are less stringent for Nod factor structure compared to intracellular infection observed in alfalfa, *M. truncatula*, *L. japonicus*, pea and beans (Goormachtig et al., 2004). Nevertheless, Nod factor production within the infection thread is necessary for proper development of intercellular bacterial infection (Den Herder et al., 2007).

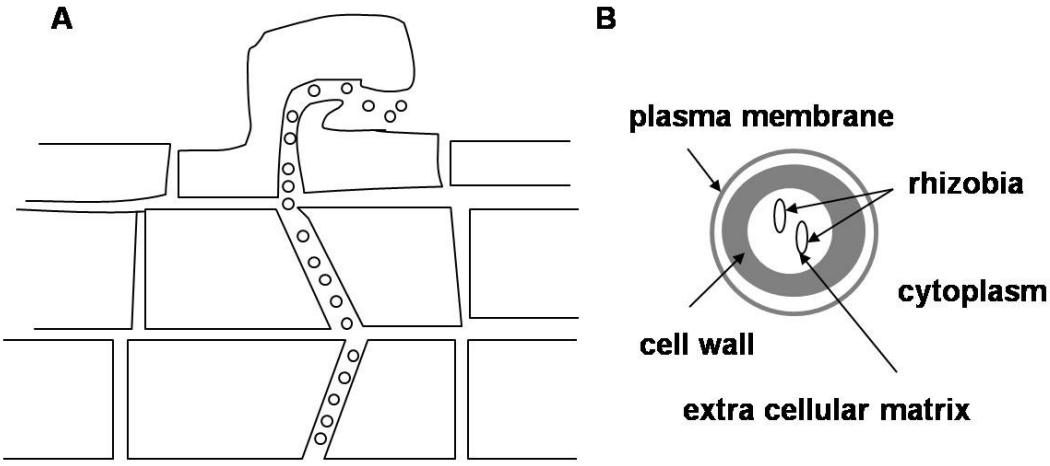


Figure 1.4. Topology of an intracellular infection thread.

A. Cartoon of tunnel-like structure intracellular infection (Infection thread) filled with column of rhizobia which are topologically exterior to the plant. The infection starts in the root hair cells and cross several cortical cell layer. B Traverse section of an infection threads filled with extracellular matrix and delimited by cell wall and plasma membrane (modified after Brewin, 2004).

Nodule Formation

Bacterial infection eventually results in the formation of nitrogen-fixing root nodules. At least, two different types of nodule development can be observed in legumes. Alfalfa, *M. truncatula* and pea form ovular "indeterminate" nodules, whereas the nodules found in *Lotus* and bean are spherical and are defined as "determinate" nodules. In indeterminate nodules, cortical cell division occurs in the inner cortex, where the nodule primordia are formed and this leads to the formation of nodules characterised by a persistent uninfected meristem responsible for nodule growth. Thereafter, the nodule is formed by a meristem at the tip and this promotes the development of an infection zone, a nitrogen fixation zone and a senescence zone. Thus indeterminate nodules grow by division of meristematic cells and the infection thread has to constantly deliver bacteria into recently divided cells just behind the meristematic zone (Libbenga and Harkes, 1973). In contrast, in determinate nodules the cortical cell divisions occur in the outer cortex and the nodules develop from a meristem which undergoes a limited number of cell divisions. During the early stage of nodule development the cells of the nodule meristem are infected. Thus, the determinate nodule grows through cell expansion, rather than cell division, and the bacterial infection spreads primarily by division of already infected cells (Rolfe and Gresshoff, 1988). Hence, although the two model legumes *M. truncatula* and *L. japonicus* share many genetic components of the Nod factor signalling pathway required for nodulation, it is worth remembering that these two species undergo two remarkably different type of nodule development.

Plant Hormones

There is increasing evidence that plant hormones play a crucial role in nodulation. Several studies have shown that auxins, cytokinins, gibberellins and brassinosteroids positively regulate different stage of nodulation. One of the first line of evidence showing the importance of auxin in nodulation come form studies demonstrating that an auxin transport inhibitors induce the formation of pseudonodules and the expression of *ENOD40*, a gene associated with induction of nodule primordia (Hirsch et al., 1989; Fang and Hirsch, 1998). Recent investigations have found that a mutation in the *L. japonicus*

LHK1 gene encoding a cytokinin receptor leads to the spontaneous formation of nodules in the absence of rhizobia (Tirichine et al., 2007); A *LHK1* loss-of-function mutant was normal for bacterial infection but failed to form nodule primordia, indicating an essential role for cytokinins in nodule organogenesis in the cortex (Murray et al., 2007).

In contrast, plant hormones such as abscissic acid, jasmonic acid, ethylene and salicylic acid play a negative role, inhibiting nodulation. For example, various levels of nodulation are differently regulated by ethylene. Ethylene inhibits the early plant responses including the initiation of calcium spiking and expression of ENOD gene (Oldroyd et al., 2001b). Thus, it has been suggested that ethylene inhibits the Nod-factor signalling pathway upstream of calcium spiking, affecting the sensitivity of the plant to Nod factor (Oldroyd et al., 2001b; Oldroyd and Downie, 2008). Ethylene is also known to regulate the number and the position of nodules. The *M. truncatula skl* mutant, defective for perception of ethylene, was initially identified as a supernodulation mutant producing approximately tenfold more nodules than wild type plants and displayed a random distribution of nodules which are normally located opposite to the protoxylem poles (Heidstra et al., 1997; Penmetsa and Cook, 1997; Penmetsa et al., 2003). Bacterial infection was also affected by ethylene. Similarly to the *lhk1* mutant, infection threads in the *skl* mutant grew in an uncontrolled fashion throughout the cortex (Penmetsa and Cook, 1997; Murray et al., 2007). Interestingly, in *S. rostrata* ethylene inhibited intracellular root hair infection but promoted intercellular infection via crack entry (Goormachtig et al., 2004). Hence, ethylene globally regulates almost each step of the nodule formation either in the epidermis and the cortex.

Recent work has shown that ABA, similarly to ethylene and jasmonic acid (Oldroyd et al., 2001b; Sun et al., 2006), inhibits calcium spiking, gene expression, bacterial infection and nodule formation (Ding et al., 2008). Given the high capability of hormones to positively or negatively affect nodulation, it is likely that an equilibrium of positive and negative hormones tightly controls the fate of nodule development. It is worth remembering that hormones are all induced in response to environmental stress such as pathogen, herbivore attack and drought. Hence, by sensing environmental change surrounding the plant, hormones might have the ability to promote or inhibit nodulation.

Mycorrhizal Symbiosis

Most terrestrial plants interact of a wide diversity of endophytic fungi, of which the best studied are those that form arbuscular mycorrhiza. A mycorrhiza is a symbiotic association between a fungus and the roots of a plant. The relationship between mycorrhizae and plants is very widespread among terrestrial vascular plants: approximately 80% of all land flowering plants (Angiosperm) are able to associate with arbuscular mycorrhizal fungi (Newman and Reddell, 1987). The mycorrhizal association is a diverse and ancient symbiosis. Fossil evidence suggests that this symbiosis initiated 460 million years ago (Wilkinson, 2001), almost 400 million years earlier than the root nodule symbiosis. In the arbuscular mycorrhiza endosymbiosis, the fungus inhabiting the root cortical cells transfers mineral nutrients, particularly phosphorus, from the soil to the cortical cells, where this is exchanged with carbon provided by the plant (Harrison, 2005). Phosphorus is an essential mineral nutrient and in many soils is limiting for plant growth (Holford, 1997). It has been demonstrated that improvements in phosphorus acquisition have a significant impact on plant growth, health, and subsequently on plant biodiversity and ecosystem productivity (van der Heijden et al., 1998). Unlike rhizobia, the arbuscular mycorrhizal fungi are obligate biotrophs and depend entirely on the plant to provide them with carbon. Consequentially, studies of these organisms have been limited by the inability to grow arbuscular mycorrhizal fungi in the absence of the plant, and in comparison with other groups of fungi, relatively little is known about them. Until now, there have been approximately 150 species of arbuscular mycorrhizal fungi described and the identification of new species is ongoing (Kramadibrata et al., 2000; Harrison, 2005). Mycorrhizae have been classified into two groups: ectomycorrhizae and endomycorrhizae. The ectomycorrhizae belong predominantly to *Basidiomyces* and form associations with woody Angiosperms and Gymnosperms. These fungi grow around the root as a mycelial sheath with some limited intercellular hyphal penetration, whereas endomycorrhizae, develop extensive intracellular fungal growth. The internal hyphae form highly branched haustoria, called arbuscules (Latin for tree). Thus, the fungi that form this type of symbiotic interaction are known as arbuscular mycorrhizae. Unlike rhizobium-legume symbioses, arbuscular mycorrhizal fungi are not restricted to interact with specific plant hosts. Nevertheless, some fungus-plant combinations have better

colonisation rates and differences in compatibility are becoming apparent. Thus, there may be levels of specificity that are not yet appreciated (Pearson and Jakobsen, 1993; Smith et al., 2004).

The establishment of arbuscular mycorrhizal symbioses, like the rhizobium-legume symbioses, starts with a chemical signal exchange between the root and arbuscular mycorrhizal fungus which occur prior to their physical interaction. In one of the first stages of host recognition, plant release into their root exudates biological molecules, called strigolactones (Akiyama et al., 2005). Strigolactones are perceived by arbuscular mycorrhizal fungi and stimulate arbuscular mycorrhizal fungi to switch from asymbiotic to presymbiotic growth, which is marked by induced branching of hyphae from germinating fungal spores (Buee et al., 2000) and elevated energy metabolism (Besserer et al., 2008). As a result of the strigolactone perception, the fungus, in turn, triggers the production of diffusible signalling molecules, which are perceived by the plant root in the absence of direct physical contact (Kosuta et al., 2003). An hypothesized diffusible compound (called Myc factor) is likely to be perceived by an arbuscular mycorrhiza-specific receptor in epidermal cells (Oldroyd et al., 2009). It has been shown that a diffusible signal from arbuscular mycorrhizal fungi can induce a transient cytosolic calcium increase in host plant cells (Navazio et al., 2007). The rapid elevation in cytosolic calcium was induced in response to the culture medium of spores of *Gigaspora margarita* germinated in the absence of a plant partner (Navazio et al., 2007). Recently, imaging of yellow cameleon indicator revealed that mycorrhizal fungi induce repetitive calcium oscillations around the nuclei of root hair cells with a signature that differs from Nod-factor-induced calcium spiking (Kosuta et al., 2008). Fungus separated from the plant root by a cellophane membrane elicits expression of *ENOD11* gene in *M. truncatula*, a Nod factor-inducible gene encoding a putative cell wall protein (Kosuta et al., 2003).

Arbuscular mycorrhiza differ profoundly to the rhizobium-legume symbiosis in terms of developmental changes induced in the plant, mechanisms of fungal or rhizobial infection and profile of genes activated by the two endosymbionts (Kistner et al., 2005). Nevertheless, surprisingly, several components of the Nod factor signalling pathway are also required for mycorrhizal association. Thus, these two different symbioses share the same symbiosis signalling (Sym) pathway, also referred as common signalling pathway

including DMI2/SYMRK, DMI1/Pollux, Castor, NUP133, NUP85 and CCaMK (Oldroyd and Downie, 2004; Oldroyd and Downie, 2006). The fact that root nodule and arbuscular mycorrhizal symbioses have conserved early-signalling genes supports the model that nodulation genes have been recruited from the more ancient arbuscular mycorrhiza symbiosis.

Once in contact with the plant root, the fungus forms an appressorium on the root epidermis through which it enters the root. Before infection, the epidermal cell assembles a transient intracellular structure with a novel cytoskeletal organization defines as prepenetration apparatus (Genre et al., 2005) that resembles the pre-infection thread structure observed in legume-rhizobia symbiosis (Parniske, 2008). After colonisation of the root surface, fungal hyphae enter through a cleft into the exodermis that opens between an exodermal cell and a cell of the outermost cortical layer (Harrison, 1998; Demchenko et al., 2004; Parniske, 2004). Inside the cortex, fungal hyphae continue to grow until they reach and penetrate the cell wall of an inner cortical cell. Subsequently, further differentiation yields highly ramified fungal hyphae, termed arbuscules (Harrison, 1997, 2005). In parallel, arbuscular mycorrhizal fungi also develop extensive hyphae outside the plant root. The intraradical and extraradical hyphae constitute a filamentous network that bridges rhizosphere and plant roots and consequently facilitates bi-directional nutrient transfer where soil nutrients move to the plant and plant photosynthesis flow to the fungus (Jakobsen, 1995; Harrison, 1997; Cavagnaro et al., 2001).

Research Objective of This Thesis

Calcium plays an essential role in legume-rhizobia symbiosis. The focus of this work was to investigate the role of Nod-factor-induced calcium flux and calcium spiking in the infection of *M. truncatula* and *L. japonicus* by rhizobia. First, a system to record and analyze Nod factor-calcium flux in *M. truncatula* root hair expressing yellow cameleon 2.1 was established and used to assess the effect of different Nod factor structure on calcium flux (Chapter 3). Secondly, *M. truncatula* and *L. japonicus* mutants affected for infection thread growth were tested for Nod-factor-induced calcium flux (Chapter 4). Third, the relationship between root hair calcium spiking and nodule organogenesis was investigated using a novel allele of the *L. japonicus SYMRK*, in Chapter 5. Fourth, the role of the *L. japonicus LNP* in the Nod factor signalling pathway was tested using calcium and genetic analysis (Chapter 6). The results presented in this thesis will provide new insight into the molecular signalling cascade that drives the infection and nodule formation in legume-rhizobia symbiosis.

This work was part of the NODPERCEPTION Marie Curie Research Training Network (MCRTN) involving eight partners from six European countries (*http://medicago.toulouse.inra.fr/NODPERCEPTION/index.htm)*. The research project concerned the establishment of the agronomically and ecologically important legume-rhizobia symbiosis. Its major aim was to identify the molecular mechanisms of Nod factor perception through an integrated and multidisciplinary research project using the model legume *M. truncatula.*

Chapter 2

Materials and Methods

Plant Material

Medicago truncatula genotype Jemalong A17 was used as wild type. The L416 line of *M. truncatula* transformed with p*MtENOD11-GUS* (Charron et al., 2004) was provided by David Barker (INRA, Toulouse, France). The *M. truncatula hcl-1*, *bit-1*, *nin* and *rit-1* mutants were provided by G. E. Oldroyd (JIC, Norwich, UK). Wild-type *Lotus japonicus* accession Gifu B-129 (Stougaard and Beuselinck, 1996) was obtained from J. Stougaard. All *L. japonicus* mutants used in this study were generated in *L. japonicus* gifu B-129. Seeds along with the source and the original description of the mutants were provided by J. Stougaard, K. Szczyglowski and M. Etzler.

Bacterial Strains

All strains used are listed in Table 2.1.

Table 2.1. Bacterial strains.

Strain	Lot no.	Plasmid	Resistance	Description	Source
S. meliloti 1021	D4594	pMH682	Str, Kan, Tet	*S. meliloti* 1021 *exo7*::Tn5 carrying *nodD3* and *syrM* on pMH682 for overproduction of Nod factor	Obtained from A. Downie
M. loti R7A	X197	-	-	Wild type	(Sullivan & Ronson, 1998)

Media

The composition of the growth media used for plant and bacterial growth are given in Table 2.2.

Table 2.2. Growth media used for bacterial and plant growth.

Solutions	Composition (1L)
Rhizobium complete medium (TY)	5 g Difco tryptone, 3 g Difco yeast extract, 1.325 g CaCl2 (15 g agar for solid medium).
Rhizobium minimal medium (Y)	0.1 g $MgSO_4$ $7H_2O$, 0.22 g $CaCl_2$ $6H_2O$, 0.22 g K_2HPO_4, 0.02 g $FeCl_3$, 1.1 g L-glutamate acid Na salt, 0.75 mg biotin, 0.75 mg aneurine HCl (Thiamine), 0.75 mg D,L-pantothenic acid Ca salt, 10 g agar, pH 6.8.
Water agar (DWA)	1.5 % Bacto agar, pH was adjusted with 1 M KOH to pH 5.7.
Fahraeus plant medium (FP)	0.1 g $CaCl_2$ $2H_2O$, 0.12 g MgSO4 7 H_2O, 0.1 g $KHPO_4$, 0.15 g Na_2HPO_4 $12H_2O$, 5 mg Ferric citrate, 2.86 mg H_3BO_3, 2.03 mg $MnSO_4$ $4H_2O$, 0.22 mg $ZnSO_4$ $7H_2O$, 0.08 mg $CuSO_4$ $5H_2O$, 0.08 mg H_2MoO_4 H_2O, 0.5% Lab M agar (for FP solid medium), pH 6.3-6.7.
Buffered nodulation medium (BNM)	390 mg MES [2-(*N*-morpholino)-ethane sulphonic acid], 344 mg $CaSO_4.2H_2O$, 122 mg $MgSO_4.7H_2O$, 68mg KH_2PO_4, 4.6 mg $ZnSO_4$ $7H_2O$, 3.1 mg H_3BO_3, 8.45 mg $MnSO_4$ H_2O, 0.25 mg Na_2MoO_4 $2H_2O$, 0.016 mg $CuSO_4$ $5H_2O$, 0.025 mg $CoCl_2$ $6H_2O$, 18.65 mg Na_2EDTA, 13.9 mg $FeSO_4$ $7H_2O$, 11.5 g (for BNM solid medium), pH 6.5.
F1 compost	Scotts® Levington F1 compost is a low-nitrogen and high-phosphorous containing compost designed for year-round propagation of sensitive subjects and where control of growth is required, e.g. in Brassicas, for steady, controlled growth (Scotts, Bramford, Suffolk, UK).

Antibiotics

Antibiotics were used for growth selection of *S. meliloti*. Stock solutions of antibiotics were dissolved in water followed by sterilisation through filtration with a 0.2 µm filter. Tetracycline was dissolved in ethanol. The final concentrations of antibiotics used for *S. meliloti* were kanamycin 50 µg/ml; tetracycline 5 µg/ml; spectinomycin 200 µg/ml.

Chemicals

Oregon Green-dextran M_r 10,000 and Texas Red-dextran M_r 10,000 dyes were supplied by Molecular Probes (Eugene, USA). All other chemical compounds were supplied by Sigma-Aldrich Company Ltd. (Poole, UK), unless stated. Synthetic Nod factor equivalent to *nodL* Nod factor (LCO IV, $C_{16:2}$, S) and *nodF nodL* Nod factor (LCO IV, $C_{18:1}$, S) were kindly provided by Eduardo Andres (CNRS, Grenoble, France).

Plant growth conditions

M. truncatula seed sterilisation and plant growth

Seeds of *M. truncatula* were scarified using sandpaper and sterilised in 0.1% sodium hypochlorite solution for two-three minutes and washed five times with sterile water. The seeds were then left to imbibe for one-two hours at room temperature or overnight at 4°C. Subsequently, seeds were left overnight in plates with water agar (DWA) medium at 4°C in the dark. The plates were then placed upside down at room temperature for one day. Seedlings with 1-2 cm long roots were transferred to Fahraeus nitrogen-free plant agar medium (Fahraeus, 1957) agar (FP) plate containing 0.1 µM L-α-(2-aminoethoxyvinyl glycine (AVG). AVG was added in order to prevent the inhibition of nodulation by ethylene accumulation in the roots. The region of the plates containing the roots was wrapped in black plastic and the plates were incubated vertically in a controlled environment (20°C/15°C, day/night cycles of 18/6 h with 32% relative humidity, 300 µmol m^{-2} s^{-1} light intensity).

Plants grown in the greenhouse were germinated on agar as described above and transferred to soil (John Innes Number 2 compost). The plants were grown in a greenhouse, watered twice daily and additional light was provided during the winter. For the first two-three days a clear glass lid was placed on top of the pots to protect the plants from drying out.

L. japonicus seed sterilisation and plant growth

Seeds of *L. japonicus* were scarified, sterilised and imbibed as described above except that were soaked in sodium hypochlorite solution for 15-20 minutes. Imbibed seeds were grown for two days on DWA water agar medium, in dark and placed upside down to allow the roots to grow vertically. Subsequently, seedlings with similar root lengths (0.5-2 cm) were selected, and transferred to FP agar medium. Filter paper (grade 0860; Schleicher and Schűll, UK) was placed between the agar and the roots to prevent the roots growing into the agar. The roots were then covered by another filter paper to keep them moist. The Petri dishes were incubated in a vertical position in a controlled

environment (see condition above) and the region of the Petri dish containing the roots was covered with black plastic.

Plants grown in the greenhouse were planted into small plastic pots in Scotts Levington F1 compost (Scotts, Bramford, Suffolk, UK) or terragreen sand. For the first two-three days a clear glass lid was placed on top of the pots to protect the plants from drying out.

Preparation of Nod Factor

Nod Factor Extraction from Liquid Medium

S. meliloti 1121 pMH682 was grown for two days on a TY agar plate. One colony was then transferred into 100 ml of Y medium containing 0.75 mg/L Thiamine, 0.75 mg/L, 0.75 mg/L Biotin, 0.75 mg/L DL-Pantothenic acid, 0.75 mg/L Calcium salt, 0.11 g/100ml NaGlu, as described on Table 2.2 and 0.1% Mannitol, and was cultured at 300 rpm at 28°C for two days. A 10 ml inoculum was then added to three flasks containing 700 ml of Y medium containing 0.3% Mannitol and 0.5 µM Luteolin. The flasks were shaken for two days at 28°C. The bacterial cultures were then centrifuged at 9000 rpm for 60 minutes at 5°C, and the clear supernatant was pumped through a C18 column (Sep-Pak) at a flow rate of 10 ml/min. The C18 column was sequentially eluted with 2.5ml of each of 20%, 40%, 60%, 80% methanol and 4 ml of 100% methanol. The fractions were analysed using root hair deformation assays and ENOD11-GUS induction as described below. The 80% methanol fraction retained the highest activity and this fraction was used for further analysis.

HPLC Analysis and Mass Spectrometry Quantification of Nod Factor (Performed by Lionel Hill, Metabolic Biology Department)

To analyze the Nod factor eluted from the Sep Pak column, 10 µl of the fraction eluted with 80% methanol was diluted five times in water and then applied to a 100 mm X 2 mm 3 µ Luna C18(2) reverse phase HPLC column (Phenomenex) using a linear

gradients of acetonitrile indicated in Table 2.3 versus 0.1% formic acid in water, at 30˚C with a flow rate of 280 µl per minute.

Table 2.3. Gradient of acetonitrile

Time	% Acetonitrile
0	5
20	95
25	95
26	5
34	5

The sample was analysed using a Thermo DecaXPplus ion trap (equipped with a Surveyor HPLC system), collecting full Mass data in negative mode using electrospray ionization. MS2 data were taken at 35% collision energy, 4.0 atomic mass unit (amu) isolation width and with dynamic exclusion, to ensure that as many ions as possible were sampled. Spray chamber conditions were 350ºC capillary temperatures, 50 units sheath gas, 5 units aux gas, 5.0kV. For the quantification of extracted Nod factor, several dilutions of the extracted Nod factor were prepared and the major natural Nod factor (1143.7amu in negative mode) was quantified to prepare a calibration curve. The standard (LCO IV $C_{16:2}$ S) was diluted to fall somewhere in the middle of the curve, and the major Nod factor was analysed (1101.7amu). Comparison of this standard with the curve gives the dilution of extract that matches standard, and hence the concentration of the extract. Other Nod factors were quantified relative to the major Nod factor of 1143.7 amu. This quantification method was a backwards version of the normal process of calibration, where dilution series of the standard rather than the extract would have been run. This method was chosen because a large amount of extract Nod factor and a low amount of standard Nod factor were available. The quantification of the Nod factors peak-areas, in extracted ion chromatograms derived from the full spectra MS data, were integrated using the Qualbrowser software and are reported in Table 2.4. Peaks identities were confirmed using data dependent MS2 spectra. The *Sm* IV $C_{16:2}$, S, Ac Nod factor concentration in the 80% methanol extract was estimated to be 1.09 mM (Figure 2.1, pick D). The Nod factor extract was kept at -20 °C.

Table 2.4. Quantification of extracted Nod factors.

Picks	Retention Time (min)	Mass (amu)	area	Identity	Concentration (M)
A	14.84	1304.6	58740588	*Sm* V $C_{16:2}$, S	0.085
B	15.53	1346.7	346551312	*Sm* V $C_{16:2}$, S, Ac	0.502
C	15.78	1101.5	108339602	*Sm* IV $C_{16:2}$, S	0.157
D	16.5	1143.6	752783376	*Sm* IV $C_{16:2}$, S, Ac	1.090
E	16.98	1145.5	159803210	*Sm* IV $C_{16:1}$, S, Ac	0.231
F	19	1173.7	57224734	*Sm* IV $C_{18:1}$, S, Ac	0.083

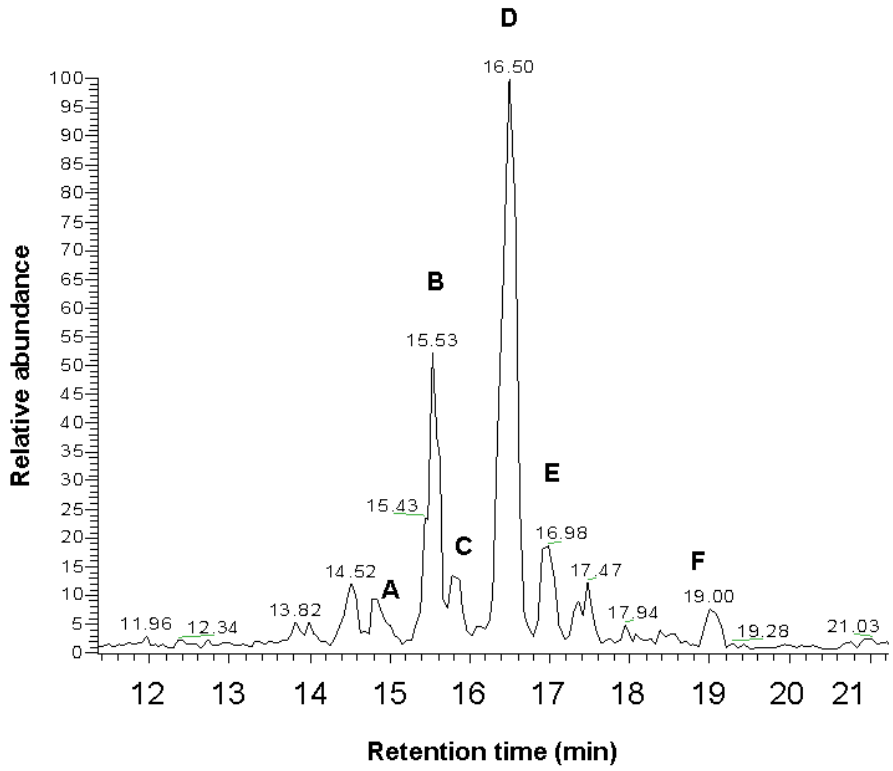


Figure 2.1. Chromatogram of the intensity of the most intense ion with a mass between 1000 and 1700, at different retention time (see Table 2.4. for identification of peaks).

Biological Estimation of Nod Factor Activity

Nod factor was analyzed by ENOD11-GUS induction assays (see below). The Nod factor extract was diluted in BNM medium to make a 10-fold dilution series of the range of 10^{-6} to 10^{-12}. Strong induction of ENOD11 was observed after 4 hours of incubation with an appropriate positive control. ENOD11-GUS was induced in *M. truncatula* roots with the fraction diluted to 10^{-10} and a weak response was observed in the sample diluted to 10^{-12}.

Physiological Techniques

Root Hair Deformation

Seedlings with root length of about 1 cm were germinated and grown for two days were transferred to Farhraeus slides filled with FP liquid medium. About one hour later Nod factor was added to a final concentration of 10 nM. For estimation of Nod factor activity following purification several dilutions were prepared. For treatment with rhizobia, *M. loti* R7A was grown for two days under agitation at 28°C in 5 ml TY liquid culture. Seedlings were inoculated with a $1x10^{-3}$ dilution of the *M. loti* culture. After Nod factor/*M. loti* was added, samples were left in the dark at room temperature for approximately 24 hours. Root hairs were examined under the light microscope and root hair deformation was scored without prior knowledge of the treatment to the seedlings (Nod factor or *M. loti* treatment or absence of Nod-factor or *M.loti*). Roots containing branching root hair cells were considered positive for this response. Images were taken using an inverted microscope with a digital camera.

ENOD11-GUS Induction Assay

Seedlings of *M. truncatula ENOD11-GUS* transgenic plants (Charron et al., 2004) were transferred into liquid BNM containing several dilution of *S. meliloti* extracted Nod factor for four hours. Subsequently, roots were imbibed in a solution containing 1 mM EDTA, pH 8.0, 50 mM Sodium Phosphate buffer pH 7.0, 0.05% v/v Triton X-100 to which 2 mM X-glcA (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide, sodium salt, Melford Laboratories Ltd) was added from a stock of 500 mM X-glcA in dimethylformamide. Roots were incubated at 37˚C overnight in dark before counting the number of blue stained roots for each treatment.

Calcium Imaging with Oregon Green

Plant Preparation

Seedlings of *M. truncatula* or *L. japonicus* were prepared as described above. A small chamber was made on a large cover glass using high vacuum grease (Dow Corning GMBH, Wiesbaden, USA) and the chamber was filled with 100 µl of liquid FP medium Seedling with 2-3 cm long root were placed into this well. The seedlings were incubated at room temperature for at least 20 min before microinjection.

Dye Preparation

Oregon Green 488 BAPTA-1-dextran 10,000 MW and Texas Red-dextran 10,000 MW (Molecular Probes, Eugene, OR, U.S.A.) were dissolved in sterile water to a final concentration of 5 mM. Texas Red (calcium insensitive dye) was used as a reference to eliminate the background fluctuation. For each experiment, 1 µl 5x injection buffer (0.75 M KCl, 0.45 M HEPES, pH 7.0) was added to 4 µl of dye solution. Due to the higher fluorescence of Texas Red, 0.4 µl aliquots of Texas Red were added to 3.6 µl of Oregon Green, and then 1 µl 5x injection buffer was added to the mixed dye solution. The solution of dye was spun at 12,000 rpm in a microcentrifuge for one minute to remove any particulate matter and the upper solution was used for microinjection.

Needle Preparation

Thin needles for microinjection were made using Borosilicate Glass Capillaries (1B120F-4; World Precision Instruments Inc.) and a computer-controlled electrode puller (model 773; Campden Instruments Ltd.). The optimized setting for pulling very thin needles was a heat intensity setting of about 16 and a pulling force setting of about 50. The tips of these needles were examined under the light microscope and only needles with thin tips were selected for use in injection.

Microinjection

The microinjection system was essentially the same as that described by Wais et al. (2000). The needle was first loaded with about 0.2 µl of dye solution containing Oregon Green and Texas Red using a long thin pipette tip (Microloader; Eppendorf) and then the needle was back-filled with 10 µl of 1 M KCl. The large cover glass containing a seedling was placed on the inverted epifluorescence microscope for microinjection. The reference electrode was placed into the FP medium in the bath. The needle was controlled by an electro-manipulator (PatchMan NP2; Eppendorf) and the tip of the needle was targeted to the apex of the growing root hair cells. The precise position of the needle tip was monitored by measuring the voltage through the needle. When the tip was in the cytoplasm, the voltage was slightly changed. Then dyes were injected into the root hair cell by iontophoresis using a direct current (DC) set at 10 nA. Injection proceeded until cells dimly fluoresced when viewed under illumination from a 100 W mercury bulb through a GFP filter block. After microinjection, root hairs were left at least 20 min before Nod-factor addition and only cells showing active cytoplasmic streaming were used for analysis. Nod factors, isolated from the reverse phase C18 column, and other compounds were added directly to the incubation chamber at the concentrations stated in the text.

Imaging by Epifluorescent Microscope

Fluorescence was imaged using a Nikon TE2000U inverted microscope coupled to a Hamamatsu Photonics digital CCD camera. The excitation wavelength was 488 nm and an 11 nm bandpass was selected using an Optoscan Monochromator (Cairn Research, Faversham, Kent, UK). An emission filter of 545 (± 15) nm was used for Oregon Green fluorescence. For dual dye imaging, an image splitter (Cairn Research, Faversham, Kent, UK) with optimised polychromatic mirror was used to monitor both Oregon Green and Texas red fluorescence and each image was sequentially collected every five seconds with a 1 s exposure using MetaFluor software. For imaging of Texas Red, an excitation wavelength of 570 nm was used and the fluorescence was monitored with an emission filter of 620 (± 20) nm. After taking a series of images, the ratiometric traces were calculated by dividing Oregon Green fluorescence by that of Texas Red at each time point.

Derivative traces represent the change in fluorescence intensity of Oregon Green/Texas Red from one point to the next ($x_{n+1} - x_n$). Traces were generated using Microsoft Excel.

Calcium Imaging with the Cameleon System YC 2.1

Seeds of the *M. truncatula* YC 2.1 transgenic line were germinated and placed in a small chamber filled with 100 µl of liquid FP as described above. Intracellular calcium concentrations were analyzed by calculating FRET using measurements of fluorescence of CFP and YFP. This was done using an epifluorescence microscope. A Nikon 20x or 40x working lens was used for imaging. The CFP component of YC2.1 was excited with a wavelength of 437 nm and an 11 nm bandpass using an Optoscan Monochromator (Cairn Research, Faversham, Kent, UK). Emitted fluorescence was separated by an image splitter with a dichroic mirror 515 nm, and then passed through an emission filter of 485 (± 20) nm for CFP fluorescence or 535 (± 15) nm for YFP fluorescence. Images were collected every five seconds with a 200 to 900 milliseconds exposure and analyzed using MetaFluor software. Values were exported into Excel (Microsoft), converted to a ration between YFP and CFP fluorescence, and plotted against time.

Calcium Imaging with the Nuclear-Targeted Cameleon NupYC2.1

Agrobacterium rhizogenes-Mediated Root Transformation

The nucleoplasmin-cameleom YC2.1 (NupYC2.1; kindly provided by D. Barker, INRA-CNRS, France) was introduced into *L. japonicus gifu* wild type roots, in *symRK-14* and *symRK-14*/har1-1 roots by hairy root transformation as described (Diaz et al., 2005) using the *Agrobacterium rhizogenes* strain AR1193. Plants were grown in plates containing B5 medium (Gamborg et al., 1968) for at least 21 days after inoculation before calcium imaging experiments.

Calcium Imaging

For calcium imaging experiments using NupYC2.1 plants transformed by hairy root transformation were transferred to plates containing Fahraeus agar medium 5-10 days before calcium analysis. Filter paper (grade 0860; Schleicher and Schűll, UK) was placed between the agar and the roots to prevent the roots growing into the agar. The roots were then covered by another filter paper to keep them moist. The Petri dishes were incubated in a vertical position in a controlled environment (20°C/15°C, day/night cycles of 18/6 h) and the region of the Petri dish containing the roots was covered with black plastic.

For microscopic observation on an inverted epifluorescent microscope, plants were placed into a chamber, made on a large cover glass using high vacuum grease (Dow Corning GMBH, Wiesbaden, Germany), and filled with liquid Fahraeus medium. Roots with high fluorescence level in root cell nuclei were selected, excised with a scalpel and transferred to a smaller chamber containing 100 ul of liquid Fahraeus medium. After addition of Nod factor at a final concentration of 100 nM changes in nuclear calcium concentrations were analyzed by calculating FRET using measurements of fluorescence of CFP and YFP as described by Miwa et al., (2006a). Images were collected every five seconds and analyzed using MetaFluor software. Values were exported into Excel (Microsoft), converted to a ration between YFP and CFP fluorescence, and plotted against time.

Molecular Biological Technique

RNA Extraction and DNaseI Treatment

For each sample, total RNA was extracted from approximately 100 mg root tissue using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. The RNA was eluted, first with 30 µl Rnase free water. Isolated total RNA was treated with Turbo DNase (Ambion) and quantified with NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies). Quality of RNA was measured using QIAxcel system (Qiagen, Ltd., Surrey, UK).

Quantitative RT-PCR

cDNA was prepared from 0.5-1 µg of total RNA using the SuperScript II first-strand synthesis system for RT-PCR (Life Technologies, Invitrogen) using oligo(dT) primers according to the manufacturer's protocol. Quantitative RT-PCR was performed using a CFX96 Real-Time System (BIO-RAD) and using SYBR Green Master Mix (Sigma). Each 10 µl PCR reaction contained 4 µl total cDNA 1:20, 5 µl SYBR Green Master Mix, (Sigma) and 0.5 µl of each primer (10 µM, designed to amplify a fragment of 50-150 bp). An initial denaturation step of 95°C for 4 min was followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. At the end of the reaction, the samples were heated at 72°C for ten minutes. Reactions were undertaken in 96-well white Microplates (Biorad). The *Polyubiquitin* gene was used as internal positive control. Primers are shown in Table 2.5. Results were expressed as a threshold cycle (CT) value. Gene expression was normalized to that of the reference gene subtracting the CT value of the *NIN* gene from that of the *POLYUBIQUITIN* gene to give a ΔCT. Fold induction was calculated by normalizing the data for each time series to that of the untreated sample taken at zero hour. Thus, the ΔCT value for treated sample was subtracted from the ΔCT of the untreated samples to give a ΔΔCT value. The data were plotted as $2^{-\Delta\Delta CT}$. The data from three technical replicates of at least three biological repetitions were plotted using standard deviation.

Table 2.5. Primers used for qPCR determination with *L. japonicus* cDNA.

Gene	Forward primer 5'-sequence-3'	Reverse primer 5'-sequence-3'
nin	ccaagcagcagtgaatgaga	aggagcccaagtgagtgcta
polyubiquitin [a]	atgcagatcttcgtcaagaccttgac	acctcccctcagacgaagga

[a] Reference gene primer from Imaizumi-Anraku et al., 2005.

CHAPTER 3

Analysis of the Effect of Nod factor Structure on Nod factor-Induced Calcium Flux

Introduction

Calcium is a highly versatile intracellular signal that controls many different cellular processes. The calcium-signalling system achieves this versatility by operating in many different ways to regulate cellular processes that function over a widely differing time scale. For example, at the synaptic junction calcium induces exocytosis in microseconds, whereas calcium operates over minutes or even hours to trigger events such as gene transcription and cell proliferation (Berridge et al., 2003). How calcium transduces so many different signals leading to specific cellular responses is still unknown. Recently, studies in animal and plant cells suggest that a calcium signal provides information to the cell through its temporal and spatial distribution, and the magnitude of the signal (Dolmetsch et al., 1998; Li et al., 1998; Allen et al., 2001; Berridge et al., 2003). Different external signals trigger unique calcium changes characterised by specific parameters in terms of magnitude, spatial and temporal distribution. Each of these distinct calcium responses is generally referred as a 'calcium signature' (or a 'code') (Luan, 2009).

In legume-rhizobia symbioses, upon recognition of the bacterial Nod factors, root hair cells, which normally function in water and nutrients absorption, reprogramme their development to accommodate the rhizobia inside the plants. In order to trigger such a change, Nod factors induce several responses such as root hair deformation, activation of phospholipase C and phospholipase D (den Hartog et al., 2001), gene induction (Journet et al., 1994; Cook et al., 1995; Charron et al., 2004) and cortical cell division (Truchet et al., 1991). When added to the legume roots, Nod factors induce at least two different

calcium responses. One is a rapid influx of calcium (calcium flux) and the second consists of oscillations in the cytosolic calcium concentration, called calcium spiking.

Within the first minutes of addition, Nod factors can induce a movement of calcium ions into the cell cytoplasm. This triggers a number of events such as the activation of anion channels through which the cells lose chloride rapidly, causing membrane depolarisation (Ehrhardt et al., 1992), followed by a cytoplasmic alkalinisation (Felle et al., 1996). The membrane depolarisation is then stopped by a charge balance due to a potassium efflux. Repolarisation of the plasma membrane is fuelled by a proton pump (Felle et al. 1998) and occurs in two phases: an initial fast phase occurring within 30 to 60 seconds and a slower phase lasting for variable times up to approximately 15 minutes (Kurkdjian, 1995). Nod factor-elicited calcium flux has been observed in several different legumes including *M. sativum* (Ehrhardt et al., 1996), *M. truncatula* (Shaw and Long, 2003), *Phaselus vulgaris* (Cardenas et al., 1999), *Pisum sativum* (Walker et al., 2000), *Lotus japonicus* (Miwa et al., 2006b) and *Vicia sativa* (de Ruijter et al., 1998), which demonstrates that this response is common, though the time of induction and the duration of the calcium flux is variable (Oldroyd and Downie, 2004).

After 10-15 minutes the legume root hair cells respond to Nod factor by showing repetitive oscillations in the cytosolic calcium concentration (calcium spiking). A typical "spike" is characterised by a rapid (1-4 seconds) increase in perinuclear calcium, and is followed immediately by a more gradual decline of calcium (approximately 30 seconds). The first part implicates the opening of calcium channels while the second phase implicates the activity of a calcium ATPase or calcium antiporter to control the movement of calcium against the electrochemical gradient, from the cytoplasm and nucleoplasm into either an internal store or extracellular space. Once initiated, spikes occur repeatedly for one-two hours with an average frequency of 60-100 seconds, depending on the position of the root hair cells along the root (Ehrhardt et al., 1992; Miwa et al., 2006a; Sieberer et al., 2009). These oscillations have been reported in alfalfa (Ehrhardt et al., 1996), *M. truncatula* (Wais et al., 2000), pea (Walker et al., 2000), *P. vulgaris* (Cardenas et al., 1999) and *L. japonicus* (Harris et al., 2003), suggesting calcium spiking is a common feature in root nodule symbiosis.

Both calcium influx and calcium spiking are induced by Nod factor, but, it is clear that calcium influx and calcium spiking can be uncoupled by different experiment

conditions. While high concentrations (> 10^{-9} M) of Nod factors induce calcium flux followed by calcium spiking, at low concentration (< 10^{-10} M) no calcium influx is observed but calcium spiking is induced (Shaw and Long, 2003). Moreover, in *P. sativum* the chitin oligomers N,N',N'',N'''-tetraacetylchitotetraose (CT4) and the N,N',N'',N'''-pentaacetylchitotetraose (CT5), which represent the two unsubstituted backbones of *Rhizobium leguminosarum* bv. *viciae* Nod factor, induced calcium spiking but not calcium flux (Walker et al., 2000). In *M. truncatula*, Nod factor lacking the sulphate group on the reducing end (purified from bacteria lacking the *nodH* gene) induced calcium spiking at 10 nM, but failed to induce reproducibly the calcium flux (Shaw and Long, 2003). Thus, in both *P. sativum* and *M. truncatula*, Nod-factor-like molecules induce calcium spiking without the induction of calcium flux (Walker et al., 2000; Shaw and Long, 2003). This evidence suggests that while calcium spiking requires a low stringency of Nod factor perception, a high concentration of specific Nod factor structure is required to elicit the calcium influx. The spatial distribution of calcium flux and calcium spiking is also different: while calcium spikes originate in the nuclear area and propagate as a wave outward, calcium flux begins at cell tip and moves towards the nucleus (Shaw and Long, 2003). Additional evidence showing that calcium influx and calcium spiking are separate responses comes from genetic analysis. Mutations in the receptor-like kinase *DMI2*/*SYMRK*, in the cation channels *DMI1*/*POLLUX, CASTOR* and nucleoporins *NUP85* and *NUP133* block calcium spiking but not calcium flux (Shaw and Long, 2003; Kanamori et al., 2006; Miwa et al., 2006b; Saito et al., 2007). These findings indicate that calcium influx and calcium spiking are independent of one other and may, thus, be involved in triggering different (but possibly overlapping) responses (Oldroyd and Downie, 2004).

Whereas several studies have shown that calcium spiking is required for Nod factor-induced gene expression and nodulation (Wais et al., 2000; Charron et al., 2004; Miwa et al., 2006a), the function of the calcium flux response remains uncertain. Analysis of infection thread initiation in vetch by nodulation mutants of *R. leguminosarum* first suggested a role for ion influx in the formation of infection threads (Walker and Downie, 2000). In those experiments the role of *nodO* in stimulating infection thread development in vetch and pea was investigated. *nodO* is a *R. leguminosarum* nodulation gene that encodes a secreted protein (Economou et al., 1990) involved in pea and vetch nodulation. This protein binds to calcium and forms cation-selective channels that allow the

movement of monovalent cations (K^+ and Na^+) across the membrane (Sutton et al., 1994). These experiments showed that the double mutant *nodE nodO* was severely impaired in its ability to form normal infection threads. It induced strong root hair deformation and formed many infection foci, yet infection threads were seldom initiated. This strain exhibited a number of novel infection-related events such as intracellular accumulation of bacteria at the base of the root hair and enlarged infection threads. NodE is required for the synthesis of the appropriate $C_{18:4}$ acyl groups to the Nod factor (Spaink et al., 1991) and in *nodE* mutants a $C_{18:1}$ acyl group is present. Interestingly, the single *nodE* and *nodO* mutants displayed infection phenotypes similar to wild type suggesting that a NodO-induced ion flux across the membrane may compensate for an infection defect associated with inappropriate Nod factor production (Walker and Downie, 2000). These results demonstrated that the initial entry of bacteria into root hairs does not require host specific substitution on the Nod factor. However, additional specificity, which can be determined by either by a modified acyl group (NodE) or by NodO encoding a secreted pore-family protein, is necessary for growth of a properly directed infection thread.

Based on the observations that high concentrations of Nod factor are required to induce the calcium flux and that a calcium-binding protein, forming a cation channel, can stimulate for infection thread growth (Sutton et al., 1994; Walker and Downie, 2000), Miwa et al. (2006) suggested that calcium flux plays a role in infection thread initiation. The model of Miwa et al. (2006) proposes that during the early stages of nodulation, low levels of Nod factor can induce root hair deformation, calcium spiking and initiate the accumulation of rhizobia in infection foci. As a result, the Nod factor levels in the foci rise, and this induces a calcium flux that can drive infection thread initiation (Miwa et al., 2006b). The observations that calcium flux can be induced in cells that are already spiking (Walker et al., 2000; Shaw and Long, 2003) are consistent with this model, which proposes that normally rhizobia induce calcium spiking prior to calcium flux.

Infection thread formation is dependent on rhizobial genes such as *nodF* and *nodL*. A *S. meliloti* mutant carrying mutations in these genes was compromised for infection thread formation (Ardourel et al., 1994). The *nodL* and the *nodF nodL* double mutants produced Nod factor with a modified structure. *S. meliloti* Nod factor normally has a backbone of four β-1,4-linked *N*-acetylglucosamine residues with an *N*-linked fatty acyl chain of 16 carbons (with one to three double bounds) attached to the non-reducing

end, a sulphate group at the reducing end and a O-acetyl group on the non-reducing terminal sugar (Figure 3.1A) (Lerouge et al., 1990; Denarie et al., 1996; Downie and Walker, 1999). The Nod factor released by the *nodF nodL* mutant lacks the acetate group at the non-reducing terminal sugar and carries an acyl chain of 18 carbons and one double bond (Figure 3.1B), whereas *S. meliloti* carrying a mutation in only *nodL* lacks the acetate group at the non-reducing terminal sugar (Figure 3.1C) but carries the C_{16} acyl chain. The aim of this chapter was to test the Nod factors structures produced by *nodL* and *nodF nodL* mutants for induction of calcium flux. For this purpose, I first established a system to reproducibly test and analyse calcium flux.

The Nod factor-induced changes in intracellular calcium have been observed using various imaging techniques. The early calcium flux in alfalfa root hairs was shown using calcium selective microelectrodes inside and outside the cell (Felle and Hepler, 1997; Felle et al., 1999b). Rapid increases in cytosolic calcium concentration have also been observed using calcium-sensitive dyes. The protein aequorin, developed in *Arabidopsis* (Johnson et al., 1995), works well for analyzing calcium changes in response to salt stress, osmotic stress, cold stress and circadian cycle in whole plants (Plieth, 2001), but its luminescent intensity is too weak to analyze calcium changes in single cells. Thus, calcium-sensitive fluorescent dyes have been microinjected into the plant cell cytoplasm to analyze calcium changes in single cells such as guard cells (Gilroy et al., 1991; Grabov and Blatt, 1998; McAinsh et al., 1990; Schroder and Hagiwara, 1990) and root hair cells (Ehrhardt et al., 1996; Walker et al., 2000). This approach has provided a tool to detect and study following cytoplasmic calcium changes, which are not recorded by the microelectrode recording technique. Nod factor-induced calcium spiking was originally described in alfalfa using calcium indicator dyes. After that, different calcium-sensitive dyes have been used for recording calcium responses in different legumes. Increases in root hair calcium were observed in *Vicia sativa* using acid loading of Indo-1 (De Ruijter et al., 1998), in *P. vulgaris* and *Vigna unguiculata* root hairs using microinjected dextran-linked Fura-2 (Gehring et al., 1997; Cardenas et al., 1999), and *M. sativa*, *M. truncatula*, *P. sativum* and *L. japonicus* using micro-injected dextran-linked Oregon green (Ehrhardt et al., 1996; Wais et al., 2000; Walker et al., 2000; Shaw and Long, 2003; Miwa et al., 2006b). Calcium Green and other single wavelength dyes allow periodicity to be observed, but a ratio-imaging technique using Dextran-coupled fluorescent calcium-insensitive dye has

been developed to unambiguously identify singular and repetitive changes in cytoplasmatic calcium ion concentration, independent from local changes in cytoplasmic volume. Therefore, a single wavelength calcium-sensitive dye (Oregon Green-dextran) has been coinjected with a calcium insensitive dye (Texas Red-dextran) for monitoring both calcium flux and calcium spiking (Shaw and Long, 2003; Miwa et al., 2006b). Microinjection of calcium-sensitive dyes has provided much information on the relative position within the cell of these changes in calcium concentration. However, due to the limitation of this technique, the analysis of calcium changes in nodulation signalling has been limited to young, growing root hair cells.

The Yellow Cameleon (YC) calcium reporter, which has been used to analyse changes in calcium in transgenic animals (Miyawaki et al., 1997, 1999), can overcome the limitation of microinjection, allowing the non-destructive and single-cell analysis of calcium in single cells. This yellow cameleon is a chimeric protein composed of a cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), linked by calmodulin and calmodulin-binding peptide. Calcium binding induces a conformational change in the cameleon proteins, which increase the efficiency of fluorescent resonance energy transfer (FRET) from CFP to YFP. Thus, as the calcium concentration increases, fluorescence from YFP increases while fluorescence from CFP decreases. This cameleon system has been successfully applied to *Arabidopsis* plants to study cytoplasmatic calcium in guard cells (Allen et al., 1999), and to analyze calcium dynamics in pollen tubes (Iwano et al., 2004). *M. truncatula* seedlings expressing the calcium-sensing yellow cameleon YC 2.1 have been used to study the Nod Factor-induced calcium spiking (Miwa et al., 2006a; Sieberer et al., 2009). Only one trace showing calcium flux was reported in that work, but a proper analysis of calcium flux using different concentrations of Nod factor had not yet been described using the cameleon calcium reporter assay.

Here, I have established a system to record and analyze calcium flux in *M. truncatula* root hair expressing yellow cameleon 2.1 and I have used this to assess the effect of different Nod factor structure on the induction of calcium flux.

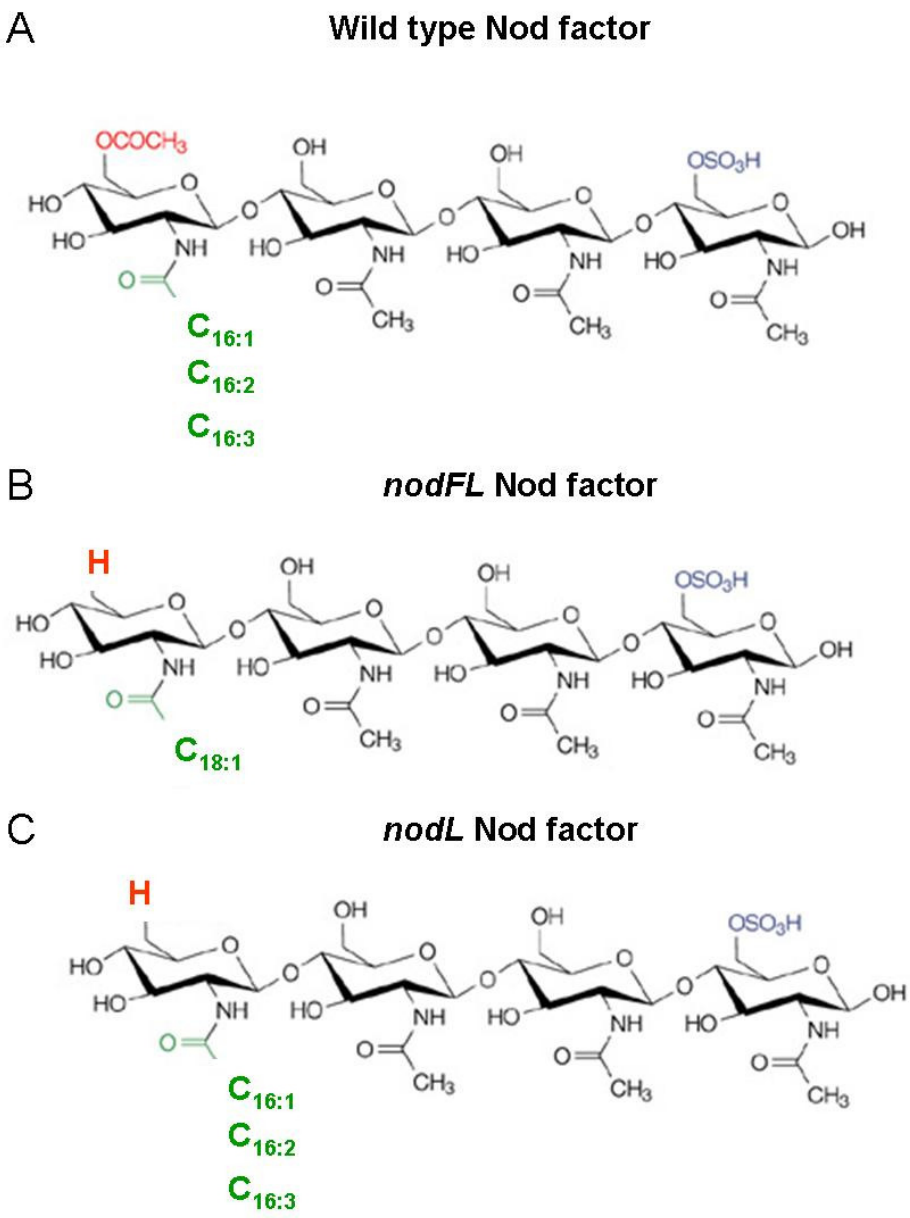


Figure 3.1. Structure of the lipooligosaccharide Nod factors assayed in this study.

A, S. meliloti wild type Nod factor has a backbone of four β-1,4-linked N-acetylglucosamine residues with an N-linked fatty acyl chain of 16 carbons with one to three double bounds, a sulphate group on the reducing sugar and an O-acetyl group on the non-reducing terminal sugar. B, The Nod factor made by the *nodF nodL* mutant lacks the acetate group and carries a fatty acyl chain of 18 carbons and one double bond. C, Nod factor released by S. meliloti carrying a mutation in nodL only lacks the acetate group at the non-reducing terminal sugar (modified from Oldroyd and Downie 2004).

Results

Analysis of Nod factor-Induced Calcium Influx in Root Hair Cells Using *M. truncatula* Plants Expressing YC 2.1.

To examine calcium flux in response to Nod factor, a seedling was mounted in a small bath (100 µl) of FP medium, set on an epifluorescence microscope. One µl of Nod factor was added to the liquid in the incubation bath to give the final desired concentration of Nod factor. Images of CFP and YFP were taken up to 30 minutes after Nod factor addition. Ratio values of YFP/CFP were plotted over time for treated plants. Wild type plants expressing YC 2.1 showed an increase in fluorescence prior to induction of calcium spiking. The calcium flux originates at the tip of the root hair cell (Shaw and Long, 2003). Therefore in order to determine whether an increase of calcium concentration was indeed a calcium flux, fluorescence imaged at the tip and at the shaft of the root hair were taken for each cell and compared to each other. The tip area was included around 10 µm from the tip of the root hair toward the base of the root hair cell, whereas the shaft area included the part of the root hair cell protruding from the root (Figure 3.2). Only those cells showing a calcium increase in the area of the root hair cell protruding from the root as well as the root hair tip were considered positive for calcium flux. In most cases the tips of root hair cells displayed a higher increase in fluorescence compared to the cell area (Figure 3.3A). However, in some case the fluorescence increase from the tip area was similar to the fluorescence increase in the cell area (Figure 3.3B). These cells were considered positive for Nod factor-induced calcium flux because both tip and cell area exhibits increase in fluorescence, whereas cells that showed calcium flux only in the tip (Figure 3.3C) or cell area were not considered positive. Representative calcium traces defective for calcium flux did not show increase in calcium either in tip or in the cell area (Figure 3.3D). Addition of water to the chamber containing the seedling did not induce any change in YFP/CFP fluorescence (data not shown).

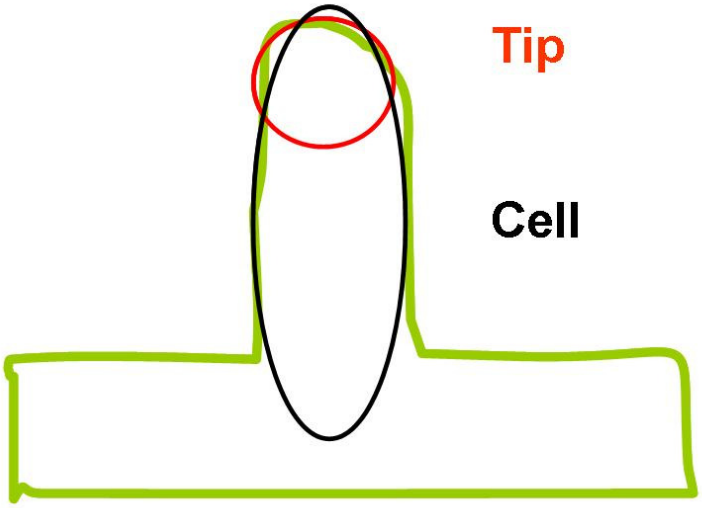


Figure 3.2. Tip and cell areas selected for the analysis of Nod factor-induced calcium changes in root hair cells.

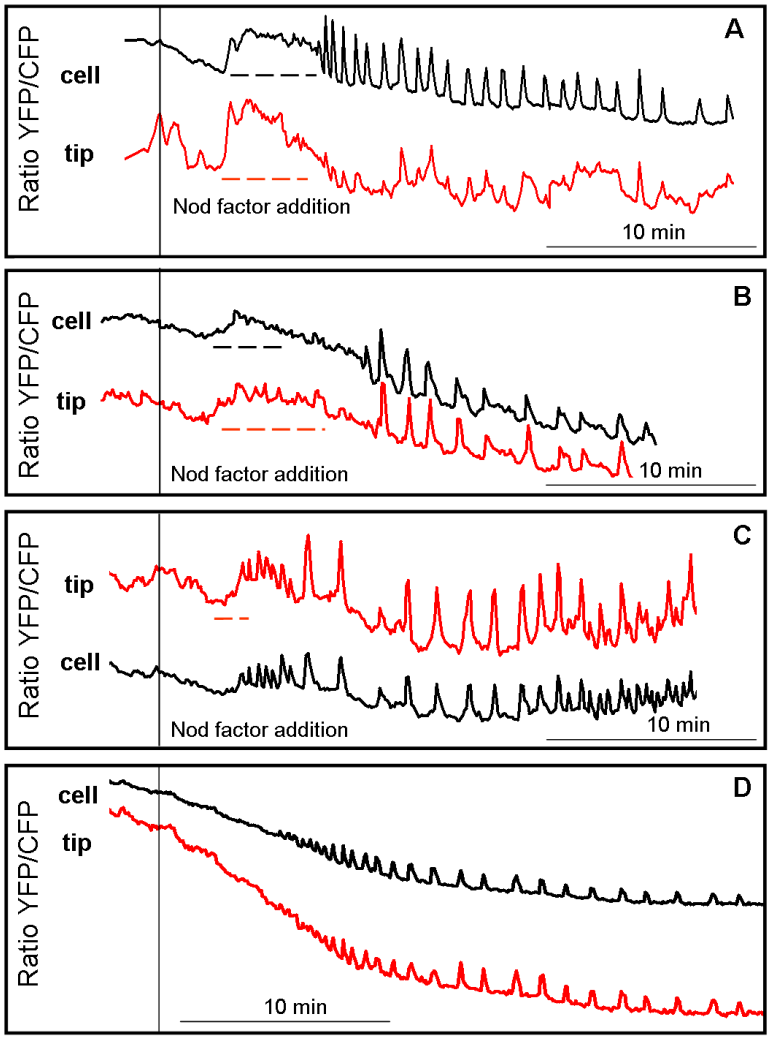


Figure 3.3. Analysis of calcium flux in root hair tip and cell area in *M. truncatula* plants expressing YC 2.1.

The ratio YFP/CFP (relative units) was plotted against time. Images were taken at 5 seconds intervals for > 30 minutes. Traces from the tip (red) and cell area (black) were analysed for each root hair cell. A, Representative traces of root hair cell showing a stronger increase in calcium in the tip compared to the cell area. B, Traces of a root hair cell showing similar increase of calcium in tip and cell area. Traces C show an increase of calcium in the tip area but not in the cell. D, Representative negative trace for calcium flux. Only those cells showing a significant increase in calcium in the tip and in the cell area were selected positive for calcium flux (A, B). The dotted horizontal lines indicate calcium flux observed in tip (red) and in the cell (black). Nod factor was added (vertical black line) at 10 nM (A,B), 1 nM (C) and 0.1 nM (D).

Titration of Nod Factor-Induced Calcium Influx and Calcium Spiking in *M. truncatula* Root Hair Cells Expressing YC 2.1.

Calcium flux has been previously studied using microinjection of calcium sensitive dyes, but a titration of Nod factor concentration for the activation of the calcium flux has not yet been described. To analyse the responsiveness of YC plants to Nod factor for induction of calcium flux, I assayed the number of root hair cells that triggered calcium flux at concentrations of Nod factor ranging from 10^{-10} M to 10^{-6} M (Figure 3.4A). A minimum of 10 root hair cells from at least 3 different seedlings was tested for each concentration of Nod factor. Cytoplasmic calcium spiking initiated 5 to 18 minutes after Nod factor addition and persisted throughout the experiment (>30 minutes). At high Nod factor concentrations, ranging from 10^{-6} M to 10^{-8} M, most of the cells were positive for calcium flux response: at 1 µM, 100 nM and 10 nM Nod factor, 15 of 16 cells (3 plants), 16 of 17 cells (3 plants) and 15 of 16 root hair cells (4 plants) were positive for calcium flux, respectively. When Nod factor was added at 1 nM, only 4 of 15 cells showed calcium flux in 3 plants tested, whereas 0 of 10 root hair cells showed a calcium flux after addition of 0.1 nM Nod factor. The concentration at which 50% of root hairs showed a calcium flux was about 3×10^{-9} M. A typical trace lacking a calcium flux is shown in Figure 3.3D.

Calcium spiking was also analysed with wild-type Nod factor using different concentrations of Nod factor ranging from 10^{-14} M to 10^{-6} M (Figure 3.4B). Oldroyd et al. (2001) previously showed a similar titration curve using microinjection of root hair cells with the calcium sensitive dye Oregon Green. The concentration at which 50% of root hairs show calcium spiking was 7×10^{-13} M. As previously reported by Shaw and Long (2003), induction of calcium spiking required a lower concentration of Nod factor; the concentration of Nod factor required for calcium flux is in the region of 4000 fold higher then that required for calcium spiking.

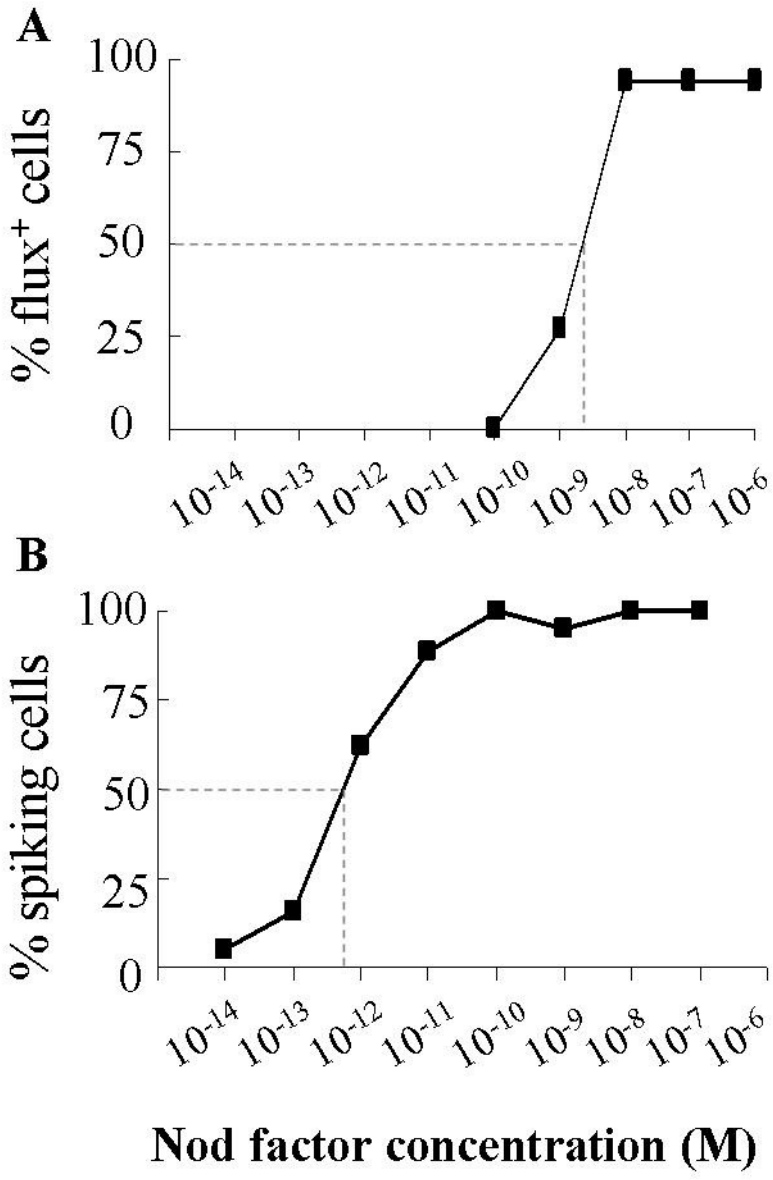


Figure 3.4. Titration curve for calcium flux and calcium spiking induced by Nod factor extracted by S. meliloti in M. truncatula plants expressing YC 2.1.

Multiple cells from wild-type plants were assessed for their calcium flux (A) and calcium spiking (B) response to different concentrations of Nod factor. The percentage of cells that showed calcium flux (A) and calcium spiking (B) is indicated. A minimum of 10 cells from at least three different plants were tested for each concentration of Nod factor plotted. Grey dotted lines indicate concentration at which 50% of root hairs showed calcium flux.

Analysis of Calcium Flux Induction by *nodFL* Nod Factor

In order to study the role of calcium flux in infection thread growth, I analysed the induction of calcium flux using a synthetic Nod factor (kindly synthesised by E. Martinez, CERMAV-CNRS, France) structurally similar to that (Figure 3.1) released by the *S. meliloti nodF nodL* mutant, which is compromised for infection thread formation (Ardourel et al., 1994). The *nodF nodL* double mutant can induce root hair deformation and can form infection foci in root hairs, but the infection threads abort at a very early stage (Ardourel et al., 1994). Synthetic Nod factor lipochitooligosaccharide IV, $C_{18:1}$, sulphate (LCO, IV, $C_{18:1}$, S), which carries a $C_{18:1}$ *N*-acyl group and lacks the acetate group (equivalent to *nodF nodL* Nod factor), did not induce calcium flux when added at 10 nM although, as expected (Oldroyd et al., 2001a), it induced calcium spiking (Figure 3.5B). LCO IV, $C_{18:1}$, S is less active at inducing calcium spiking than wild-type Nod factor (Oldroyd et al., 2001a). Therefore, I tested if a similar reduction in activity was observed for the induction of calcium flux. Even using a 100 times greater concentration (1 µM) of LCO IV, $C_{18:1}$, S (*nodF nodL* Nod factor), most of the cells were still defective for calcium influx (Figure 3.6A): only two of 16 root hair cells showed calcium flux. A trace showing calcium flux induced by 1 µM of LCO IV, $C_{18:1}$, S is shown in Figure 3.7. No difference was observed in the type of calcium flux induced in cells stimulated by low or high Nod factor concentration.

Calcium spiking was also analysed using different concentrations of *nodF nodL* Nod factor. As previously shown (Oldroyd et al., 2001a) *nodF nodL* Nod factor was less active in inducing calcium spiking compared to wild type Nod factor (Figure 3.6B). The concentration at which 50% of root hairs show calcium flux was 8×10^{-10} M, compared to 7×10^{-13} M for wild type Nod factor. This indicates that *nodF nodL* Nod factor was approximately 1000 time less active than wild-type Nod factor for induction of calcium spiking.

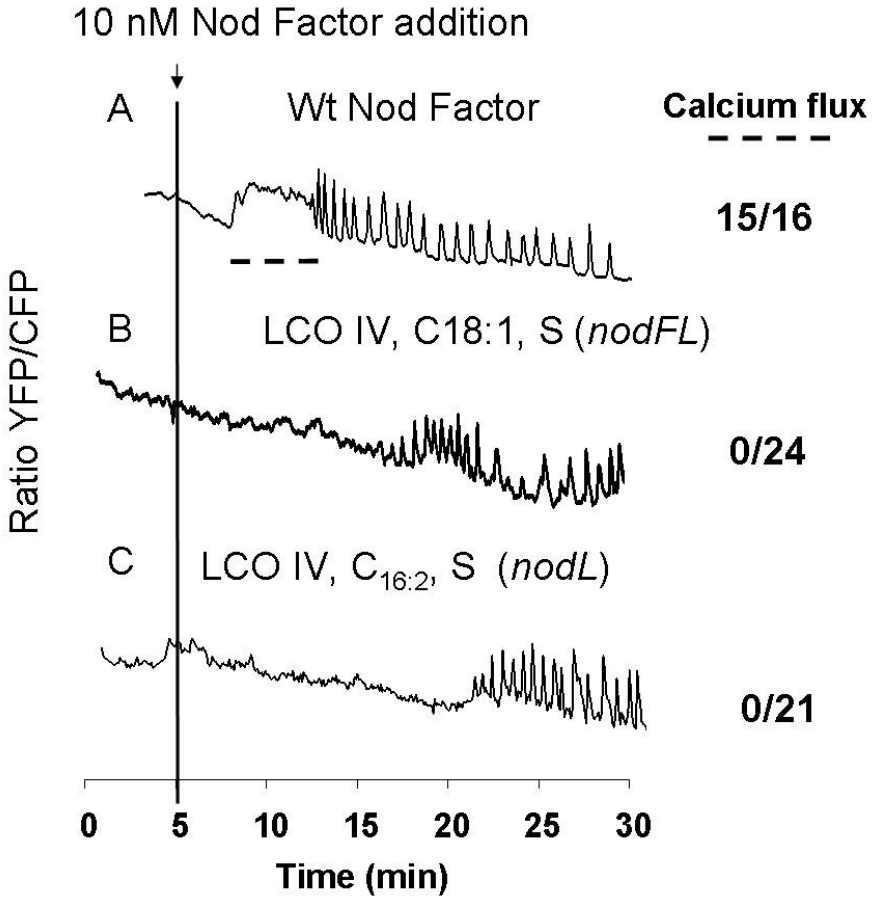


Figure 3.5. *nodFL* and *nodL* Nod factors do not induce calcium flux.

Change of intracellular calcium in wild-type *M. truncatula* root hairs treated with 10 nM Nod factor equivalent to that produced by wild type (A), the *nodF nodL* mutant (B) and *nodL* mutant (C). Root hairs were imaged using cameleon. The black vertical line indicates addition of Nod factor. The horizontal dashed black line shows calcium flux. The numbers indicate number of cells with calcium flux/total number of cells analysed with each Nod factor. A minimum of three plants has been tested in each treatment. The traces show data of changes in fluorescence of ratio of YFP:CFP fluorescence (arbitrary units).

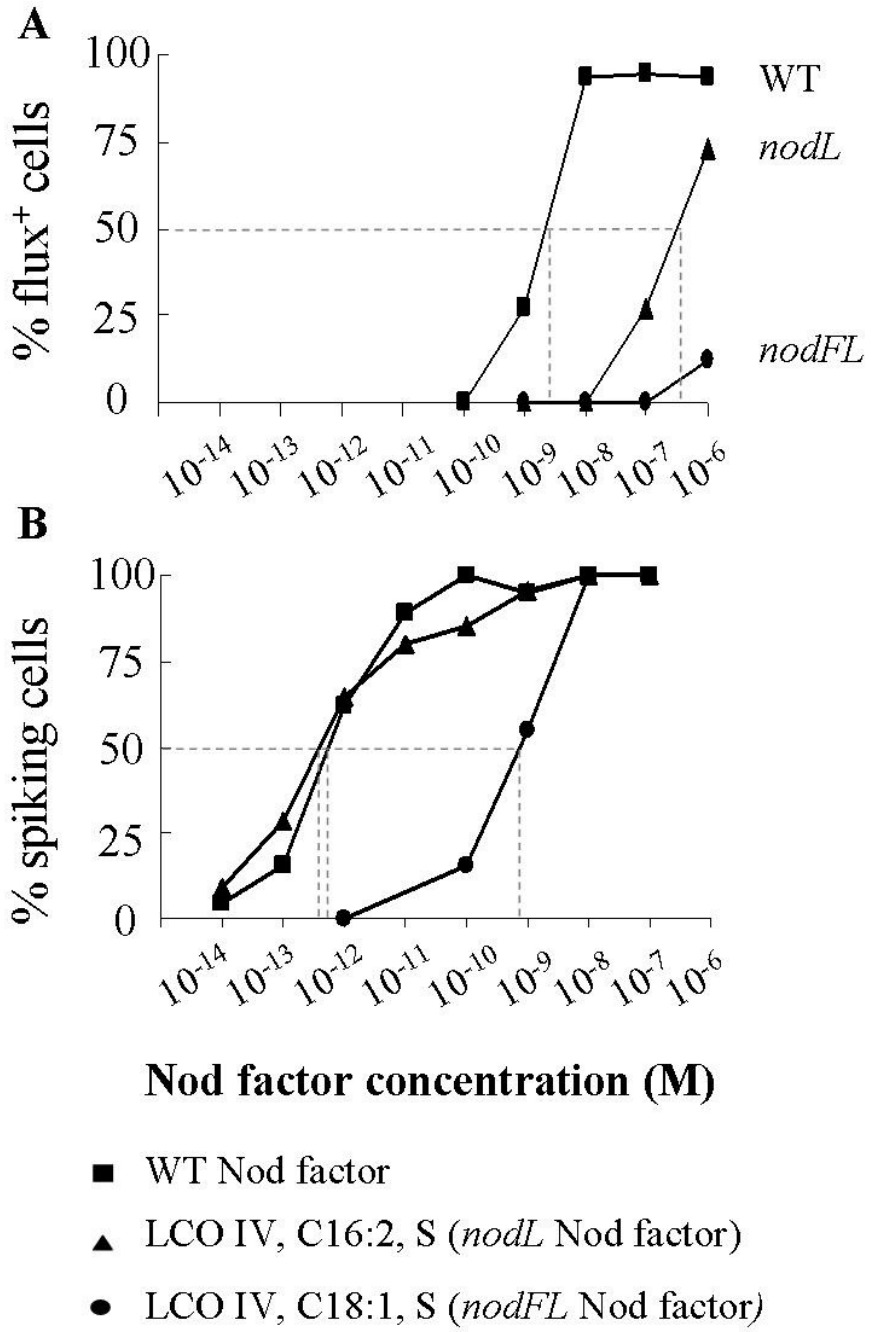


Figure 3.6. Dose response curves for calcium flux and calcium spiking induced by different Nod factors on wild-type *M. truncatula*.

Multiple cells from wild type plants were assessed for their calcium flux (A) and calcium spiking (B) responses to different concentrations of wild type Nod factor (square, shown for comparison), synthetic *nodL* Nod factor (triangle) and synthetic *nodFL* Nod factor (circle). The percentage of cells that showed calcium flux (A) and calcium spiking (B) is indicated. A minimum of ten root hair cells from at least three different plants were tested for each in each treatment.

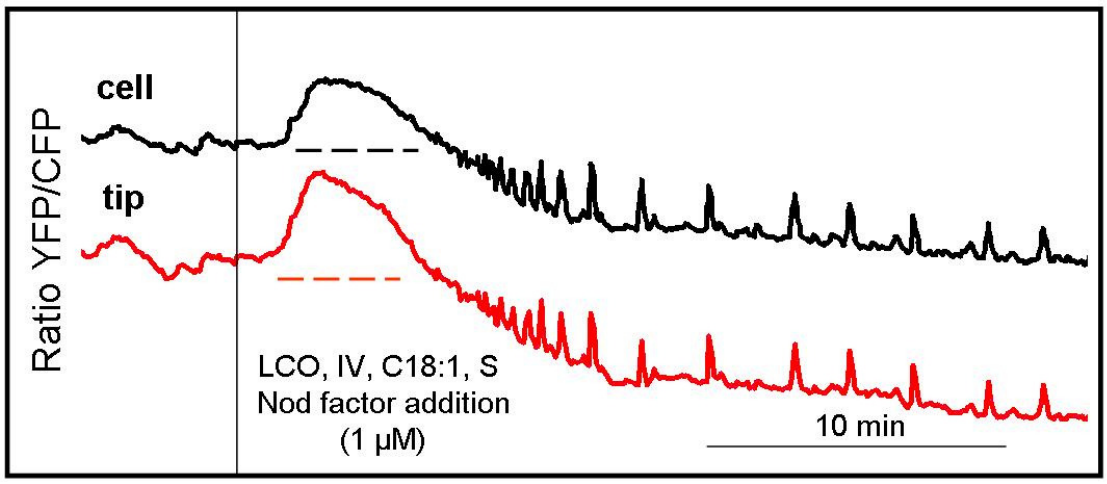


Figure 3.7. LCO, IV, C18:1, S **(**equivalent to nodFL Nod factor) induced calcium flux only in 2 of 16 cells treated at 1 µM concentration.

The ratio YFP/CFP was plotted against time. Images were taken at 5 seconds intervals for > 30 minutes. Traces from the tip (red) and cell area (black) were analysed. The dotted horizontal lines indicate calcium flux observed in tip (red) and in the cell (black).

Analysis of Calcium Flux Induction by *nodL* Nod Factor

The inability of the *nodFL* Nod factor to induce calcium flux could be due to the lack of the acetyl group or to the presence of the $C_{18:1}$ instead of the $C_{16:2}$ acyl group. To test this, I analysed the induction of calcium flux by LCO IV, $C_{16:2}$, S (equivalent to that formed by a *nodL* mutant, synthesised by E. Martinez, CERMAV-CNRS, France), which lacks the acetyl group but carries a wild-type acyl group (Figure 3.1C). No calcium flux was induced by LCO IV, $C_{16:2}$, S (*nodL*) Nod factor (10 nM) (Figure 3.5C). It required a 100-fold higher concentration of the *nodL* Nod factor than the wild type Nod factor to induce a calcium flux (Figure 3.6A). The concentration at which 50% of root hairs show calcium flux was 5×10^{-7} M, compared to 3×10^{-9} M for wild type Nod factor. The *nodL* mutant can induce infection thread formation although at a lower level then wild type. Wild-type *Sm* IV Nod factor ($C_{16:2}$, Ac, S) induced calcium flux before calcium spiking in almost all of the cells tested (10 nM, Figure 3.5A).

Different concentrations of *nodL* Nod factor were also tested for induction of calcium spiking. Calcium spiking was normal for *nodL* Nod factor (Figure 3.6B). The concentration at which 50% of root hairs showed calcium flux was 5×10^{-13} M. This was similar to the 7×10^{-13} M found for 50% activity with the wild type Nod factor. These results are consistent with the previous report showing a titration of calcium spiking for different concentrations of wild type and *nodL* Nod factor using microinjection technique (Oldroyd et al., 2001a).

Discussion

Calcium flux is one of the earliest responses induced by Nod factor in root hair cells. In this present study, I showed that Nod factors equivalent to those produced by *S. meliloti nodF nodL and nodL* mutants affected for infection were greatly decreased for induction of calcium flux. These observations provide support for the hypothesis that calcium flux is required during the formation of infection threads (Miwa et al., 2006b). The Nod factor released by the *nodF nodL bacterial* mutant is unable to induce infection thread formation (Ardourel et al., 1994). Here, I show that the synthetic LCO IV, $C_{18:1}$, S Nod factor, structurally equivalent to Nod factor released by *nodF nodL* bacterial mutant was defective for calcium flux even when Nod factor was added at micromolar concentrations. As previously shown, LCO IV, $C_{18:1}$, S was less active at inducing calcium spiking than wild-type Nod factor (Oldroyd et al., 2001a).

The Nod factor released by the *nodF nodL* bacterial mutant lacks the acetate group on the non reducing terminal sugar and carries a $C_{18:1}$ fatty acyl chain instead of the $C_{16:2}$ of wild type Nod factor. To determine which of these structural changes was responsible for the lack of calcium flux I tested LCO IV, $C_{16:2}$, S (equivalent to that formed by a *nodL* mutant), which lacks the acetyl group but carries a wild-type acyl group and I found that it was affected for induction of calcium flux. This indicates that plant recognition of the acetate group at the non-reducing terminal sugar in the Nod factor structure is a key element required for the induction of calcium flux. The synthesis of Nod factor carrying a $C_{18:1}$ fatty acyl chain and carrying the acetate group at the non reducing terminal sugar (equivalent the Nod factor released by the *S. meliloti nodF* mutant) is technically very difficult therefore it was not possible to test the induction of this Nod factor for induction of calcium flux. The results showing that 10 nM of *nodL* and *nodFL* Nod factors induce calcium spiking but not calcium flux support that calcium spiking and calcium flux are two different and separable responses.

The Nod factor equivalent to that produced by *nodL* mutant was shown to be normal for the induction of calcium spiking, but reduced and delayed for the growth of infection threads (Ardourel et al., 1994). These results suggest that, when calcium spiking is induced normally but calcium flux is not, the plant can still induce infection thread formation but the process is reduced and delayed. This is the case of the infection

phenotype induced by *nodL* Nod factor. However, when both calcium responses are abolished, as we observed with *nodFL* Nod factor, no infection threads are formed. Taken together, these results suggest that the two calcium responses induced by Nod factor, calcium flux and calcium spiking, are both required for the initiation of infection thread growth.

In this study, I used cameleon calcium sensor to observe calcium spiking and singular rises in cytoplasmic calcium concentration resulting from exposure to Nod factor. Titration curves of calcium spiking using different concentration of wild type, *nodFL* and *nodL* Nod factor obtained using cameleon imaging were similar to previous titration curves recorded using microinjection of the calcium-sensitive dye Oregon green (Oldroyd et al., 2001a). This is consistent with previous comparisons of the two calcium imaging techniques (Miwa et al., 2006a).

In summary, a key novel observation is that LCO IV, $C_{16:2}$, S, equivalent to that formed by a *nodL* mutant, is greatly reduced for induction of the calcium flux. Previous observations (Oldroyd et al., 2001a) showed that this Nod factor is wild-type for induction of calcium spiking. Thus, there is a link between Nod factor structure, calcium flux, and the lack of initiation of normal infection. Earlier publications suggested a role for calcium influx in the infection of legume by rhizobia, but such a role was only based on the observations that a high concentration of Nod factor is required to induce calcium flux and that a calcium-binding protein forming a cation channel can stimulate infection (Walker and Downie, 2000). The results presented in this chapter provide the missing link by directly showing that Nod factor released by bacterial mutants unable to infect legumes are impaired for calcium flux. These results strongly suggest that calcium flux correlates with infection thread formation.

Downstream of calcium spiking, several components have been identified and shown to be required for the expression of early nodulation genes. On the other hand, genes induced downstream of calcium flux are still unknown. The only evidence we have are the results here presented with the structurally modified Nod factors which correlate calcium flux with the formation of infection threads. However, a future challenge is the identification of genes induced downstream of calcium flux. These results have shown that the *nodL* mutant Nod factor induces calcium spiking in the same way as wild type Nod factor across a range of concentrations. However, at 10 nM, *nodL* Nod factor induces

no flux, whereas wild type Nod factor induces 100% flux. This opens the opportunity to do a microarray to identify genes expressed by 10 nM wild type Nod factor but not induced by 10 nM *nodL* Nod factor. These future experiments could identify genes specifically induced by calcium flux and reveal exciting new events in the infection of legume by rhizobia.

CHAPTER 4

Analysis of Nod Factor-Induced Calcium Responses in the *Medicago truncatula* and *Lotus japonicus* Mutants Affected for Infection Thread Formation

Introduction

Several lines of evidence suggest that calcium spiking is central to the Nod factor signalling pathway and is essential for the expression of early nodulin genes required for nodulation. Pharmacological antagonists shown to block Nod factor-elicited calcium spiking are also efficient inhibitors of early nodulin gene induction (Charron et al., 2004). Moreover, several nodulation mutants, as *M. truncatula* (*Mt*) *nfp/ L. japonicus* (*Lj*) *nfr5*, *Lj nfr1, Mtdmi2/Ljsymrk, Mtdmi1/Ljpollux, castor, Ljnup85* and *Ljnup133*, are also impaired for induction of calcium spiking and are unable to trigger gene expression of early nodulin genes (Wais et al., 2000; Amor et al., 2003).

Although several genes have been identified to be required for the induction of calcium oscillations, so far the only nodulation mutants affected for induction of calcium flux are the Nod-factor receptor mutants *nfp* in *M. truncatula* and *nfr1* and *nfr5* in *L. japonicus*, which are defective for all Nod factor-induced responses. The absence of nodulation mutants compromised for calcium flux has kept the role of this calcium response unclear. It has been previously suggested that calcium flux could play a role during the formation of infection thread by rhizobia (Miwa et al., 2006b). Analysis of infection threads induced by the *nodFL* and *nodL S. meliloti* mutants revealed that Nod factor structural specificity is required for the initiation and development of infection threads. The results presented in Chapter 3 show that the synthetic Nod factors, chemically equivalent to those produced by the *nodFL* and *nodL S. meliloti* mutants, were greatly reduced for the ability to induce calcium flux. These data support the hypothesis that calcium flux is required for infection thread growth. The formation of infection

threads is dependent on plant genes such as *HCL*, *NIN*, and *BIT-1*. The plant mutants *hcl*, *nin* and *bit-1* are unable to develop infection threads when inoculated with *S. meliloti* (Limpens et al., 2003; Marsh et al., 2007; Smit et al., 2007).

The genes *L. japonicus NAP1* (for Nck-associated protein 1) and *PIR1* (for 121F-specific p53 inducible RNA) have been recently characterised (Yokota et al., 2009; Miyahara et al., 2010). *NAP1* and *PIR1* were found to be essential for infection thread formation and colonisation of *L. japonicus* roots by its bacterial symbiont, *M. loti* (Yokota et al., 2009). *NAP1* and *PIR1* were previously shown to be involved in actin rearrangements (Li et al., 2004). The first observable events after adding purified bacterial Nod factor to roots occur in root hairs within minutes; changes in rates of cytoplasmic streaming, nuclear movements, and changes in the shape of the vacuole are all observed. Within ten minutes, the endoplasmic reticulum shifts position towards the tip of the root hair (Allen and Bennett, 1996; Miller et al., 2000). This alteration of the cytoarchitecture is accompanied by changes of the actin and microtubule cytoskeleton (Cardenas et al., 1998; de Ruijter et al., 1999; Weerasinghe et al., 2003; Vassileva et al., 2005; Weerasinghe et al., 2005). In at least four species, bean (*Phaseolus vulgaris*), alfalfa (*Medicago sativa*), common vetch (*Vicia sativa*) and lotus, treatment of root hairs with Nod factor leads to rapid changes (within three to five minutes), in the polymerization pattern of actin (Cardenas et al., 1998; Weerasinghe et al., 2005). In growth-arresting root hairs, those that show root hair deformation in response to Nod factor, endoplasmic microtubules disappear shortly after Nod factor addition, but reform within 20 minutes (Sieberer et al., 2005). Nod factor was shown to trigger fragmentation of long actin bundles extending into the root hair apical tips, and subsequently fine bundles of filaments accumulate in the apical/subapical region in responding root hairs (Cardenas et al., 1998; de Ruijter et al., 1999; Weerasinghe et al., 2005). Nod-factor signalling elicits several other early responses in compatible host root-hair cells, including membrane depolarisation, ion fluxes across the membrane in the tip of the root hairs, and calcium spiking around the nuclear area (Ehrhardt et al., 1992; Ehrhardt et al., 1996; Felle et al., 1998, 1999a; Walker et al., 2000; Shaw and Long, 2003). The temporal relationship between Nod factor-induced actin rearrangement and calcium responses and how these responses are mechanically linked is still unknown.

When infected with *M. loti*, the *nap1* and *pir1* mutants developed an excess of uncolonised nodule primordia, indicating that these two genes were not necessary for the initiation of nodule organogenesis *per se*. Nevertheless, the formation of infection threads and their subsequent progression into the root cortex were significantly impaired in these mutants. Root hair cells of the *nap1* and *pir1* mutants were short and had mostly transverse or web-like actin filaments, while, in wild-type root hairs, bundles of actin filaments were mainly longitudinal. Consistent with these observations, differences in the temporal and spatial organisation of actin filament were also observed between wild-type and mutant root hairs after Nod factor treatment (Yokota et al., 2009).

The availability of mutants that induce root hair deformation but lack infection thread growth allowed an analysis of calcium responses in mutants predicted to retain initial Nod factor perception but possibly lack the subsequent Nod factor signalling that could lead to initiation of infection. The available mutants would also enable an analysis of the relationship between actin rearrangement and calcium responses.

Results

Analysis of Calcium Flux Using Microinjection of Oregon Green and Texas Red

Microinjection of cells with calcium sensitive dyes, as Oregon Green, allow an accurate recording of changes in cytoplasmic calcium levels and is commonly used to analyse calcium responses in animal and plant cells. However, cytoplasmic streaming can influence the changes in fluorescence recorded using Oregon Green because it is a single wavelength dye. Unlike calcium spiking, calcium flux is a unique event which occurs a few minutes after the addition of Nod factor, and, because of this, it can be easily confused with a non-specific increase in recorded fluorescence. Co-injection of Oregon Green with a calcium insensitive reference dye greatly reduces non-specific signals. Therefore, ratiometric analysis of the fluorescence derived from the two dyes has been developed and used for analysis of Nod factor-induced calcium flux in *M. truncatula* and *L. japonicus* root hair cells (Shaw and Long, 2003; Miwa et al., 2006b).

Although, ratiometric analysis with Oregon Green and Texas Red provide a better analysis of calcium flux compared to single wavelength dye, the baseline fluorescence of untreated cells can present sporadic increases in fluorescence, generally called background noise, which make difficult the analysis of specific calcium response to Nod factor. Calcium flux is known to originate at the tip of root hair cells (Shaw and Long, 2003). Therefore, in order to prevent confusion in the analysis of calcium changes after Nod factor addition, the fluorescence obtained from the tip and the cell was compared for each cell microinjected. These data were analysed in the same way as described for analysis of calcium flux using yellow cameleon in Chapter 3. Figure 4.1A shows an example of a calcium trace, obtained after microinjection with Oregon Green and Texas Red. This trace shows background noise in the baseline fluorescence prior to and after the addition of Nod factor. Thus, based on this trace it is difficult to establish if this cell is positive for Nod factor-induced calcium flux. Nevertheless, when this trace is compared with the fluorescence of the tip of the root hair (Figure 4.1B) it is clear that this root hair is positive for calcium flux, which was induced within one minute of the addition of Nod factor.

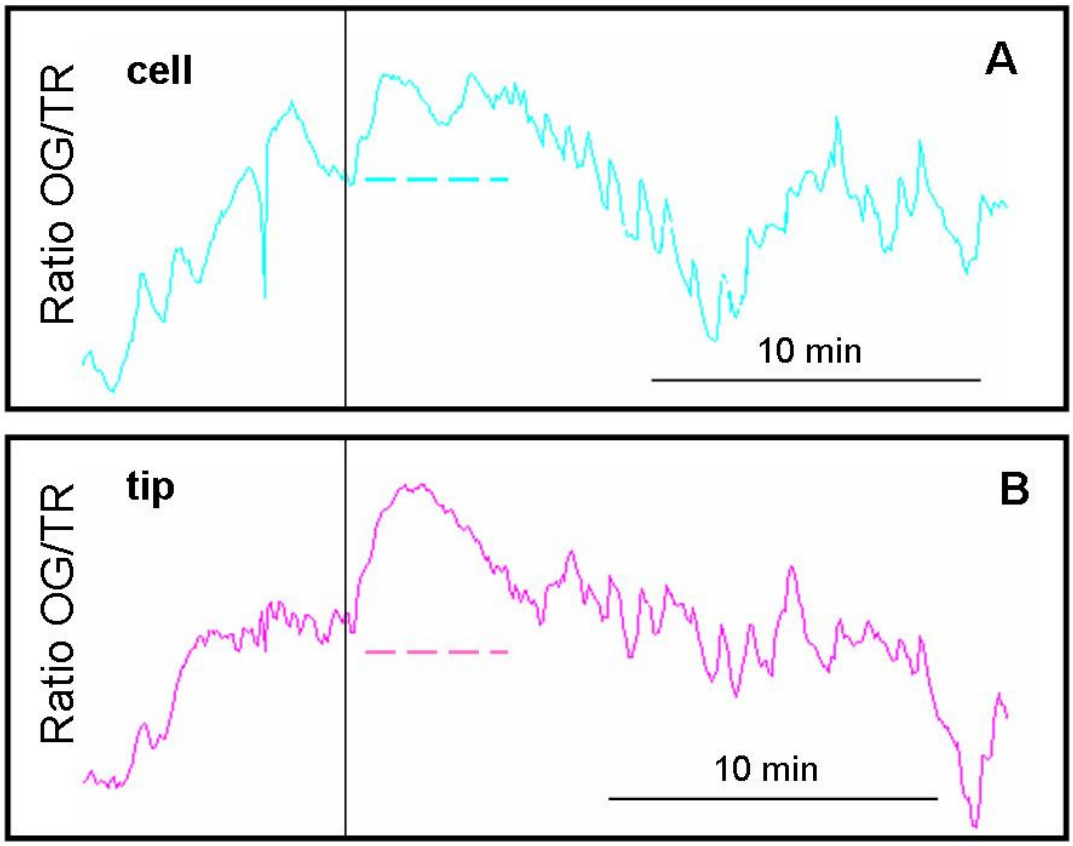


Figure 4.1 Example of a calcium trace, obtained after microinjection with Oregon Green and Texas Red presenting a background noise.

The ratio Oregon Green/Texas Red (OG/TR) was plotted against time. Images were taken at 5 seconds intervals for 30 minutes. Trace A represents calcium changes in cell area. Based on this trace it is difficult to establish weather this cell is positive for Nod factor-induced calcium flux. However when this is compared with the fluorescence of the tip of the root hair (B), it is clear that this root hair is positive for calcium flux. Dashed lines indicate calcium flux. Addition of water to the chamber containing the seedling did not induce any change in Oregon Green/Texas Red fluorescence (data not shown).

Titration of Nod factor-induced Calcium Flux Using Microinjection of Oregon Green and Texas Red

Calcium flux has been observed in root hair cells treated with 10 and 1nM of Nod Factor, but a titration curve of calcium flux in root hair microinjectected with calcium sensitive dye, over a wide range of Nod factor concentration has not been shown. To assay the responsiveness of *M. truncatula* plants for calcium flux, several root hair cells were microinjected and tested for this response after addition of Nod factor concentrations ranging from 10^{-12} to 10^{-7} M. For each root hair cell, calcium levels from tip and cells were analysed. A minimum of 10 cells from at least three different plants were tested for each concentration of Nod factor. The concentration at which Nod-factor-induced calcium flux in 50% of the root hair tested was 5×10^{-10} M. Curiously, as shown in Figure 4.2 the titration curve never reached 0% cells responding. Concentrations of Nod factor ranging from 10^{-12} to 10^{-10} M appeared to trigger calcium flux in approximately 25% of the root hair cells tested. Such a high baseline of the titration curve observed with microinjection was not observed using yellow cameleon (Figure 3.4A). The cameleon calcium sensor provides a better ratiometric analysis (YFP/CFP) compared to the ratiometric analysis of Oregon Green and Texas Red (Oregon Green/ Texas Red), in which the amount of the two dyes can vary in each microinjection. As a result, the calcium traces recorded with yellow cameleon are typically very flat with low background noise. This suggests that the background noise observed with microinjection is probably causing a false detection of calcium flux in approximately 25% of the root hair cells tested. Indeed, most of the root hair cells (6 of 8) that were found to be positive for calcium flux at low Nod factor concentrations (10^{-12} to 10^{-10} M) showed some noise in the fluorescence baseline before Nod factor addition. An example of a trace showing calcium flux in the tip and the cell area of a root hair treated with 10 pM Nod factor is shown in Figure 4.3.

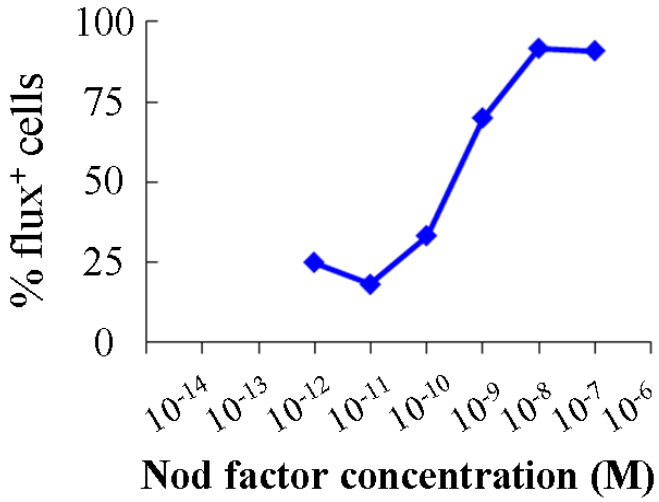


Figure 4.2. Titration curve of Nod factor-induced calcium flux in root hair cells microinjected with Oregon Green and Texas Red.

Multiple cells from wild-type plants were assessed for calcium response induced by different concentrations of Nod factor. The percentage of cells that showed calcium flux is indicated. A minimum of 10 cells from at least three different plants were tested for each concentration of Nod factor.

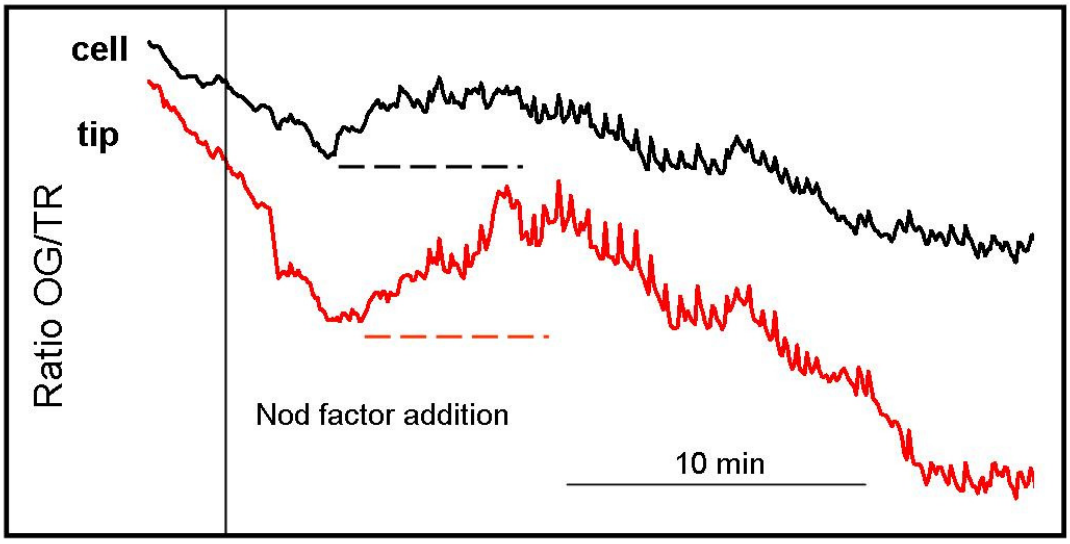


Figure 4.3 Example of a calcium trace of a root hair microinjected with Oregon Green and Texas Red and treated with 10 pM Nod Factor scored positive for Nod factor-induced calcium flux.

The ratio Oregon Green/Texas Red (OG/TR) was plotted against time. Images were taken at 5 seconds intervals for 30 minutes. The dashed horizontal lines indicate calcium flux observed in the cell (black) and in the tip (red) area.

Analysis of Calcium Flux in *M. truncatula* Mutants Lacking Infection Threads

Several legume genes have been identified as being required for calcium spiking but not for calcium flux. The mutants *dmi1*/*pollux*, *dmi2*/*symrk*, *castor*, *nup85*, *nup133, nena* and *cyclops* are all defective for calcium spiking but retain calcium flux (Shaw and Long, 2003; Miwa et al., 2006b; Groth et al., 2010). However, genes required for calcium flux but not for calcium spiking have not yet been identified. As calcium flux has been proposed to be involved in infection thread formation, such mutants might be defective for growth of infection threads. Good candidates to be tested for this phenotype are the Nod-factor-receptor mutants. Several genes have been identified as potential Nod-factor-receptors. In fact, there appears to be redundancy in predicted Nod factor receptor genes. The *hcl* mutant is compromised for root hair curling and infection thread formation. It has been suggested that HCL could be a Nod factor receptor required for infection thread growth (Smit et al., 2007). If this is the case, and if calcium flux is required for infection thread growth, one might expect the *hcl* mutant to be compromised for calcium flux. In the *hcl* mutant 16 of 24 root hair cells tested in 11 plants were positive for induction of flux in response to 10 nM Nod factor (Figure 4.4B). When the same Nod factor concentration was added to wild-type plants, eleven of twelve root hairs induced calcium flux in 6 plants (Figure 4.4A). Chi square analysis revealed that although fewer cells were positive for calcium flux, this reduction was not statistically significant. To further asses the responsiveness of the *hcl* mutant for calcium flux, Nod factor concentrations of 10^{-10}, 10^{-9} and 10^{-7} M were tested for calcium flux in this mutant. At these concentrations there was no difference between calcium flux response in the *hcl* mutant and wild type plants (Figure 4.5).

In Chapter 3 I showed that one hundred times more *nodL* Nod factor than wild-type Nod factor was required to induce calcium flux in wild-type plants (Figure 3.6A). HCL has been proposed to be the Nod-factor receptor that recognises structural specificity of Nod factors (Smit et al., 2007) and so the *hcl-1* mutant was tested for induction of calcium flux by Nod factor lacking the *nodL*-determined acetate on the non reducing terminal sugar. Calcium flux was similarly induced by *nodL* and wild-type Nod factor in the *hcl* mutant (Figure 4.6). Chi square analysis of the data collected at each Nod factor concentration

revealed that the number of root hair cells positive for calcium flux induced by *nodL* Nod factor, in the *hcl-1* mutant, was not significantly different from calcium flux induction by the wild type Nod factor (p 0,05) at any concentration point. These results suggest that, for the induction of calcium flux, the *hcl-1* mutant is unable to discriminate the structural differences between wild-type and *nodL* Nod factor. This is consistent with previous findings indicating that the HCL receptor is able to recognize Nod factor structure specificity (Smit et al., 2007).

The observation that the *hcl* mutant was not blocked for calcium flux indicates that mutation of this gene is not sufficient to block this response and suggests that other genes must be involved in the induction of calcium flux. Thus, other *M. truncatula* mutants compromised for infection thread growth were analysed. The *nin* mutants undergo excessive root hair curling, but are impaired in infection and fail to form nodules following inoculation with *S. meliloti* (Marsh et al., 2007). As previously shown, this mutant showed normal calcium spiking (Marsh et al., 2007). Calcium flux was observed in only 6 of 13 root hair cells tested (4 plants). Compared to wild type plants a significant reduction (p 0,05) in the number of root hair cells showing calcium flux was observed (Figure 4.4C).

The infection mutant *bit-1* was also tested for calcium flux. *BIT-1* encodes ERN, a transcription factor that is necessary for nodulation. Mutation in this gene blocks the initiation of the infection thread (Middleton et al., 2007). The *bit-1* mutant was positive for calcium spiking, but compromised for Nod-factor induced calcium flux: 6 of 13 root hairs was positive for calcium flux whereas in wild type 11 of 12 root hairs tested exhibited calcium flux, (Figure 4.4D). Chi square analysis of the data collected with the *bit-1* infection mutants revealed that the number of root hairs positive for calcium was significantly reduced compared to wild type plants (p 0,05). These results show that despite showing normal calcium spiking, both the infection mutants *nin* and *bit-1* may be reduced for calcium flux responses compared to wild type plants.

The GRAS transcriptional factor NSP2 acts downstream of CCAMK and is required for nodule organogenesis (Gleason et al., 2006). Mutation in this gene causes absence of infection and cortical cell division following inoculation with *S. meliloti* (Oldroyd and Long, 2003). The *M. truncatula* mutant *nsp2-2* was tested for induction of calcium flux. Twelve out of 15 root hair cells exhibited calcium flux in 7 different *nsp2-2* plants (Figure 4.4 F).

Chi squared analysis revealed no significant difference between the number of cells positive for calcium flux in the *nsp2-2* mutant compared to wild-type. As previously reported, calcium spiking was normal in the mutant plants (Oldroyd and Long, 2003).

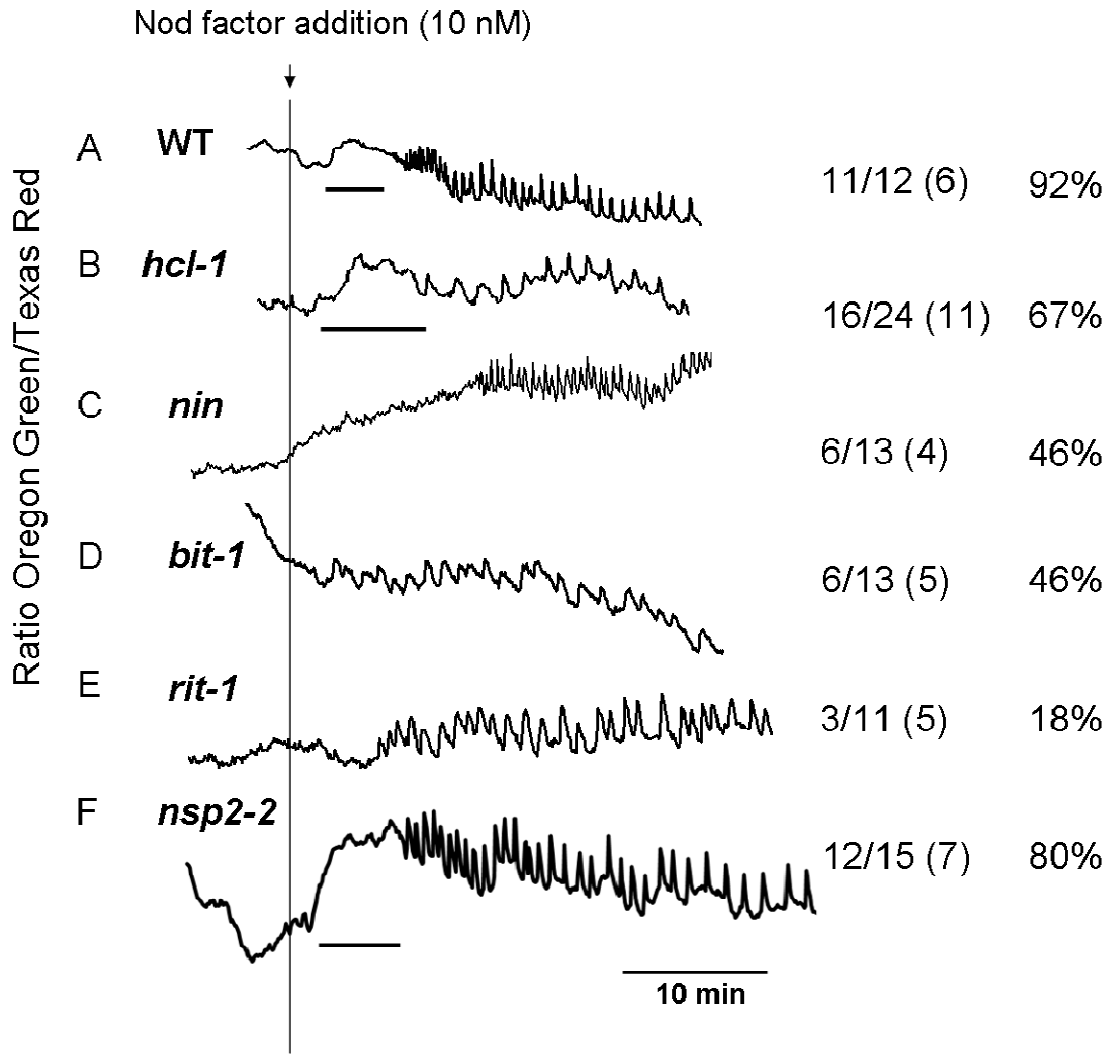


Figure 4.4. Changes in intracellular calcium in *M. truncatula* wild-type, *hcl, nin, bit-1, rit-1* and *nsp2-2* root hairs imaged using ratiometric fluorescence of Oregon Green and Texas Red.

Nod factor was added as indicated to a final concentration of 10 nM to seedlings and the ratio (arbitrary units) of fluorescence of Oregon Green (calcium sensitive-dye) and Texas Red (calcium-insensitive dye) was calculated every 5 seconds for more than 40 minutes. Representative traces are shown for wild-type (A), *hcl* (B)*, nin* (C)*, bit-1* (D), *rit-1 (E)* and *nsp2-2* (F). The horizontal black line indicate region of the trace where there is a significant transient increase in cellular calcium at the root tip and cell area. The number of root hair positive for calcium flux divided for the total number of root hair cell tested for each mutant is reported next to each trace. In parenthesis is indicated the number of plants tested.

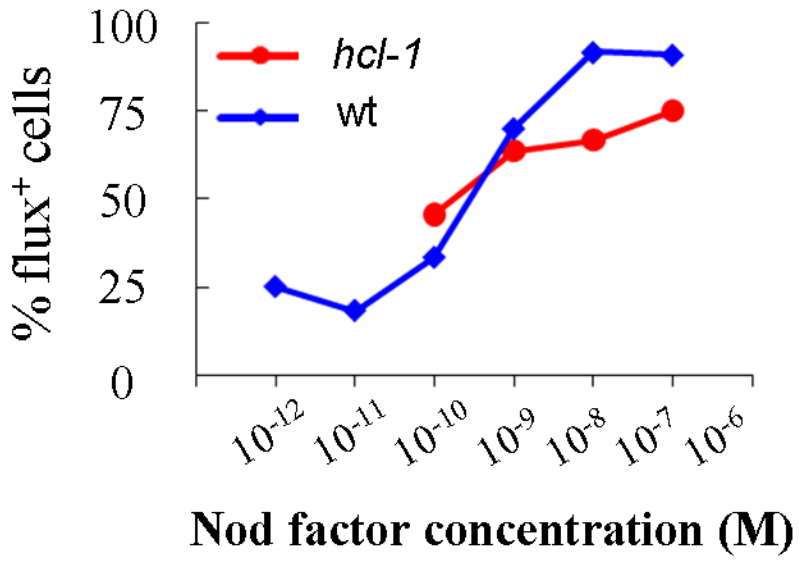


Figure 4.5. Induction of Nod factor-induced calcium flux in the *hcl* mutant.

Multiple cells of the *hcl* mutant (red circles) were assessed for calcium response induced by different concentrations of Nod factor. For comparison the titration curve observed for wild-type (blue diamonds) is presented in Figure 4.2. The percentage of cells that showed calcium flux is indicated. At least 10 cells from at least three different plants were tested for each concentration of Nod factor.

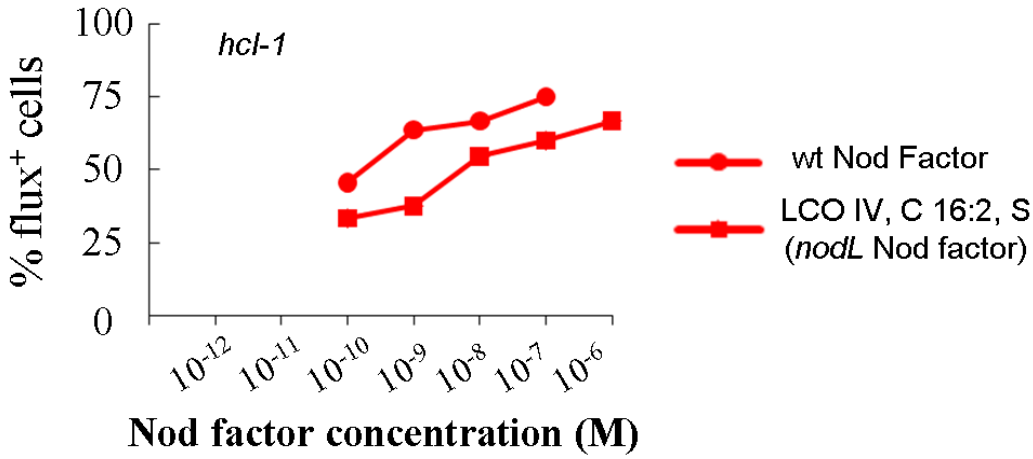


Figure 4.6. Dose response curves for *M. truncatula hcl-1* with *nodL* and wild-type Nod factor (shown for comparison).

Multiple cells from the *hcl* mutants were assessed for their calcium flux response to different concentrations of LCO IV, C 16:2, S (equivalent to *nodL* Nod factor (red squares). For comparison calcium flux response induced by wild type Nod factor (red circles) is shown. The percentage of cells that show calcium flux is indicated. At least 10 cells from at least three different plants were tested for each concentration of Nod factor.

Analysis of Calcium Responses in the *Mt rit1 and Lj nap1-1, pir1-1, and nap1-1/pir1-1* Mutants

Screening of mutagenised *M. truncatula* and *L. japonicus* populations identified an unusual class of mutants showing both aberrant symbiotic and trichome phenotypes (Tansengco et al., 2003; Ooki et al., 2005). Our collaborators (Yokota et al.) characterised the *nap1* and *pir1* mutants and cloned the corresponding genes that encode proteins that are most likely involved in the assembly of F-actin filaments (Yokota et al., 2009). *nap1* and *pir1* mutants were identified as small nitrogen-starved plants when grown on nitrogen-deficient nutrient medium. Inoculation of plants carrying *nap1-1* and *pir1-1* alleles with *M. loti* triggered the formation of small white nodules. The empty nodules formed on *nap1* and *pir1* roots suggested that the infection process might be perturbed in these mutants. To investigate this possibility, our collaborators examined *nap1-1* and *pir1-1* roots following inoculation with *M. loti*. A significantly decreased number of infection threads was observed in mutants compared with wild-type although *nap1* and *pir1* mutants were capable of forming some infection threads extending through the root hair. Nevertheless, wild-type-looking infection threads were very rarely observed (Yokota et al., 2009).

The function of NAP and PIR proteins has been investigated in detail in animals, protists, and *Arabidopsis*. Both proteins are part of the SCAR/WAVE complex that mediates actin dynamics. In root hairs of *nap1-1* and *pir1-1* in mutants, actin filaments were significantly more transverse and less longitudinally aligned (Yokota et al., 2009). In wild type plants, Nod factor caused a rapid rearrangement of actin filaments, characterised by an accumulation of actin bundle in the tip of the root hair treated for 30 minutes with Nod factor. In the *nap1-1* and *pir1-1* mutants no accumulation of diffuse actin was observed (Figure 4.7, modified form Yokota et al., 2009).

Nod factor–induced calcium influx coincides temporally and spatially with actin rearrangement and was, hence, proposed to be involved in the rapid reorganisation of actin in responding root hairs (Sanchez et al., 1991; de Ruijter et al., 1999). Calcium influx and calcium spiking were therefore measured in *nap1-1* and *pir1-1* mutants to investigate the relationship between the early electrophysiological and cellular changes observed in root hairs. Ratios of fluorescence of Oregon Green to Texas Red were determined after

Nod factor application (Miwa et al., 2006b), and, surprisingly, as seen in Figure 4.8, no significant differences in calcium influx or calcium spiking were observed between the wild type and the *nap1-1* or *pir1-1* mutants. These results suggest that the genes *NAP1* and *PIR1* are not required for the induction of Nod factor-induced calcium influx and calcium spiking.

An alternative explanation is that *NAP1* and *PIR1* are redundant in function for the activation of calcium responses. To test this possibility, the induction of calcium responses in the double mutant *nap1-1/ pir1-1* was analysed. The double mutant *nap1-1/ pir1-1* developed empty nodules upon inoculation with *M. loti*, and, occasionally, a few pink nodules were found (Yokota et al., 2009). This was associated with the impairment in the initiation and/or progression of infection threads (Yokota et al., 2009). The overall growth of the double mutant was affected to the similar extent as observed in the corresponding single mutants (Yokota et al., 2009). When the double mutant was tested for Nod factor-induced calcium responses no differences were seen in calcium spiking or calcium influx (Figure 4.8) (Yokota et al., 2009). This suggests that Nod factor–induced actin rearrangement mediated in *L. japonicus* by *NAP1* and *PIR1* act downstream or in parallel to calcium influx and spiking.

During the course of this work I analysed calcium responses in the *rit-1* (Root hair Infection and Trichome) mutant. The *rit-1* mutant also has a phenotype characterised by short thick root hairs. Infection of this mutant with *S. meliloti* results in arrested growth of infection threads and few nodules are formed (Miyahara et al., 2010). I assessed the ability of this mutant to induce Nod factor-induced calcium spiking and calcium flux. When Nod factor (10 nM) was added to root hairs of the *rit-1* mutant, Nod factor induced normal calcium spiking. 24 of 30 root hair cells showed calcium spiking in 10 plants tested. However, when tested for induction of calcium flux, only 4 of 12 root hair cells tested were positive for this response (Figure 4.4E). Thus, *rit-1* was normal for calcium spiking, but appeared to be compromised for induction of calcium flux. This suggests that RIT-1 may play a role in the response to calcium flux. Recently, the *rit-1* mutant has been found to carry a chromosomal translocation between chromosome 3 and chromosome 4, disrupting the normal function of *RIT1,* which is the putative orthologues of the *L. japonicus NAP1* gene (Miyahara et al., 2010). This left me with the difficulty of reconciling the results obtained with the *L. japonicus nap-1* mutant, normal for calcium flux (Figure

4.8), with that of the *M. truncatula rit-1* mutant showing a significant decrease of induction of calcium flux (Figure 4.4 E).

Given the discordance between the responsiveness for calcium flux in the orthologous mutant *rit-1* and *nap1*, *rit-1* was tested for calcium flux at higher Nod factor concentration. When the concentration of Nod factor was increased from 10 nM to 100 nM, 4 out of 10 root hairs (7 plants) showed influx in the *rit-1* mutant, whereas, in wild type, 9 of 10 cells (4 plants) were positive for flux indicating that even at high concentration of Nod factor the *rit-1* mutant was impaired for initiation of calcium flux. Chi square analysis revealed that the number of root hairs positive for calcium was significantly reduced compared to wild type plants (p 0,05). Further comments on the different ability to induce calcium flux observed in the *Mt rit-1* and *Lj nap-1* mutants are provided in the discussion section of this Chapter.

Figure taken from our collaborators (Yokota et al., 2009).

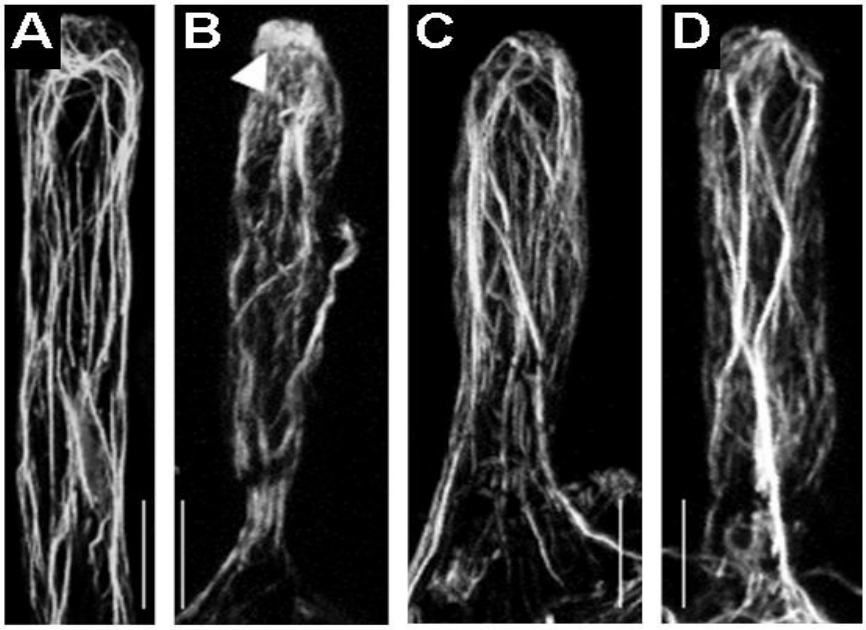


Figure 4.7. Actin Cytoskeleton of Root Hairs, visualized by Alexa-Phalloidin Staining or Expression of the 35S:GFP-ABD2-GFP F-Actin Reporter in Transgenic Roots (from our collaborators Yokota et al., modified form Yokota et al. 2009).

(A) Wild-type root hair before Nod factor application, phalloidin. (B) Wild-type root hair 30 minutes after Nod factor application. Note the zone of diffuse actin accumulation at the tip of the root hair (arrowhead), phalloidin. (C) *pir1-1* root hair 30 minutes after application of Nod factor, phalloidin. (D) *nap1-1* root hair 30 minutes after application of Nod factor, phalloidin. Bars = 20 µm.

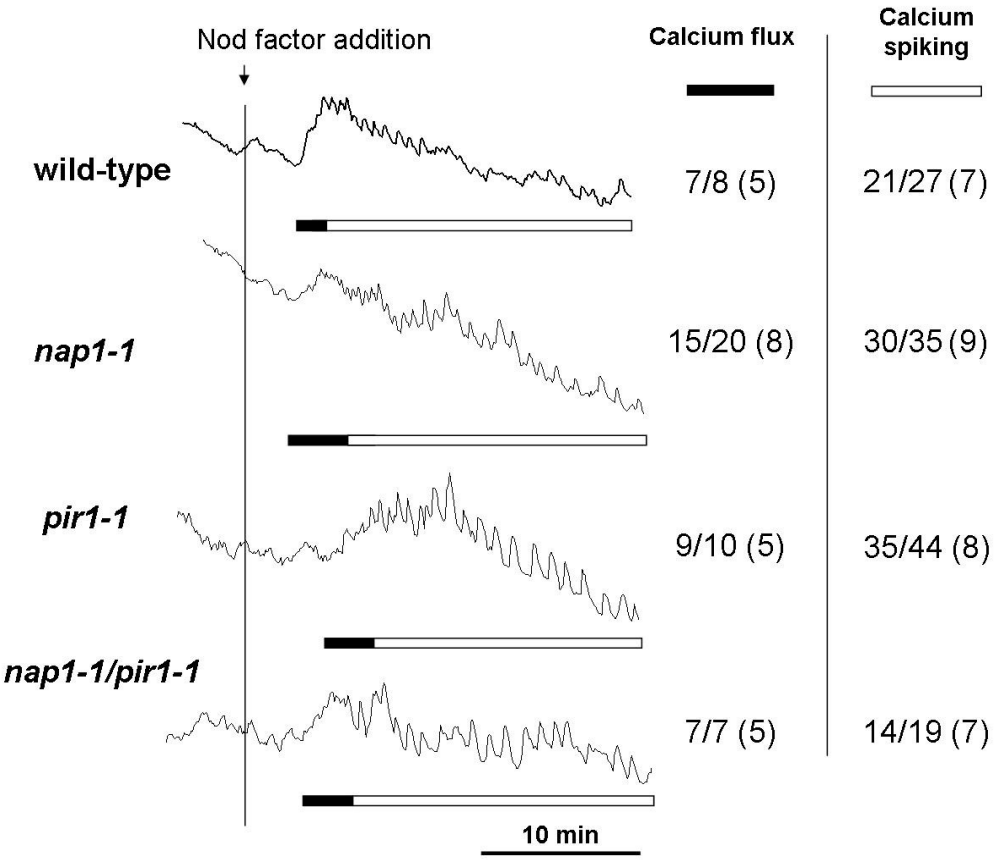


Figure 4.8. Nod Factor–induced calcium influx and calcium spiking in *L. japonicus nap1-1*, *pir1-1*, and *nap1-1/pir1-1* mutants (modified from Yokota et al. 2009).

Calcium levels were monitored in individual root hairs of the wild type and *nap1-1*, *pir1-1*, and *nap1-1/pir1-1* mutants following addition of 100 nM *M. loti* Nod factor (black vertical line). The ratios (arbitrary units) of fluorescence of Oregon Green (calcium sensitive) to Texas Red (calcium insensitive) were recorded every 5 s for >30 minutes. The number of cells showing calcium spiking or calcium influx is shown in the inset table as a fraction of the total number of cells analysed (with the total number of plants tested in parentheses). Solid bars indicate region of the trace where there is a significant transient increase in cellular calcium in the root tip and cell area, and the open bars indicate the parts of the traces showing nuclear associated calcium spiking.

Discussion

In this Chapter, the induction of calcium flux in microinjected root hairs of *M. truncatula* and *L. japonicus* wild type plans and infection mutants was analysed. As described in Chapter 3, the analysis of calcium flux was based on a comparison among cytoplasmic calcium levels recorded in the cell and tip of root hairs. This method was particularly useful to distinguish specific singular increases in calcium level from non-specific increases in background fluorescence, often observed in traces of microinjected root hair cells. Nevertheless, approximately 25% of root hair cells always show calcium flux even when they were treated with 100 to 1 pM Nod factor. At these concentrations calcium flux was not observed when analysed with yellow cameleon (Chapter 3). Given that most of the traces positive for calcium flux at the lowest Nod factor concentrations showed some background noise before the addition of Nod factor, it is likely that they are false positives, probably due to increases in the background fluorescence.

The baseline fluorescence of traces obtained with yellow cameleon are much less noisy. A possible explanation for this difference is that and the perforation of cell wall and plasma membrane, and the injection of dyes into the cell cytoplasm could cause induce changes in resting calcium levels. However, it is important to point out that only root hair cells showing an active cytoplasmic streaming were analysed for calcium responses. In addition, the ratio of YFP/CFP calculated with yellow cameleon produces a much better ratio than the Oregon Green/Texas Red ratio used in injection experiments, in which the amount of each dye can vary for each injection. I believe that the difference between the ratiometric accuracy is responsible for the great amount of noise observed using injection.

Previously, the only two genes shown to be required for Nod factor-induced calcium flux were genes encoding the putative Nod factor receptor NFP in *M. truncatula* (Amor et al., 2003) and *NFR-1* and *NFR-5* in *L. japonicus* (Radutoiu et al., 2003). *HCL* encodes the LYK3 receptor. A weak allele of this gene blocks infection thread growth and it has been suggested that HCL is the Nod factor receptor required for infection thread growth (Smit et al., 2007). Therefore, the *hcl-1* mutant was predicted to be defective for Nod factor-induced calcium flux. The data show that in the *hcl* mutant a lower number of

root hair cells induced calcium flux compared to wild-type plants. However, more than a half of the root-hairs tested were positive for calcium flux. This indicates that mutation of the *HCL* gene is not sufficient to block this response. This suggests that other genes must be required for the induction of calcium flux. Indeed, there seems to be a redundancy of different LYK's genes in *M. truncatula*. *MtLYK2*, for example, is also in the NFR1-LYK3 syntenic region, groups together with LYK3 and NFR1 in a phylogenetic tree, and is 80% identical and 90% similar to *Mt*LYK3 (Limpens et al., 2003; Smit et al., 2007). Thus, Nod factor-induced calcium flux observed in the *hcl* mutant could be explained by redundancy function of LYK3-like genes in the induction of calcium flux.

Analysis of calcium flux with Nod factor equivalent to those from *nodL* and wild-type Nod factor in the *hcl* mutant revealed that, when the *HCL* gene is mutated, the plant loses its ability to discriminate between the two Nod factor structures for the induction of calcium flux. This is consistent with previous findings indicating that the HCL receptor is able to recognize Nod factor structure specificity (Smit et al., 2007). *hcl-4* is a weak allele of *HCL* that controls infection in a manner dependent on Nod factor structure. The number of nodules formed on *hcl-4* roots is reduced more than forty fold when inoculated by *S. meliloti nodF nodE* mutant, and nodules are completely absent after inoculation with the *S. meliloti nodL* mutant, indicating that *MtLYK3* is involved, directly or indirectly, in recognition of Nod factors during infection. The observation that the *hcl* mutant is unable to discriminate between wild type and *nodL* Nod factor reinforces the finding that LYK3 acts in a Nod factor structure dependent fashion.

NAP1 and *PIR1* encode proteins involved in actin rearrangement. Fundamental processes of legume-*Rhizobium* symbiosis, such as root hair deformation and formation of infection threads, depend on dynamic rearrangements of the microtubule and actin networks of the cytoskeleton. A significant cytoskeletal reorganisation is associated with the initial swelling and subsequent re-initiation of polar root hair tip growth, as well as with the initiation and subsequent inward oriented extension of infection threads. Indeed, in the *nap1* and *pir1* mutants Nod factor failed to trigger actin bundle accumulation, normally observed in the tip of root hair cell after 30 minutes of application, and root hair deformation (Yokota et al., 2009). Our results show that the

nap1 and *pir1* mutants were normal for both calcium flux and calcium spiking, indicating that NAP1 and PIR1 are not required for these responses and suggest that Nod factor–induced actin rearrangement mediated in *L. japonicus* by *NAP1* and *PIR1* act downstream or in parallel to calcium influx and spiking.

Nod factor-induced calcium flux and calcium spiking are initiated within 20 minutes of the addition of Nod factor whereas the *NAP1*- and *PIR*-mediated rearrangements were first observed 30 minutes after Nod factor addition. Thus, I can not role out the possibility that calcium responses occur upstream of the actin rearrangement mediated by *NAP1* and *PIR.* Nevertheless, this seems unlikely given the evidence suggesting that calcium spiking, calcium flux and root hair deformation are three independent events induced by Nod factor. 1. Mutants blocked for the induction of calcium spiking or calcium flux were found to be positive for root hair deformation (Esseling et al., 2004; Miwa et al., 2006b); 2. Initiation of cytoskeletal changes, such as actin remodelling start three to six minutes after Nod factor addition (Allen and Bennett, 1996; Cardenas et al., 1998; Esseling et al., 2003, 2004) whereas calcium spiking is normally induced later (15-20 minutes after Nod factor application). 3. Low Nod factor concentrations trigger calcium spiking and root hair deformation but not calcium flux (Shaw and Long, 2003; Miwa et al., 2006b) or the calcium flux–associated alkalinization measured using microelectrodes (Felle et al., 1998; Radutoiu et al., 2003). The observations reported in this chapter are consistent with the calcium responses occurring in parallel with the NAP1- and PIR1-induced cytoskeletal changes required for the Nod factor-induced deformation of root hairs, although I cannot exclude the possibility of a low level or localised change in calcium that cannot be measured in root hairs using existing techniques.

Calcium influx has been suggested to occur at the initial stage of infection thread formation when a high concentration of Nod factor is generated by the growing bacteria entrapped in the infection focus, (Miwa et al., 2006b). In *nap-1* and *pir-1* mutants*,* the rare infection threads that did form underwent rapid disintegration, and very few infection threads were able to extend to the base of the epidermal cell. The lack of infection thread formation within the root cortex suggested that *NAP1* and *PIR1* are essential for the progression of the infection process beyond the root epidermis (Yokota et al., 2009). Thus, it is possible that calcium flux play a role upstream of *NAP1*

and *PIR1* during the formation of the infection threads and this could explain the results showing that *NAP1* and *PIR1* are required for the development of infection threads but are not required for induction of calcium influx.

M. truncatula nin, bit-1 and *rit-1* mutants are defective for infection thread growth. Here, I found that these genes are required for calcium flux but not for calcium spiking as the mutants *nin, rit-1,* and *bit-1* were impaired for induction of calcium flux but normal for induction of calcium spiking. Conversely, in *L. japonicus*, mutations in *NIN* (Miwa et al., 2006b) and *RIT*-1 (orthologous *NAP1)* resulted in plants that did induce calcium flux, whereas I observed that mutation in the orthologous *M. truncatula* genes reduced calcium flux compared to wild-type when Nod factor was added at 10 nM. Among all the infection mutants tested for calcium flux the *rit-1* mutant was the most impaired for this response. Because of the discrepancy in the results obtained between the *rit-1* and *nap1* mutants, the responsiveness to higher Nod factor concentration for calcium flux was tested in the *rit-1* mutant. When Nod factor was added at 100 nM, significantly fewer root hairs were positive for calcium flux compared to wild type plants, confirming that the *rit-1* mutant is compromised for calcium flux. Corroborating this observation, if, as discussed above, approximately 25% of the root hairs positive for calcium flux are due to the background noise, the number of root hairs positive for a specific calcium flux in the *rit-1* mutant would be almost zero.

One of the common phenotypic characteristics of *L. japonicus nap1* and *M. truncatula rit-1* mutants is the arrested trichome development. The elongated, filamentous trichomes are visible on the sepals, the abaxial midribs of leaves, and leaf stalks of wild type plants. By contrast, trichome formed in the sepals, leaves leafs, and leaf stalks on the *nap1* and *rit-1* mutants are distinctively shorter and deformed (Yokota et al., 2009) Miyara et al., 2010, in press). Root hair development is also affected in the *Lotus* and *Medicago* mutants, although a difference between the *nap1* and *rit-1* mutants was observed for the root hair cells phenotype. In the *nap1* mutant the root hair phenotype was rather subtle. Its severity in terms of length and number of root hairs formed varied substantially even between individuals of the same genotype, precluding quantification (Yokota et al., 2009). Reduced root hair development was observed in old

root hairs in the upper part of the root of the *nap1* seedlings, whereas the young root hairs that are selected for injection were not much different from wild type (Figure 4.9). In contrast, the root hairs of the *M. truncatula rit-1* mutant showed a much more severe phenotype. Root hairs along the length of the root (including root hairs selected for injection experiments) were typically short and thick (Figure 4.9). This difference in the root hair phenotype indicates that mutations in *Medicago RIT-1* and orthologous *Lotus NAP1* have a different effect on the development of young root hair cells. Infection threads are polar growing structures like trichomes, root hairs and pollen tubes. Therefore, it is possible to speculate that *Medicago RIT-1* and the *Lotus NAP1* genes might play slightly different roles during the formation of infection thread and this could reflect the different calcium flux responses observed in the two species. Indeed, the development programme of infection thread and nodule in *L. japonicus* and *M. truncatula* are very different. *L. japonicus* develops determinate nodules, characterised by the absence of persistent meristems, whereas *M. truncatula* develops indeterminate nodules with continuous meristem growth. The function of infection thread in legume species developing determinate nodules, such as *Lotus*, is to deposit bacteria at the site of cortical division, whereas infection threads of indeterminate nodules, as in *M. truncatula,* must reach the inner cortical cortex where cell division originating in the pericycle lead to the development of persistent meristem. The rare infection threads formed in *L. japonicus* plants carrying the mutant *nap1* allele underwent rapid disintegration, and only sporadically infection threads that extended to the base of epidermal cells were observed. The total lack of infection thread formation within the root cortex suggested that NAP1 is essential for the progression of infection process beyond the root epidermis (Yokota et al., 2009). At this late development stage of infection thread it is very likely that *L. japonicus* and *M. truncatula* undergo two different developmental processes. Therefore, may not be so surprising to find that orthologous protein involved in infection thread formation induce different responses in the two plant species. Nevertheless, it is worth remembering that the mutation which was induced by fast neutron mutagenesis in the *M. truncatula rit-1* mutant involves a complex rearrangement (Miyahara et al., 2010) and, as a consequence, it is possible that more than one gene is mutated in the *M. truncatula rit-1* mutant. To confirm the reduction of calcium flux in the *M. truncatula rit-1* mutant other alleles should be tested.

Nod factor is known to induce early nodulin gene expression, cortical cell divisions and promote nodule morphogenesis (Spaink et al., 1991; Truchet et al., 1991). Gene expression changes induced by Nod factor are abolished in the Nod factor signalling mutants *nfp*, *dmi1*, *dmi2*, *dmi3*, *nsp1, nsp2* and *bit-1* (Catoira et al., 2000; Mitra et al., 2004a; Marsh et al., 2007; Middleton et al., 2007). An activated *CCAMK* is sufficient to induce the formation of spontaneous nodules in the absence symbiont bacteria in both *M. truncatula* and *L. japonicus* (Gleason et al., 2006; Tirichine et al., 2006a). In *M. truncatula* this depends on the transcriptional factors *NSP1* and *NSP2*, *NIN* and *BIT-1* (Gleason et al., 2006; Marsh et al., 2007; Middleton et al., 2007). This strongly argues that *NFP*, *DMI1*, *DMI2*, *DMI3*, *NSP1, NSP2* and *BIT-1* are on a linear pathway with respect to gene regulation and induction of nodule morphogenesis. The finding that mutations in *NFP*, *DMI1* and *DMI2* block calcium spiking and that antagonist of calcium spiking also block gene induction clearly indicates that calcium spiking is central to this pathway.

The *BIT* and *NIN* genes, were found to be required for efficient induction of calcium flux. One possible explanation is that NIN and BIT, activated by the calcium spiking signalling pathway are also recruited at a later stage to play a role in the induction of calcium flux during the formation of infection thread. This is consistent with earlier reports showing that most of the genes (e.g. *CCAMK)* that take part in the Nod factor signalling pathway are also required for infection thread formation upon rhizobial infection (Catoira et al., 2000; Kistner et al., 2005).

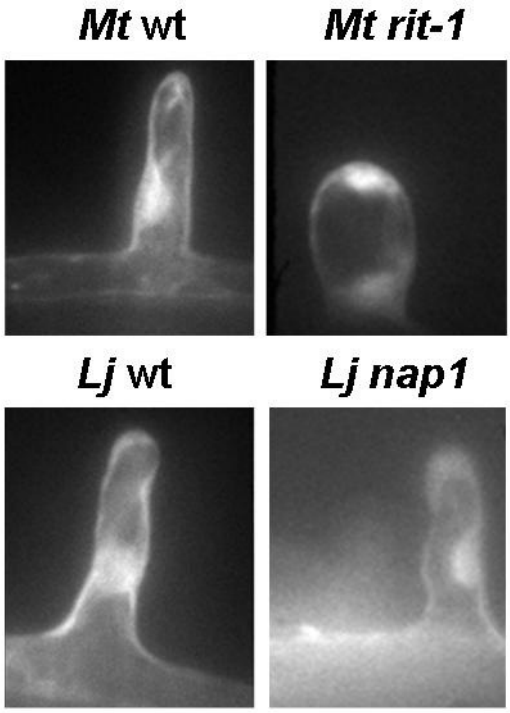


Figure 4.9. Root hair phenotype of *M. truncatula rit-1* and *L. japonicus nap1* mutants.

Root hair cell microinjected with Oregon Green and Texas Red are shown. The root hairs of *M. truncatula rit-1* are short and thick, whereas the *L. japonicus nap1* mutant has root hair cells similar to wild type.

Chapter 5

Lotus japonicus symrk-14 Forms Nodules but Is Severely Impaired for Nod Factor-Induced Calcium Spiking

Introduction

Calcium oscillations play an important role in several biological processes in animals and plants. In animals, calcium modulates diverse cellular activities ranging from fertilisation to cell death (Berridge et al., 2000). In plants calcium oscillations have been shown to regulate stomatal aperture (Allen et al., 2000), circadian clock (Dodd et al., 2007) and legume-rhizobia symbiosis (Ehrhardt et al., 1996). In legume-rhizobia symbioses perception of the bacterially-derived Nod factor by the plant leads to the induction of calcium oscillation around the nucleus which last for several hours (Ehrhardt et al., 1996). Mutation in many of several genes required for nodule formation, as the genes encoding the Nod factor receptors NFR1 and NFR5, the LRR-receptor kinase SYMRK, the ion channel-like proteins CASTOR and POLLUX, and the nucleoporins NUP85 NUP133 and NENA, are required for Nod factor-induced calcium spiking. A plant-specific calcium/calmodulin-dependent kinase (CCaMK) encoded by *DMI3* in *M. truncatula* and *L. japonicus* is likely decode the Nod factor-generated calcium spiking signal (Levy et al., 2004; Mitra et al., 2004b; Tirichine et al., 2006b). A single amino-acid replacement in the kinase domain or an autoactive CCaMK, containing only the kinase domain, is sufficient to induce expression of early nodulin genes and is responsible for the spontaneous nodulation phenotype observed in absence of *Sinorhizobium meliloti* (Gleason et al., 2006; Tirichine et al., 2006b; Tirichine et al., 2006a). Pharmacological antagonists shown to block Nod factor-elicited calcium spiking are also efficient inhibitor of early nodulin gene induction (Engstrom et al., 2002; Charron et al., 2004). All together this evidence

clearly indicates that calcium spiking is central to the Nod factor signalling pathway that activates nodulation.

Nod-factor perception by the plant induces two parallel responses: one in the epidermis and one in the root cortex (Oldroyd and Downie, 2008). In the epidermis, Nod factor is necessary for bacterial infection via infection thread growth, while in the cortex, Nod-factor perception induces cortical cell division and nodule morphogenesis. Analysis of legume mutants with defects in components of Nod factor signalling highlighted differences between induction of epidermal responses and induction of cortical cell divisions. Recently, Madsen at al., showed that inoculation of the double mutants carrying the gain-of-function *CCAMK* and a mutation in one of the genes required upstream of calcium spiking, but downstream of Nod factor receptors, resulted in infected nodules. This suggests that activation of calcium spiking is not directly required for bacterial infection. The *L. japonicus* gain-of–function allele *snf-2* encoding the cytokinin receptor LHK1 leads to the formation of spontaneous nodules (Murray et al., 2007; Tirichine et al., 2007). Inoculation of the double mutant *snf2 ccamk* did not resulted in the formation of infected nodules (Madsen et al., 2010). These results indicate that activation of CCAMK, which is likely to occur through calcium spiking, is not only required for nodule organogenesis but is also required for bacterial infection. Whether Nod factor-induced calcium spiking plays a role in the epidermal and/or cortical programme is still an area of debate.

Mutation in either *L. japonicus NUP85* or *NUP133* blocked Nod factor-induced calcium spiking, but some cortical cell divisions occurred (Kanamori et al., 2006; Saito et al., 2007). Inoculation of *nup133-2*, *nup133-3*, and *nup133-4* with *M. loti* revealed a temperature-sensitive nodulation deficiency: when inoculated at 26°C, *nup133-2*, *nup133-3*, and *nup133-4* did not form nodules, whereas at permissive temperatures (22°C) they developed small ineffective nodules. The root nodules formed, were ineffective and electron microscopy showed that rhizobia were not released from infection threads. Analysis of ion fluxes using a calcium-sensitive dye revealed that calcium all four allelic *nup133* mutants were deficient for Nod factor-induced calcium spiking (Kanamori et al., 2006). Similarly, *nup85-1* and *nup85-2* mutants failed to induce calcium spiking in response to Nod factor addition (Miwa et al., 2006b; Saito et al., 2007). Nevertheless, these mutants do form a few effective nodules (Szczyglowski et al., 1998;

Kawaguchi et al., 2002; Kistner et al., 2005). Recently an other *L. japonicus* mutant carrying a mutation the a nucleoporin gene *NENA* has been identified. Similarly to *nup85* and *nup133,* the *nena* mutant form nodules in a temperature dependent fashion and is impaired in perinuclear calcium spiking (Groth et al., 2010). These results suggest a situation in which nodule morphogenesis appears to occur in the absence of calcium spiking, pointing toward the possibility that it may be possible to break the link between calcium spiking and nodule morphogenesis.

Microinjection of *L. japonicus* root hairs is laborious and technically demanding, so this technique does not lend itself to identification of rare or old uninjecteable cells that might induce calcium spiking. Therefore, given the few number of nodules formed in the *nup* mutants, it is possible that occasional nodulation events are due to the occasional activation of calcium spiking, not observed in the microinjection assays. However, an unresolved challenge is to explain the *NUP133*/*NUP85*-dependent calcium spiking mounted by wild-type plants 10 min after Nod-factor application and the intriguing lack of calcium spiking in *nup133* and *nup85* mutants carrying weaker alleles that unexpectedly initiate nodule development albeit at reduced frequency (Kanamori et al., 2006; Miwa et al., 2006b; Saito et al., 2007).

L. japonicus har1 mutants respond to inoculation with *Mesorhizobium loti* by forming an excessive number of nodules due to genetic lesions in the *HAR1* autoregulatory receptor kinase gene (Krusell et al., 2002; Nishimura et al., 2002). Autoregulation controls the extent of nodulation events in the roots and, thus, serves to preserve the homeostasis of the nitrogen-fixing symbiosis. Screening for suppressors of the *L. japonicus har1-1* hypernodulation phenotype identified, among many mutant lines, two alleles of *L. japonicus SYMRK*: *symrk-14* and *symrk-13* (Murray et al., 2006).

SYMRK is a transmembrane leucine-rich-repeat-receptor-kinase containing an extracellular leucine-rich-repeat region (LRR), a transmembrane domain and an intracellular protein kinase (Figure 5.1) (Endre et al., 2002; Stracke et al., 2002). This gene is essential for nodulation and is very highly-conserved among legumes, though the function of the protein is not yet fully known. Given that LRR have been shown to mediate specific protein-protein interaction (Jones and Jones, 1997) and that *Lotus SYMRK* is identical to *RECEPTOR-LIKE KINASE* (*RLK*) (Stracke et al., 2002), it has been proposed that SYMRK might interact with an unidentified extracellular protein and

mediate the phosphorylation of some components yet to be identified. Indeed, recent work showed that DMI2, the *M. truncatula* orthologous of SYMRK, interacts with HMGR1, a key enzyme in synthesis of mevalonate and that the interaction required the cytosolic active domain of DMI2 (Kevei et al., 2007). SYMRK is not thought to be a Nod factor receptor as *SYMRK* mutants do show some responses to Nod factor, including root hair deformation (Stracke et al., 2002) and calcium flux (Miwa et al., 2006b).

Most *SYMRK* mutations (e.g. *symrk-13/har1-1*) completely block nodulation and mycorrhization. However, a novel allele was identified during the screening for suppressors of the *L. japonicus har1-1* hypernodulation phenotype; *symrk-14* contains a single amino acid change (Proline to Threonine) in the GDPC motif just upstream of the leucine rich repeat (Figure 5.1). The *symrk-14/har1-1* mutant was reduced for nodules and was also defective for mycorrhization, although it had a more subtle nodulation defect. Root hairs of the *symrk-14/har1-1* mutant responded to inoculation with *M. loti* by extensive root hair deformation and initiation of nodule primordia. The *symrk-14/har1-1* mutant formed a higher number of nodule primordia/white bumps that did not develop into nodules compared to *har1-1* mutant, and was, hence, classified as a Nod^+ hyperinfected mutant (Murray et al., 2006).

The intriguing location of the *symrk-14* mutation and its unique symbiotic phenotype spurred our collaborators (S. Kosuta and K. Szczyglowski) to further characterise this mutant. Analysis of bacterial infection in *symrk-14/har1-1* mutant revealed that bacterial infection thread formation in the mutant was blocked (S. Kosuta, personal communication). Thus, *symrk-14/har1-1* mutant showed a very interesting phenotype. The mutant does not form symbiotic relationships with arbuscular mycorrhiza, and it does not form infection threads with symbiotic rhizobia; therefore it is a loss-of-function mutant. Surprisingly, however, the *symrk-14/har1-1* mutant forms nodules (Murray et al., 2006). This ability to nodulate without root hair infection threads enabled me to use this mutant to ask the question: is root hair calcium spiking required for nodule organogenesis?

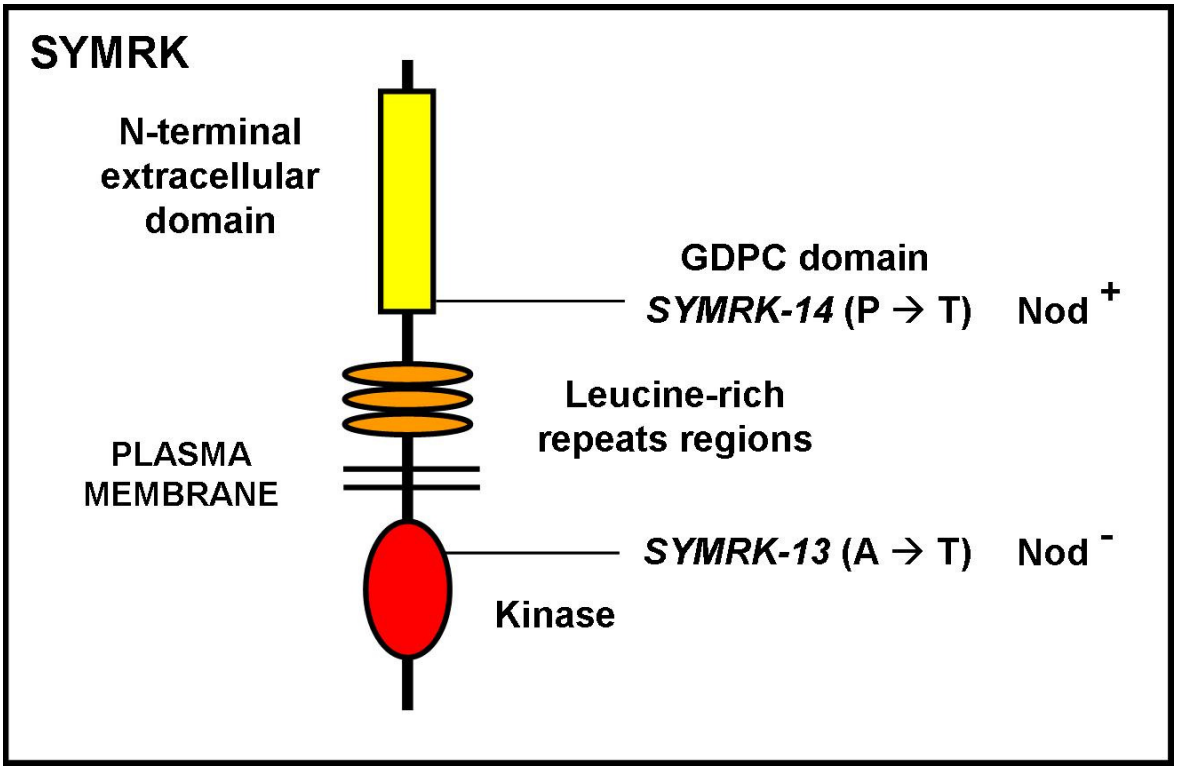


Figure 5.1. Schematic structure of SYMRK protein and relative position of the amino acid changes in the *symrk-14* and *symrk-13* alleles.

Results

Ljsymrk-14 is I*mpaired* for Nod Factor-Induced Calcium S*piking*

Screening for suppressors of the *L. japonicus har1-1* hypernodulation phenotype identified two alleles of *L. japonicus SYMRK*: *symrk-14* and *symrk-13* (Murray et al., 2006). Both *symrk-14* and *symrk-13* mutants failed to form symbiotic relationships with arbuscular mycorrhiza. The *symrk-13* mutant was also completely defective for nodule formation whereas the *symrk-14* mutant formed nodules (Murray et al., 2006).

The phenotype of the *symrk-14* mutant was further characterised by our collaborator (S. Kosuta et al.). Analysis of infection threads revealed that *symrk-14/har1-1,* such as *symrk-13/har1-1,* did not form infection thread after inoculation with *M. loti* (S. Kosuta, personal communication). The *symrk-14/har1-1* mutant produced fewer nodules but more nodule primordia than the har1-1 mutant (Figure 5.2A), giving a total nodulation events (nodule primordia plus nodules) not statistically different from *har1-1* plants, (Figure 5.2B). The observation that the *symrk-14/har1-1* mutant is strongly defective for infection thread formation indicates that *symrk-14* is a loss-of-function mutant.

This mutant provided a tool to test if root-hair calcium spiking is required for nodulation, independently of infection thread growth. Calcium spiking was tested in root hair cells of the *symrk-14* mutant using microinjected Oregon Green. In order to maximize nodulation, the experiments were carried out in the high nodulation background *har1-1*. Surprisingly, as shown in Figure 5.3, 5.4 and in Table 5.1, the *symrk-14* mutant completely failed to show calcium spiking. None of the 38 root hair cells tested in 16 plants of the *symrk-14/har1-1* mutant were positive for calcium spiking although these mutants formed 25.5 nodules per plant 21 days post inoculation. The single *har1-1* mutant was used as a positive control; this mutant formed 52.2 nodules per plant 21 day post inoculation (Figure 5.2 and Table 5.1) and was positive for induction of Nod factor-induced calcium spiking: seven of eight root hair cells tested in five different plants displayed calcium spiking (Figure 5.3, 5.4 and Table 5.1). The null mutant *symrk-13/har1-1*, which is completely defective for nodulation, was used as a negative control. As expected, this allele blocked calcium spiking. None out of 8 root hair cells showed calcium spiking (6 plants tested, Figure 5.3, 5.4 and Table 5.1).

Analysis of the original traces of calcium level recorded in the *symrk-14/har1-1* mutant revealed that about one third of the traces had a high variation in fluorescence (calcium) background observed also in the absence of Nod factor (Figure 5.4 and Table 5.1). Ten of 38 root hair cells showed a "noisy" background while the remaining 28 root hair cells tested had quiet traces (Figure 5.4 and Table 5.1). The traces represent ratios of Oregon green (calcium-sensitive dye) and Texas red (calcium-insensitive dye) both co-injected in root hair cells, so there seem to be real changes occurring in calcium levels in some cells (the noisy ones), but not what we would call calcium spiking. Calcium spiking is a specific type of signalling event characterised by regular oscillations that form "spikes". The spike refers to the shape of the calcium elevation, a very sudden increase in calcium followed by a more gradual decline. Secondly, calcium spiking consist in repetitive calcium oscillations which last for several hours, the repetitive nature of these oscillations and their regular period represent clear marks of calcium spiking. A third feature is the presence of a lag time between addition of bacteria or Nod factor and initiation of calcium spiking. The calcium "noise" observed in the *L. japonicus symrk-14/har1-1* root hair cells did not exhibit any of these three hallmarks of calcium spiking (Figure 5.4).

Microinjection of *symrk-14/har1-1* root hairs was more difficult compared to wild-type, *symrk-13/har1*-1, or *har-1* mutants, indicating an enhanced touch response of this mutant. Cells tended to burst very easily when the needle was taken away from the root hair cell after an injection. For this reason a more delicate microinjection and finer needle were made in order to microinject root hair cells without damage. Only the root hair cells showing good cytoplasmic streaming were selected for calcium analysis. Thus, all the 38 cells tested were carefully selected cells showing active cytoplasmic streaming. The *symrk-13/har1-1* and *har1-1* mutants were similar to wild type in terms of these observations (noise, bursting, injectability). Even considering the noisy traces of *symrk-14* mutant, none of the root hairs tested showed activation of calcium spiking after Nod factor treatment.

Microinjection of root hairs has the disadvantage that only a limited number of cells can be analysed. To analyse a larger number of cells, the nuclear-targeted cameleon (NupYC2.1) (Sieberer et al., 2009) was introduced into wild-type Gifu, *symRK-14* and *symRK-14 /har1-1* roots via *Agrobacterium rhizogenes*-mediated transformation. Root hairs with high fluorescence levels in root cell nuclei were selected (Figure 5.5). In the

wild-type, regular calcium spiking was observed in 63 out of 285 epidermal cells examined (Figure 5.6a). In addition, a few calcium spikes, as illustrated in Figure 5.6b, were also detected in 16 cells. The *symRK-14* mutant has a similar nodulation phenotype to that observed with the *symRK-14 /har1-1* mutant. Inoculation with *M. loti* resulted in the formation of fewer nodules but more nodule primordia than wild-type plants and no infection threads were formed (S. Kosuta, personal communication). When transgenic roots of the *symRK-14* mutant were analysed for calcium spiking using Nup-YC2.1, only 2 cells (out of 376) showed clear calcium spiking (Figure 5.6c) and, in addition, a few calcium spikes, as shown in Figure 5.6d, were observed in 10 cells. Few calcium spikes were also observed in 3 of 115 cells in the *symRK-14 /har1-1* mutant (Figure 5.6e). The occasional calcium spiking was previously reported with one of the weak alleles (*dmi2-2*) of orthologue gene in *M. truncatula* (Wais et al., 2000).

Figure kindly given by Sonja Kosuta, personal communication.

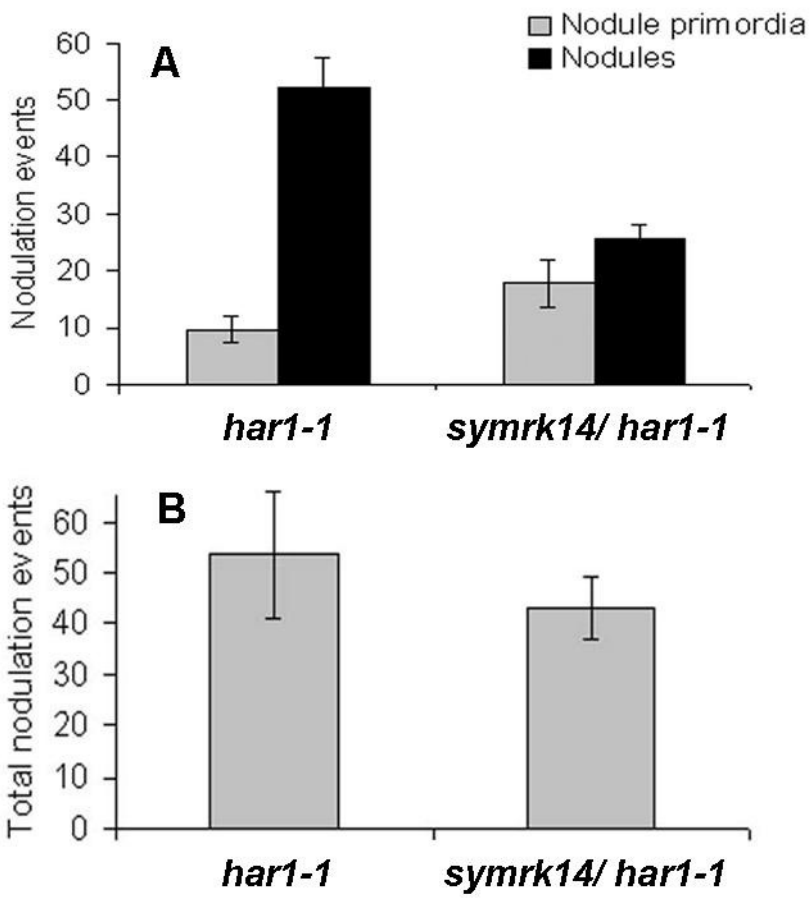


Figure 5.2. Nodulation phenotype of the *L. japonicus har1-1* and *symrk-14/har1-1* (S. Kosuta, personal communication).

A. Histograms of nodule primordia and nodule number formed on *har1-1* and *symrk-14/har1-1* 21 days after inoculation with *M. loti*. B. Histograms of total nodulation events (nodules plus nodule primordia) in *har1-1* and *symrk-14/har1-1* (A), 21 days after inoculation with *M. loti*. Error bars represent Standard Deviation.

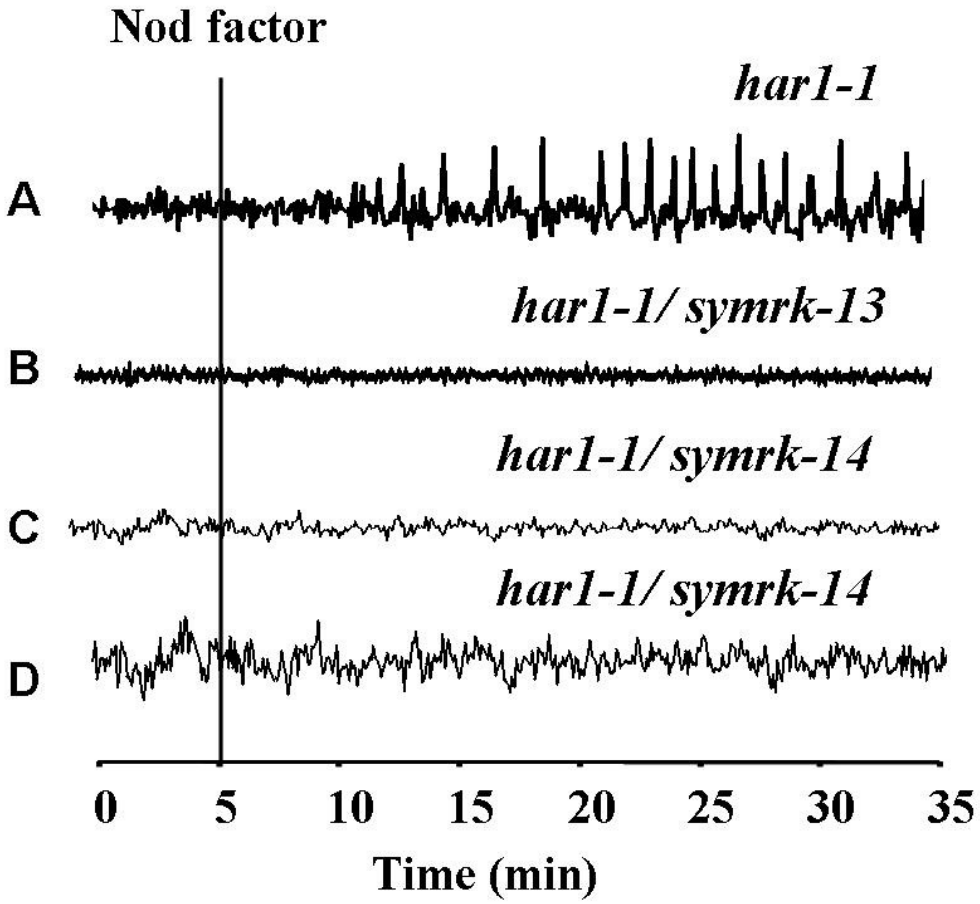


Figure 5.3. Changes in cytoplasmic calcium concentration in mutants of *L. japonicus* after the addition of Nod factor (100 nM).

Data are presented as derivative traces representing the change in fluorescence intensity of Oregon Green/Texas Red from one point to the next ($x_{n+1}- x_n$). The traces show representative cells for the three mutants of *L. japonicus; har1-1* (A), *har1-1*/*symrk-13* (B) and *har1-1/symrk-14* (C and D).

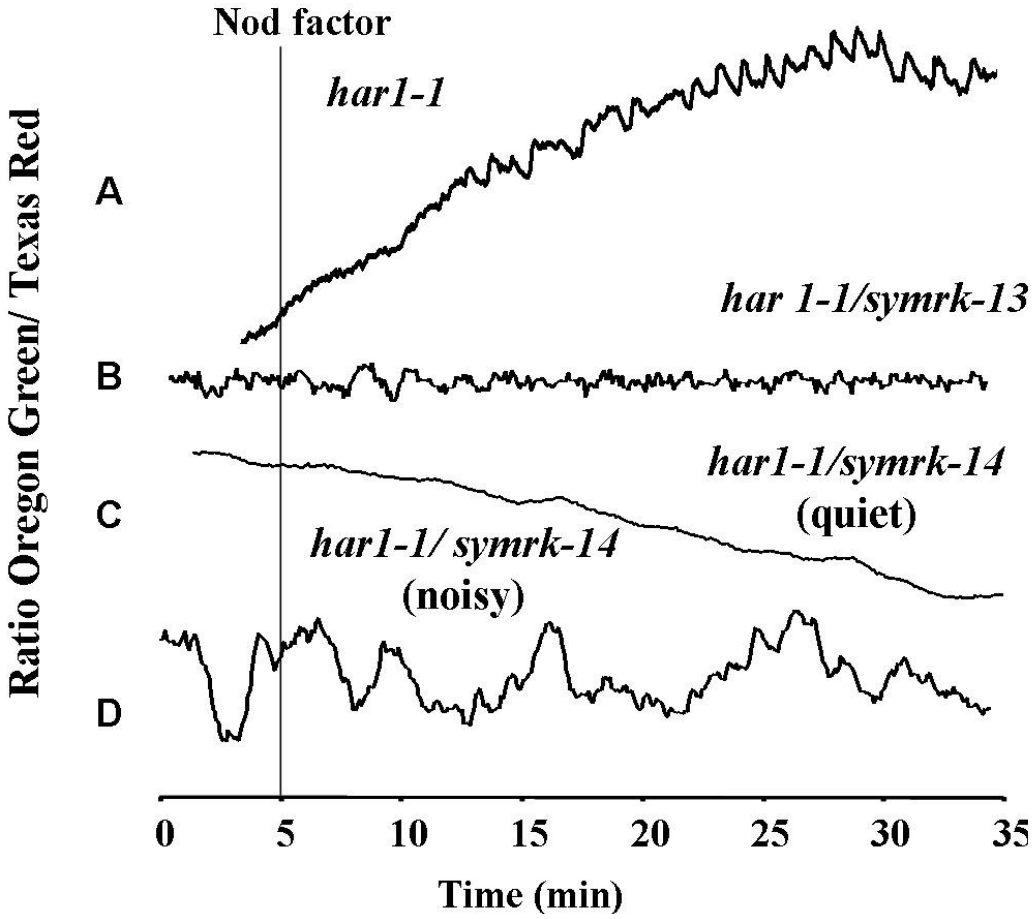


Figure 5.4. Original traces showing changes in cytoplasmic calcium concentration in mutants of *L. japonicus* after the addition of 100 nM Nod factor.

Data present the original traces of the mutants of *L. japonicus* root hair cells *har1-1* (A), *har1-1*/*symrk-13* (B) and *har1-1/symrk-14* (C and D) from Figure 5.3.

Table 5.1. Number of nodules formed per plant per each mutant 21 day post inoculation (S. Kosuta, personal communication) and number of root hair cells positive for calcium spiking/total number of root hairs tested in *L. japonicus* mutants. A minimum of three plants for each mutant were tested for calcium spiking experiments

	Nodules per plant	**No. spiking cells/ No. cells tested**	
har1-1	52.2	7/8	
har1-1/ symrk-13	0	0/8	
har1-1/ symrk-14	25.5	0/38	0/28 quiet 0/10 noisy

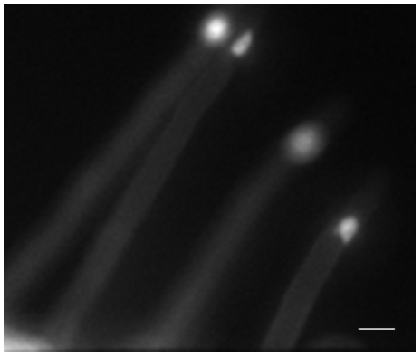


Figure 5.5. Root hair cells of wild-type *L. japonicus* expressing cameleon NupYC2.1.

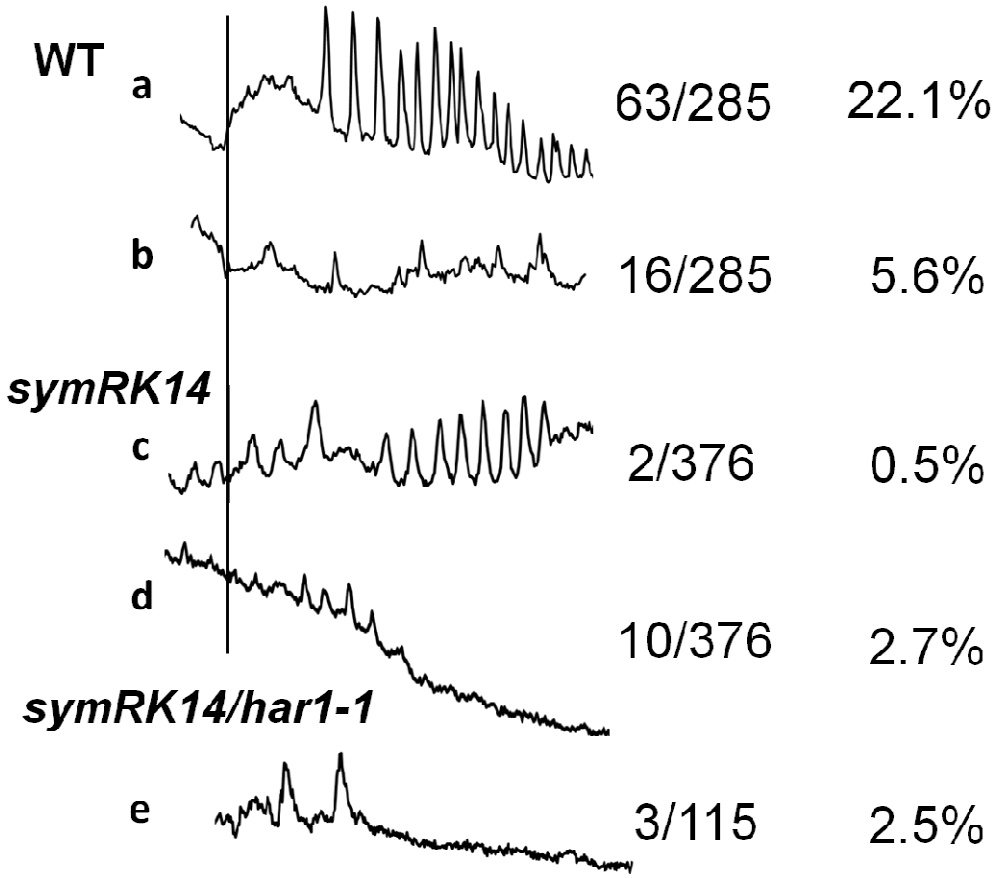


Figure 5.6. Nuclear calcium oscillation in response to *M. loti* Nod factor monitored in *L. japonicus* wild type and *symRK-14* and *symRK-14/har1-1* roots expressing cameleon NupYC2.1

Changes in nuclear calcium were recorded following the addition of 100 nM Nod factor (vertical line) in *L. japonicus* wild type Gifu (a and b), *symRK-14* (c and d) and *symRK-14/har1-1* (e) epidermal cells. The ratio YFP/CFP was plotted against time. Images were taken at 5 seconds intervals for > 30 minutes. The traces show representative cells positive for calcium spiking (a and c) and representative cells showing a few spikes (b, d and e). A minimun of 10 roots from 10 different wt and *symRK-14* mutant plants were tested. Four roots from three different *symRK-14/har1-1* plants were tested. The number of epidermal cells positive for calcium oscillation divided by the total number of cells tested and the relative percentage is indicated next to each trace.

Discussion

Mutational analysis has identified several genes required for both calcium spiking and nodule formation in the Nod factor signalling pathway and this led to the idea that calcium spiking plays a central role in nodule formation. However, three *Lotus japonicus* mutants, *nup85 nup133* and *nena*, form nodules despite being impaired in Nod-factor-induced calcium spiking. Microinjection of *symrk-14/har1-1* root hair cells with Oregon Green/Texas Red revealed that although the *symrk-14/har1-1* mutant forms nodules to the same extent as wild-type, calcium spiking was not induced after addition of Nod factor. One explanation was that only a small proportion of root hair cells are capable of inducing calcium spiking. This would have been similar to what was proposed for the *nup85* and *nup133* mutants which were nodulated but showed no calcium spiking. Microinjection of *L. japonicus* root hairs does not lend itself to identification of rare cells that might induce calcium spiking. Hence, either a lack of requirement for calcium spiking or calcium spiking induced occasionally in some cells, possibly among cell types where root-hair calcium measurements are not possible using the microinjection approach, could be responsible for the occasional infection and nodulation events found with these mutants. In addition, the possibility that calcium spiking occurs in other cells or other cell types (e.g. root epidermal not-hairs cells, cortical cells developing into root hair cells) could not be excluded.

To analyse a larger number of cells, and to analyse different types of epidermal cells (e.g. root epidermal not-hairs cells and old root hair) the nuclear-targeted cameleon (NupYC2.1) (Sieberer et al., 2009) was introduced into wild-type Gifu, *symRK-14* and *symRK-14*/har1-1 roots via *Agrobacterium rhizogenes*-mediated transformation. This analysis revealed occasional Nod-factor-induced calcium spiking in the *symRK-14* mutant. Calcium spiking was observed in two of nearly 400 cells in the *symrk-14* mutant corresponding to about a fortyfold reduction compared with wild-type plants. These results suggest that in roots containing thousands of cells, tens of cells may be able to spike, activate CCAMK and nodule organogenesis in the cortex. Thus, calcium spiking occasionally induced in few root hair cells could explain the nodule organogenesis events observed in the *symRK-14* mutant. No clear calcium spiking was observed in the *symrk-14/har1-1* with 3 of 115 cells showing a few spikes. The total number of root hair cells

tested with this mutant was considerably lower than the total number of root hair cells tested in the *symRK-14* mutant. Therefore it is possible that by doubling the number of cells tested cells positive for calcium spiking could be found.

Using transgenic *L. japonicus* wild-type plants expressing NupYC2.1, 22% of the cells tested responded to 100 nm Nod factor with calcium spiking. When the same concentration of Nod factor was added to microinjected root hair cells of *L. japonicus* wild-type plants, 87% cells were positive for spiking. The root hairs selected for microinjection of *L. japonicus* were typically young with an active cytoplasm streaming and were close to the tip of the seedlings. Root hair cells imaged using NupYC2.1 included a heterogeneous group of long and short root hairs, and non-root hair epidermal cells. I considered the possibilities that 1. Non-root hair epidermal cells might be less responsive to Nod factor for calcium spiking than root hair cell and 2. That young root hair cells with active cytoarchitecture might be more responsive to Nod factor for calcium spiking than root hair cells not showing an active cytoarchitecture. Neverthess I found that 1. by only considering root hair cells (and not not-root hair epidermal cells), the percentage of cells positive for spiking was 19.5% and 2. That even by considering only those root hair cells with active cytoarchitecture (by selecting the root hairs with the nucleus in close proximity to the tip) 25.7% of cells were positive for spiking. Thus, the type of cells did not seem to be a determining factor in the reduction of cell positive for calcium spiking. A considerable difference between the two techniques was the age of the plant tested. Calcium imaging using NupYC2.1 was performed in transgenic plants grown on plate for at least 21 days, while 2 days old seedling were used for microinjection. This difference together with the use of “hairy roots” rather than seedling roots might have had an impact in the responsiveness of root hair cells to Nod factor.

Microinjection experiments revealed that root hair cells of the *symrk-14/har1-1* were more sensitive to touch. This is consistent with the Esseling et al. (2004) observation that *DMI2* alleles in *M. truncatula, M. sativa* and *L. japonicus* are hypersensitive to touch. At this regard, interestingly, it has been proposed that involvement of SYMRK in reduction of touch sensitivity of root hairs (Esseling et al., 2004) may rely on the N-terminal extracellular domain (Markmann et al., 2008) where the *SYMRK-14* mutation occur. This could easily explain the particular hypersensitivity to touch of the *symrk-14* mutant. One could argue that the microinjection is damaging the root hair so much that

they are unable to spike. However, given the hypersensitive to touch of the *symrk-14/har1-1* root hair cells, a more delicate microinjection with finer needles was performed in order to not damage the *symrk-14/har1-1* root hair cells. Only microinjected cells showing active cytoplasmic streaming were selected for calcium analysis. Therefore, in all cells tested for spiking no cell damage was observed which could have prevented cells to spike.

Analysis of calcium levels with microinjection of Oregon Green/Texas red also shows that *symrk-14/har1-1* root hair cells had a high degree of background noise. The 'noise' was not dissimilar to that which Wais et al. (2000) noted in some of the cells carrying a weak allele of the *M. truncatula* ortholog of *SYMRK*, *DMI2*. *dmi2-2* (*P1*) is a weak allele of *DMI2* and the mutant shows some initiation of nodule primordia and some mycorrhizal invasion. P1 also had 'noisy' calcium that looks similar to *symrk-14/har1-1*, except that it sometimes (20%) showed spiking. Occasional low-amplitude calcium increases before addition of Nod factor was also observed in *M. truncatula dmi2-1* and *dmi2-3* mutants (Shaw and Long, 2003). However, it was concluded that the *dmi2* mutant was blocked for calcium spiking. It is possible that, in the "noisy" mutants *symrk-14* and *dmi2*, the noise itself is sufficient to trigger nodule morphogenesis, however fluctuations in basal calcium are present before Nod factor addition either in the *symrk-14/har1-1* mutant and in the *M. truncatula DMI2* alleles (Wais et al., 2000; Shaw and Long, 2003). Moreover, if variable calcium levels were sufficient to trigger nodule morphogenesis, we would expect to see some spontaneous nodulation in *symrk-14/har1-1* mutant, but this is not the case. When nuclear calcium concentrations were monitored with NupYC2.1, "noisy" calcium levels were not observed. Given the touch-sensitivity of the *symrk* mutant, it is possible that the stress caused by the microinjection of the root hair cells altered the resting calcium levels generating a noisy calcium signature. An alternative explanation is that the noise background observed with microinjection derived by calcium fluctuations in the cytoplasm, which can not be detected by nuclear-targeted YC 2.1.

An interpretation of the results showing that nodule morphogenesis occurs with few root hair calcium spiking could be that calcium spiking is not essential for signalling in nodulation. However, this interpretation is inconsistent with several other pieces of data suggesting the opposite. The observation that antagonists that block calcium spiking also block expression of genes downstream in the Nod factor signalling pathway and the

observation that mutations that block calcium spiking also block all signalling events downstream of calcium spiking clearly suggest that calcium spiking plays an important role in Nod factor signalling. It will be of interest to determine whether the *symrk-14/har1-1* is normal for Nod factor induction of early nodulin genes, such as *NIN*, downstream of calcium spiking.

One possibility is that *SYMRK* is required for the integrity of root hairs, but has less of a requirement in other cells. In this way, a partially functional *SYMRK-14* allele might cause impairment of calcium spiking in root hairs (possibly even as an indirect effect) but not in other cell types. If this occurred it might be possible to induce nodule morphogenesis but essentially no infections. There are various modifications of such a model; for example cells other than root hairs might express a protein that helps stabilise the SYMRK protein, or lack a protease so it is more degraded in root hairs. However, further experiments have to be done in order to test such a hypothesis.

Interestingly, all mutants lacking calcium spiking also lack infection threads. It is still not known how infection thread growth is regulated during signalling. The mutant *symrk-14/har1-1* is very strongly defective for infection thread formation. However, interestingly, bacteria do get in as intercellular infection threads have been observed between infected cortical cells (S. Kosuta, personal communication). The lack of intracellular infection threads and calcium spiking in the *symrk-14* root hair cells suggests that calcium spiking is necessary for root hair infection. Therefore, I postulate that Nod factor signalling, including calcium spiking, is required each time the infection thread enters a new cell. Because in *har1-1 symRK-14* calcium spiking is absent in root hair cells, infection threads can not start in these cells. Nevertheless, uncolonised nodule primordia with patches of bacteria at the surface observed in the *symrk-14/har1-1* mutant suggest that the bacteria are entering by alternative means. Infection threads are by definition intracellular. Although intercellular infection may occur (i.e. crack entry), in the *symrk-14/har1-1* and *symrk-14* mutants, the bacteria do not enter the epidermis by way of an infection thread. What we see in the *symrk-14/har1-1*, *symrk-14* and other infection mutants, is that once the bacteria get to the cortex, they are able to enter the cells, sometimes forming infection threads in the cortex. This suggests two possibilities: 1. that entry requirements in the cortex may be less stringent than for epidermal cells and may not require calcium spiking, and/or 2. that the requirement for calcium spiking in cortical

cells may be less stringent than in epidermal cells. We can't role out the possibility that calcium spiking occurs in cortical cells where it could triggers infection thread formation among these cells.

I considered the possibility that the lack of infection thread formation in *symrk-14* might be a result of the heightened touch-sensitivity described by Esseling et al. (2004). Esseling et al. (2004) show that *dmi2* root hairs curl in response to Nod factor, but stop when the tip meets the shank of the root hair, preventing a true shepherd's crook from forming. In *symrk-14*, the root hairs curl excessively (often twice around e.g. 720 degrees), which would not be possible if the root hairs stopped when the tip touched the shank. Despite the double curl, the bacteria aren't properly entrapped, which may be why infection threads are so rare in this mutant. In this case, SYMRK may have a specific role in microcolony formation, as was proposed for *hcl* (another excessive root hair curling mutant) (Catoira et al., 2001; Smit et al., 2007). The data indicating that symbiotic entry of arbuscular mycorrhizal fungi is also blocked in the *symrk-14* mutant suggests a specific role of SYMRK in intracellular accommodation of symbiotic microbes.

Based on these observations, I propose a model with a dual pathway downstream of the initial Nod factor perception at epidermal cells. Nod factor perceptions by specific receptors would trigger calcium spiking in root hair cells which is essential for formation of intracellular infection thread. In parallel, Nod factor perception activates secondary signals that are essential for outer cortex colonisation. Nod factor effect in cortical cells are generated from a distance because it is very unlikely that Nod factor moves through symplastic or apoplastic as they are immobilized in the plant cell walls (Goedhart et al., 2000). Thus, Nod factor probably generates secondary signals that, together with signals coming from the epidermis, drive the development of nodules. Plausible mediators of these primary cortical invasion events are cytokinins and auxin. There is increasing evidence showing that cytokinins and auxin play important role in the activation of cortical responses. It has been known for decades that auxin transport inhibitors can induce the formation of pseudonodules and the expression of ENOD40, a gene associated with nodule primordia (Hirsch et al., 1989; Hirsch and Fang, 1994; Fang and Hirsch, 1998; Mathesius et al., 2000). Moreover, rhizobia, or even to *E. coli* transformed in order to be able to make transzeatine (a cytokinin) can activate nodulations in legumes (Cooper and Long, 1994). Furthermore, a cytokinin-responsive promoter has been found to be induced

during the development of nodule meristem. In addition, genetic studies in *L. japonicus* and *M. truncatula* have highlighted the crucial role of cytokinin in nodulation. Mutation in a *Lotus* histidine kinase (LHK1) which functions as a cytokinin receptor (Tirichine et al., 2007) leads to spontaneous nodulation, whereas loss-of-function mutation in *LHK1* (Murray et al., 2007) or RNAi-mediate downregulation of its ortholog in *M. truncatula* (Gonzalez-Rizzo et al., 2006) causes a dramatic reduction in nodule formation. *lhk1* mutants are normal for the initiation of bacterial infection, but the are unable to form nodule primordia (Murray et al., 2007). This, together with our results showing that the *symrk-14/har1-1* mutant induces nodule primordia without epidermal bacterial infection, indicates that the ability to form nodule primordia is not essential for bacterial infection. SYMRK could be a branch point in the signalling leading to epidermal versus cortical processes. Recognition by the extracellular domain of some unknown rhizobial signals might be essential for the activation of epidermal responses such as a proper root hair deformation, calcium spiking and bacterial infection whereas cortical responses are maybe less stringent for this external interaction. In this way, a mutation in the extracellular GDPC domain, such as the *SYMRK14* mutation, would prevent activation of epidermal responses whereas cortical responses would be triggered.

Plant leucine-rich repeat-receptor like kinases (LRR-RLKs) connect the plant cell to the surrounding environment; outside stimuli are perceived by the extracellular LRR domain and are converted into cellular responses by the intracellular kinase. SYMRK is a LRR-RLK required for symbiotic association of legumes with nitrogen-fixing rhizobia and phosphate-acquiring arbuscular mycorrhizal fungi. Recently, it has been shown that SYMRK is also required for actinorhizal symbiosis in the tree *Casuarina glauca* (Gherbi et al., 2008) and in the cucurbit *Datisca glomerata* with actinobacteria of the genus *Frankia*, revealing a common genetic basis for the forms of plant root endosymbiois (Markmann et al., 2008). RNAi-mediated silencing of SYMRK blocked nodulation and arbuscular mycorrhizal symbiois in *Casuarina glauca* and in *Datisca glomerata*. These reports also show that *CgSYMRK* could restore root endosymbiois in *Lotus symrk* mutants (Gherbi et al., 2008). Introduction of *SYMRK* from rice and tomato, containing only two LRR domains, was sufficient to restore arbuscular mycorrhizal symbiosis but not nodulation in *Lotus symrk* mutant (Markmann et al., 2008). These results, thus, revealed that *SYMRK* is an essential component of the genetic basis for both plant-fungal and plant-bacterial

endosymbiois and is conserved between legumes and actinorhiza-forming plants (Gherbi et al., 2008).

Here, I presented a new *SYMRK* allele with a novel symbiotic phenotype: normal nodulation and blocked or aborted rhizobial and mycorrhizal infection. The *SYMRK-14* mutation causes a single amino acid change, P to T, in the GDPC motif just upstream of the LRR region which is thought to be important for protein-protein interactions. Phylogenetic analysis indicated that the GDPC motif is highly-conserved in LRR-RLKs of evolutionarily-divergent land plants, including the moss *Physcomitrella patens* and the liverwort *Marchantia polymorpha*, but is absent from those of more distant plant relatives such as green and brown algae, and volvox (Sonja Kosuta, personal communication). The requirement of the GDPC motif for root endosymbiosis and the fact that the mycorrhizal symbiosis coincides with the appearance of land plants (Wilkinson, 2001), indicate that the GDPC motif may have been vital for the evolution of both arbuscular mycorrhizal symbiosis and the transition of plants to a terrestrial lifestyle (Sonja Kosuta, personal communication).

Although studies (Esseling et al., 2004; Capoen et al., 2005; Gherbi et al., 2008; Markmann et al., 2008) have recently given new insight into the function of SYMRK, it remains unclear what signal is perceived by the extracellular domain of SYMRK. The ability of *L. japonicus SYMRK* to restore nodulation of *Medicago* with *S. meliloti* indicates that *SYMRK* is not directly involved in determining the legume-rhizobium specificity (Markmann et al., 2008). An attractive possibility is the proposed involvement of SYMRK in processes such as reduction of touch sensitivity of root hairs (Esseling et al., 2004). In this regard, it is interestingly to note that it has been proposed that the involvement of SYMRK in reduction of touch sensitivity of root hairs may rely on the N-terminal extracellular domain of SYMRK (Markmann et al., 2008) and this is the region where the SYMRK14 mutation occurs. Such a function could explain why this protein is required, and has been selected, for the establishment of endosymbiois with arbuscular mycorrhizal fungi, rhizobia and actinorhiza bacteria. Although very different from each other, arbuscular mycorrhizal fungi, rhizobia and actinorhiza bacteria all have to overcome the touch sensitivity of root hair cells to successfully penetrate and infect cortical cells.

In summary, *symrk14* data indicate that residual calcium spiking in the epidermis may be sufficient to induce nodule organogenesis and also supports the corollary, that

infection threads in the epidermis are associated with calcium spiking. The study of nodulation-defective mutants has shown that it is possible to separate epidermal responses form cortical responses: bacterial infection can take place in the absence of nodule organogenesis (Murray et al., 2007), and conversely nodule organogenesis can be accomplished without of bacterial infection (Gleason et al., 2006; Tirichine et al., 2006b). Here, an additional example where nodule morphogenesis can form without bacterial infection is shown. Coming back to the original question: is calcium spiking in root hairs really a good indicator of nodule morphogenesis? These results suggest that even when calcium spiking is induced in a small number of epidermal cells nodule morphogenesis can be initiated. The lack of infection threads and calcium spiking in the *symrk-14* and *symRK-14/har1-1* mutant suggests that calcium spiking in the epidermis is more closely associated with induction of infection rather than with cortical cell division. It will be now essential to understand how the Nod factor-signalling pathway is transduced from the epidermal to the cortical cells to discover the mechanism that underpins the coordination of nodule morphogenesis with rhizobial infection in legumes.

CHAPTER 6

Lectin-Nucleotide Phosphohydrolase (LNP) Is a New Component Required for Nod-Factor Signalling in *Lotus japonicus*

Introduction

Adenosine triphosphate (ATP) is not only an energy source but also a signalling molecule (Roux and Steinebrunner, 2007; Liu et al., 2008). Two groups of enzymes have the ability to hydrolyze ATP: ATPases and apyrases. There are substantial differences among these enzymes. The ATPases have high substrate specificity and hydrolyze ATP to adenosine diphosphate (ADP) and orthophosphate. They use the energy stored in ATP for various processes including metabolism, protein phosphorylation and ion transport. In contrast, apyrases (nucleotide phosphohydrolases [NTPases]) have low substrate specificity and are insensitive to ATPase inhibitors. Apyrases are highly active, nonenergy-coupled enzymes that catalyze the hydrolysis of different nucleoside triphosphates (NTPs) and nucleoside diphosphates yielding nucleoside-monophosphates and orthophosphates (Komoszynski and Wojtczak, 1996).

Apyrases can be dived into two major categories: ecto-apyrases and endo-apyrases. Ecto-apyrases have an extracellular catalytic domain whereas endo-apyrases have an intracellular catalytic domain (Komoszynski and Wojtczak, 1996). Apyrases are found in all prokaryotic and eukaryotic organisms (Steinebrunner et al., 2003). For instance, in yeast (*Saccharomyces cerevisiae*) two endo-apyrase, encoded by the *GDA1* and *YND1* genes, mediate the turnover of GTP to GMP and are required for the N- and O-glycosylation of proteins in the Golgi lumen (Abeijon et al., 1993; Gao et al., 1999). In animals, several studies have highlighted the important regulatory roles of apyrases in the control of signalling events, such as neurotransmission (Edwards et al., 1992;

Komoszynski and Wojtczak, 1996; Nedeljkovic et al., 2005), blood platelet aggregation (Marcus and Safier, 1993), and ATP-mediated immunoresponses (Idzko et al., 2007).

Interestingly, ecto-apyrases function in animals in neurotransmission at the synaptic junction. In the nervous system ATP acts as an excitatory neurotransmitter (Burnstock, 2007). In many synaptic contacts formed in the in central nervous system, ATP is released from neurons where it interacts with P_2 purinoreceptors (Figure 6.1). These receptors have a high affinity for ATP and ADP, but a much lower affinity for AMP. Extracellular ATP is rapidly hydrolysed by the conjugated action of the nucleotidases: Apyrase and ATPase. These enzymes have recently been purified and characterised (Kukulski and Komoszynski, 2003). Whereas ATPase catalyses the hydrolysis of ATP to ADP (Heine et al., 1999), apyrases hydrolyse ATP and ADP equally well directly to adenosine monophosphate (AMP). Since AMP has much lower affinity for the P_2 purinoreceptors, the receptor stimulation is broken. Subsequently, 5'-nucleotidase, an enzyme abundant in the synaptic space, hydrolyzes AMP to adenosine (Todorov et al., 1997). Adenosine is a more potent neurotransmitter and neuromodulator than ATP, it penetrates cell membranes where it is phosphorylated, restoring the cellular ATP pool. Adenosine also has the effect of stimulating adenyl cyclase, causing an increase in the secondary messenger cyclic AMP (cAMP) (Komoszynski and Wojtczak, 1996). Thus, apyrases have the role of reducing one signal (ATP) contributing to the generation of an alternative signal (adenosine), consequently controlling the levels of adenine nucleotides, and the duration and extent of their respective receptor activation.

In plants, apyrases have been implicated in numerous different systems. For example, it has been shown that apyrases from pea are involved in plant phosphate nutrition (Thomas et al., 1999). A yeast (*Saccharomyces cerevisiae*) phosphate-transport mutant was complemented by an ecto-apyrase from pea (*Pisum sativum*) and the overexpression of this gene significantly increased the amount of phosphate uptake (Thomas et al., 1999). These transgenic lines also displayed higher resistance to toxins suggesting an additional role of apyrase in toxin resistance (Thomas et al., 2000). Moreover, the finding that the same pea apyrase copurified with the cytoskeleton led to the hypothesis that apyrase plays a role in mRNA transport along the cytoskeleton (Shibata et al., 1999). It is also know that Calmodulin and Casein Kinase II regulates a pea nuclear endo-apyrase (Hsieh et al., 2000). Riewe at al. (2008) have shown that a potato-

specific apyrase is localised to the apoplast and is involved in gene expression, growth and development (Riewe et al., 2008). In *Arabidopsis,* single T-DNA knockout mutants of two ecto-apyrase genes (*AtAPY1* or *AtAPY2*) did not exhibit a discernible phenotype, while the double mutant displayed complete inhibition of pollen germination, revealing a role of apyrase in the sexual reproduction of plants (Steinebrunner et al., 2003; Wolf et al., 2007).

Ecto-apyrases were first implicated to have a role in nodulation when a unique lectin (Lectin-nucleotide phosphohydrolase, LNP), originally isolated from the legume root *Dolichos biflorus* (Quinn and Etzler, 1987), was shown to be an apyrase and to bind Nod factor (Etzler et al., 1999). Preincubation of LNP with Nod factors that are recognised by its carbohydrate-binding site resulted in an increase in the V_{max} of this enzyme showing that Nod factor binding increased the catalytic activity of the apyrase (Etzler et al., 1999). Subsequent studies showed that *Db*-LNP localises predominately in the root hair cell surface of young roots and that *Db*-LNP redistributes to the tips of the root hair cells after exposure to Nod factor (Kalsi and Etzler, 2000). Root hair cells are known to be receptive to Nod factor and so this finding is consistent with a role for *Db*-LNP in Nod factor perception. Further evidence for *Db*-LNP being involved in nodulation comes from studies of roots treated with antiserum against *Db*-LNP that caused nodulation and root hair deformation to be inhibited (Etzler et al., 1999).

The finding that LNP is involved in nodulation in *D. biflorus* has stimulated recent research on this protein in legumes and now cloned genes and sequences are available from alfalfa (*Medicago sativa)*, *Medicago truncatula*, *Lotus japonicus*, soybean (*Glycine soya*), and pea (*Pisum sativum*) (Roberts et al., 1999; Day et al., 2000; Cohn et al., 2001; Shibata et al., 2001). Phylogenetic analysis revealed that these sequences fall into the legume-specific clade containing *Db*-LNP and another clade that contains two Arabidopsis apyrase-like proteins and a second apyrase identified in *D. biflorus, Db*-apyrase-2 (Roberts et al., 1999) (Figure 6.2.). Roberts et al. (1999) have shown that the legume-specific alfalfa and pea proteins have a carbohydrate binding activity and, hence, can be referred to as LNPs. It has been proposed that the nodulation-implicated apyrase came into being following a gene duplication event (Roberts et al., 1999).

In *M. truncatula* the identification of apyrases and the analysis of their putative role in nodulation are shown by two separate reports which gave contradictory results.

Cohn et al. (2001) identified four putative apyrase genes *MTAPY1, 2, 3* and *4*; two of which (*MTAPY1* and *MTAPY4)* are inducible within 3 to 6 hours after inoculatation with *Sinorhizobium meliloti,* while mRNA levels of *MTAPY2* and *MTAPY3* remained unaffected by rhizobial inoculation. *MTAPY1* was not expressed in roots of two symbiotic mutants *dmi1* and *pdl* even after inoculation with *S. meliloti* suggesting a role for apyrases early in nodulation (Cohn et al., 2001). Navarro-Gochicoa et al. (2003) characterised six apyrase-like genes in *M. truncatula*. Comparison with the *M. truncatula* sequence revealed that two of the clones corresponded to *MTAPY2* and *MTAPY4* (Cohn et al., 2001), two other clones represented new genes, whereas the remaining two clones were identical in either the 5' or 3' moiety to *MTAPY1*. Inspection of the cluster containing *MTAPY1* in the TIGR *M. truncatula* Gene Index database (Quackenbush et al., 2001) suggested that *MTAPY1* cDNA originally identified by Cohn at al. (2001) probably represents a chimera produced during the 3'- and 5' RACE used to obtain this clone. Given the clear separation of the *M. truncatula* genes and their encoded proteins into two phylogenetic classes, *Mt-apyrase* genes were renamed as genes *APY1* (the legume-specific class) and genes *APY2* (legume-non specific class). *APY1* is multigene family which include *MtAPY1;1* (corresponding to 3' moiety of *MTAPY1)*, *MtAPY1;2* (new gene), *MtAPY1;3* (corresponding to 5' moiety of *MTAPY1* and 3'of *MTAPY3*), *MtAPY1;4* (corresponding to *Mtapy4*) and *MtAPY1;5* (new gene) (Navarro-Gochicoa et al., 2003). In contrast to what was observed by Cohn et al. (2001), Navarro-Gochicoa et al. (2003) saw a transient induction of the *APY1* mRNA in wild type after inoculation with *S. meliloti nodA* mutant (incapable of forming symbiosis), as well as in the *M. truncatula* nodulation defective mutant, *dmi1.* Analysis of induction of apyrase mRNA at 6 hours in other symbiotic mutants (*dmi2*, *dmi3*, *hcl* and *nsp*) did not exhibit any clear differences in the expression of the apyrase genes in any of the symbiotic mutants. All together the results of this second report indicate that *APY1* genes are not induced specifically by rhizobia or rhizobial Nod factor.

Soybean also contains at least two apyrases: an endo-apyrase found in the Golgi apparatus, GS50; and an ectoapryase localised in the plasma membrane, GS52 (Day et al., 2000). When applied to roots, antibodies against GS52 blocked nodulation, while those against GS50 had no effect. RT-PCR supports the hypothesis that only GS52 is important in nodulation, as this gene, but not GS50, is up-regulated after treatment with the bacterial symbiont (Day et al., 2000). Recent work has shown that silencing GS52 reduced

nodulation, and, more interestingly, the addition of ADP to silenced roots restored the wild type phenotype (Govindarajulu et al., 2009). When the soybean GS52 gene is expressed in *L. japonicus* the frequency of infection events increases and the number of nodules doubles (McAlvin and Stacey, 2005). Perhaps not surprisingly, based on these observations, GS52 is orthologous to the *LNP* in the *D. biflorus* and *Pisum sativum*, whereas GS50 belongs to the subfamily of apyrase which includes apyrases from *Arabidopsis thaliana* and the *apyrase-2* from *D. biflorus* (Day et al., 2000).

This chapter examines the role of a *L. japonicus* apyrase that shows homology to *DbLNP*. Our collaborators (Roberts NJ, Kalsi G., Rose A., Stiller J., Gresshoff P. and Etzler M.) have silenced *LjLNP* using antisense technology – these plants are unable to form nodules. Clearly, LNP is key to nodulation, the observation that *DbLNP* binds Nod factor suggests that LNP is involved in the Nod factor signalling pathway, however evidence of LNP being actually required for Nod factor signalling has not yet been found. Here, we prove that *LNP* is indeed a component of the Nod factor signal transduction pathway, being essential for Nod factor-induced calcium spiking and calcium influx, and for associated downstream gene induction.

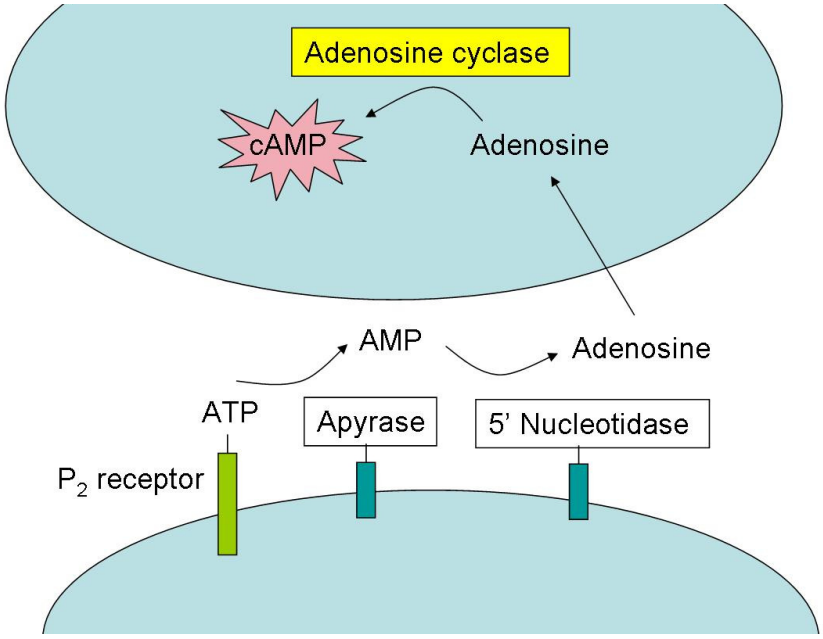


Figure 6.1. Nucleotide reaction pathway inside the synaptic space during neurotransmission process.

Hydrolysis of receptor-bound ATP, catalysed by apyrase, release the P_2 receptor and AMP. 5'-nucleotidase further hydrolyze AMP producing Adenosine. Adenosine penetrates into the cells and activates Adenosine cyclase realising cAMP. Figure modified from Komoszynski and Wojtczak (1996).

Image taken from Navarro-Gochicoa et al., (2003).

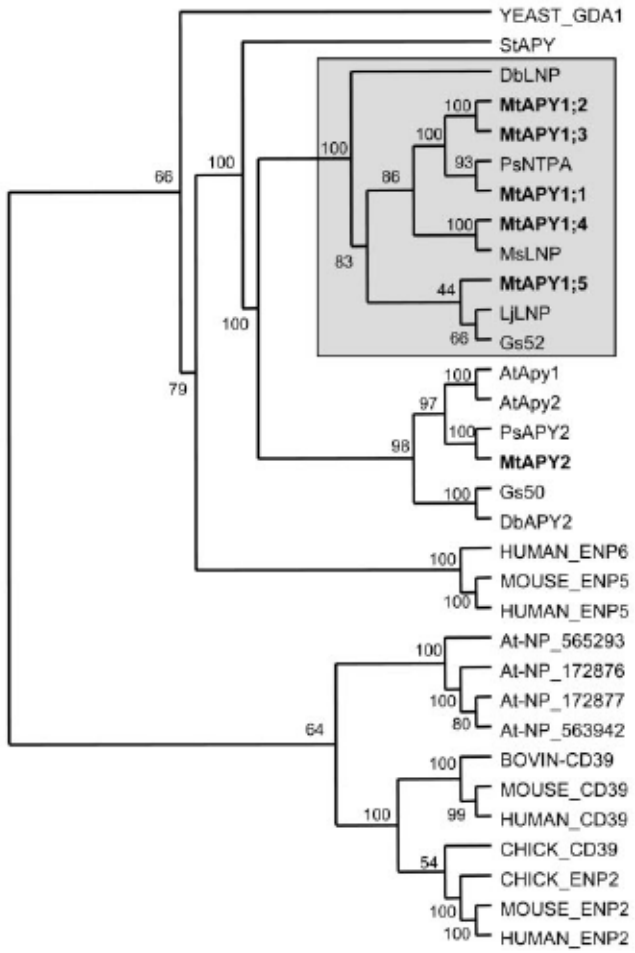


Figure 6.2. Phylogenetic tree of various apyrase-related proteins of *M. truncatula* (MtAPY), Arabidopsis (At), potato (St), soybean (Gs), *D.biflorus* (Db), pea (Ps), *L. japonicus* (Lj), chicken, human, bovine, mouse, and yeast. Image taken from Navarro-Gochicoa et al., (2003).

Results

Antisense Suppression of LNP Expression Results in a Nod$^-$ and Myc$^-$ Phenotype (Roberts et al., Personal Communication)

To investigate the role of *LNP* in *L. japonicus* during nodulation, our collaborators chose to induce post-transcriptional silencing of LNP using an antisense gene construct driven by the constitutive 35S promoter. Using *Agrobacterium tumefaciens* and a pBIN19 binary plasmid vector (Bevan, 1984), *L. japonicus* were transformed with a constructs carrying the 5' (nucleotides 1-719) and 3' (nucleotides 536-1383) cDNA, inserted in antisense orientations between the cauliflower mosaic virus 35S promoter and terminator sequence.

Immunoblotting by Roberts et al. (personal communication) showed the depletion of LNP in uninoculated 8-day old roots from LNP three of the transformed antisense lines, named 5'D, 5'R and 3'O (generated using the 5' or 3' constructs). The three LNP transformed antisense lines had a substantially reduced level of immunoreactive material. In comparison, the vector control had approximately wild type levels of LNP (Figure 6.3A).

Previously it has been shown that LNP is present in the surface of epidermal cells and root hairs (Etzler et al., 1999). To examine the specific effect of *LNP* antisense suppression on the level of LNP on surface, our collaborators employed whole mount confocal immunofluorescence microscopy. This analysis revealed that the surface of the roots of lines 5'D, 5'R and 3'O lines had no detectable LNP in comparison to the wild type and vector control antisense line (Figure 6.3 B-M). The agreement of the results obtained by confocal immunofluorescence microscopy and immunoblot analyses shows that expression of root surface LNP was successfully suppressed in the transgenic lines.

Three weeks after inoculation with *M. loti*, the stable LNP antisense lines did not form any nodules (Roberts at al., personal communication, Figure 6.4). The observation that the 5'D LNP antisense line inhibited for LNP production is unable to form nodules when inoculated with *M. loti* strongly indicates that *L. japonicus LNP* is necessary for nodulation. This is consistent with the observation that RNAi-mediated silencing of *GS52,*

orthologous of *LjLNP,* results in severe suppression of nodule formation (Govindarajulu et al., 2009).

The inability of many early nodulation mutants (nod^-) to form symbioses with arbuscular mycorrhizal fungi (Harrison, 2005) prompted Roberts at al., to investigate the ability of the 5'D 5'R and 3'O antisense lines to be colonised by these fungi. Excessive fungal hyphal branching was observed on the epidermal cells of LNP^- lines compared to wild type and vector control 15 days after inoculation, however fungal hyphae were unable to invade the epidermis of the LNP silenced roots (Roberts at al., personal communication). These results show that the LNP antisense lines are unable to establish symbiotic association with mycorrhizal fungi and they show that this block is, primarily, at the epidermal cell surface.

Figure from N. Roberts et al., personal communication.

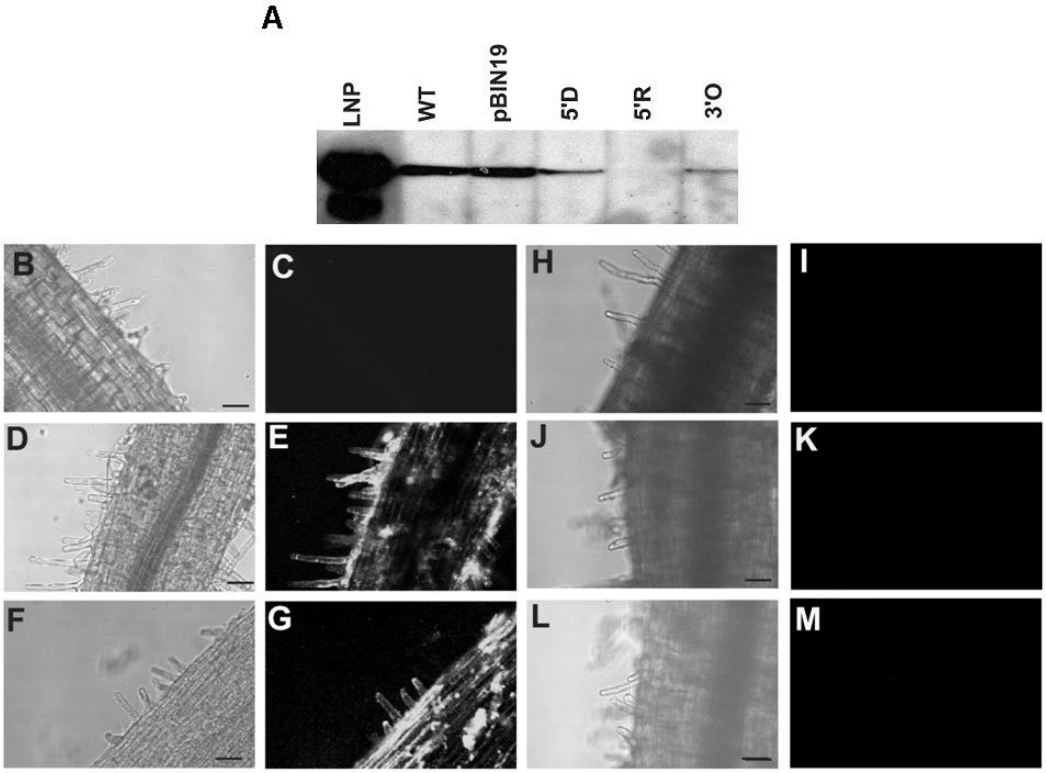


Figure 6.3. 5'D, 5'R and 3'O *LNP* antisense line have reduced level of LNP (N. Roberts, personal communication).

(A) Immunoblot analysis of LNP from wild type, control vector and transgenic antisense line using antiserum prepared against recombinant LNP. The single immunoreactive band corresponds to approximately 46 kDa, the predicted size of Lj-LNP. **(B-M)** Confocal immunofluorescence microscopy was performed using preimmunization serum **(B, C)**; or anti-LNP serum **(D-M)**; on whole mounts of fixed 7-day old uninoculated roots from the following *L. japonicus* lines, **(B-E)** wild type; **(F,G)** vector control; **(H,I)** 5'D; **(J,K)** 5'R; **(L,M)** 3'O. Scale bar = 50 µm

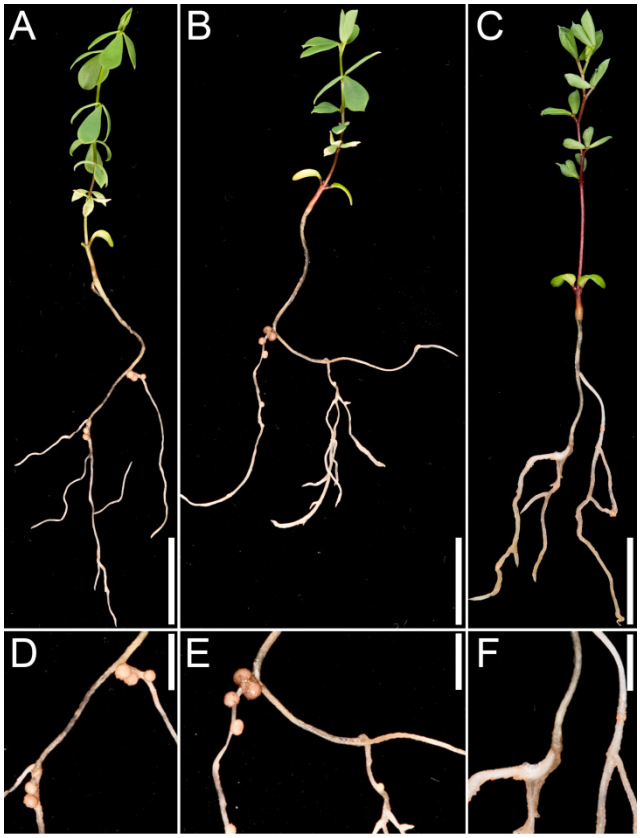


Figure 6.4. Root nodule phenotype of the *L. japonicus* 5'D LNP antisense line.

Nodules were formed in wild type plants (A and D) and vector control (B and E) three weeks after inoculation with *M. loti*. No nodules were observed in the 5'D LNP antisense line (C and F). Scale bar = 10 mm (A-C) and 5 mm (D-F).

Expression of *NIN* is Impaired in *LNP* Antisense Line

The lack of nodulation in the LNP antisense lines suggested that LNP may play a role in the Nod factor signalling pathway. Although there is evidence showing that *Db*-LNP binds to Nod factor, it has not yet been tested if LNP is involved in the transduction of the Nod factor signal. In *L. japonicus*, transcription of *NIN* is induced after a few hours of Nod factor treatment. The transcript abundance of this gene represents a useful indicator of Nod factor signalling. The observations that the induction of *NIN* requires genes necessary for the activation and the decoding of calcium oscillations (*NFR1, NFR5*, *DMI1*, *DMI2, DMI3, NSP1* and *NSP2)* provides strong evidence for a causal link between calcium spiking and *NIN* induction (Radutoiu et al., 2003; Imaizumi-Anraku et al., 2005; Miwa et al., 2006b; Marsh et al., 2007). To test if LNP is required for *NIN* induction, *NIN* expression was assayed in the 5'D *LNP* antisense line after Nod factor treatment. *NIN* transcript levels in the *5'D* LNP antisense line were not significantly induced after 12, 24 or 48 hours of Nod factor treatment (Figure 6.5). In contrast, in the wild type, and antisense vector control seedlings, *NIN* was clearly induced 24 hours after Nod factor addition (Figure 6.5). These data indicate that *LNP* is required for full transcriptional activation of *NIN* suggesting that LNP is a component of the Nod factor signalling pathway.

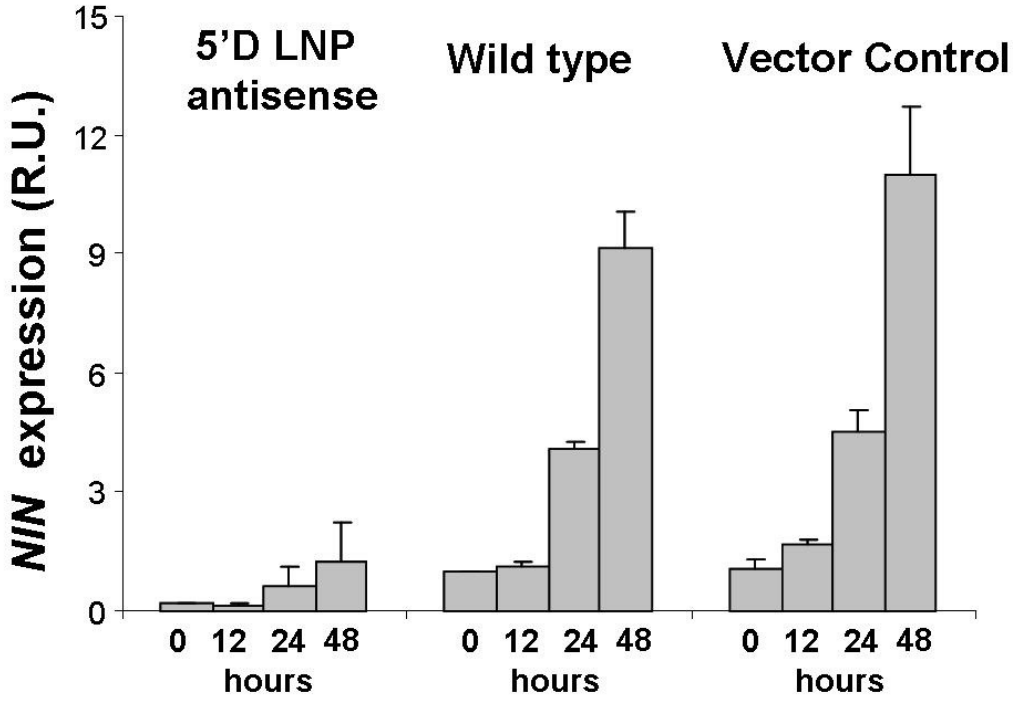


Figure 6.5. The *L. japonicus* 5'D LNP antisense line does not induce *NIN* expression after Nod factor treatment (100 nM).

Quantitative RT-PCR analysis of *NIN* expression in 5'D LNP antisense line, wild type and vector control plants at 0, 12, 24 and 48 hours after Nod factor treatment. *Polyubiquitin* was used as internal control. All data are from three technical replicates of at least three biological repetitions. Mean values ± SD are shown.

LNP is Required for Activation of Nod factor-Induced Calcium Spiking

In *L. japonicus* the perception of Nod factor by NFR1 and NFR5 leads to the generation of calcium oscillation around the nucleus. Perception of calcium spiking by a calcium/calmodulin-dependent protein kinase is then associated with induction of early nodulin genes necessary for nodulation (Oldroyd and Downie, 2004; Gleason et al., 2006; Hirsch et al., 2009). To determine if LNP is required for Nod-factor-induction of calcium spiking, calcium signalling was analysed in the 5'D *LNP* antisense line. The 5'D line was completely defective for Nod factor induced calcium spiking: no cells showed calcium spiking in 43 root hairs tested on 13 plants. In contrast, wild-type plants and the empty vector control plants, treated in parallel to the antisense line, were positive for Nod factor-induced calcium spiking showing calcium spiking in 30 out of 36, and 30 out of 38 root hair cells tested respectively (Figure 6.6). These data indicate that *LNP*, like *NFR1*, *NFR5* and components of the symbiosis signalling pathway is required for Nod factor-induced calcium spiking and are consistent with the observation that LNP is required for *NIN* induction.

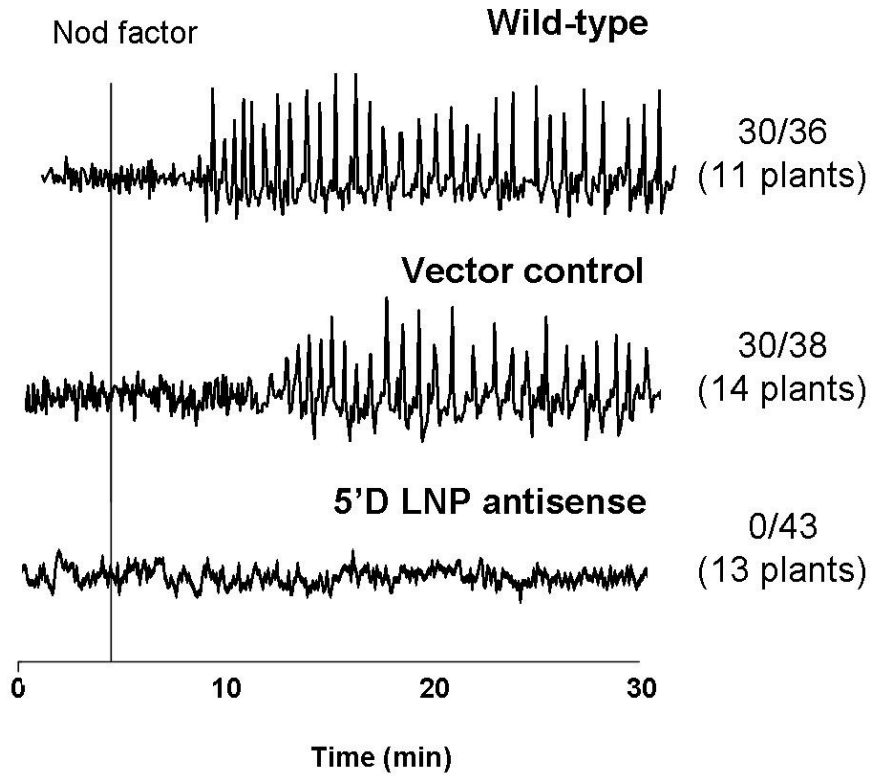


Figure 6.6. The *L. japonicus* 5'D LNP antisense line is defective for Nod factor-induced calcium spiking.

Roots of wild-type, vector control plants and 5'D LNP antisense line were treated with 100 nM *M. loti* Nod factor (black vertical bar). Data are presented as derivative traces representing the change in fluorescence intensity of Oregon Green/Texas Red from one point to the next ($x_{n+1} - x_n$). The number of cells showing calcium spiking is shown as a fraction of the total number of cells analysed (with the number of total plants tested in parentheses).

The 5'D LNP Antisense Line is Defective for Calcium Flux

LNP may act either as a Nod factor receptor, such as *NFR1* and *NFR5,* or as a component of the signalling pathway shared by rhizobia and mycorrhizal fungi, such as *SYMRK, NUP133, NUP85, CASTOR* and *POLLUX*, downstream of the Nod factor receptor. To discriminate between these possibilities I exploited the observation that *nfr1* and *nfr5* mutants are completely defective for all Nod factor-induced responses, while *symRK*, *nup133, nup85, castor* and *pollux* mutants are defective for calcium spiking and gene induction but show some Nod factor responses, such as Nod factor-induced calcium influx and root hair deformation (Miwa et al., 2006b). Thus, the 5'D LNP antisense line was tested for Nod factor-induced calcium influx. In wild type, the calcium influx response is induced within the first few minutes of Nod factor application. Using the dual-dye pseudo-ratiometric calcium imaging system, a rapid rise in cytosolic free calcium was detected in *L. japonicus* in response to Nod factors (Miwa et al., 2006b). As the calcium influx response is localised in the tip of the root hair (Shaw and Long, 2003), changes in intracellular calcium were analysed in the cells and in the tip of every root hair tested as described in Chapter 4; only those cells which induced a significant transient increase in tip calcium and in the shaft area of root hair cells were considered positive for influx. No perceptible changes in cytoplasmic calcium concentration in the 5'D LNP antisense line were observed (n= 26 cells tested from ten plants), whereas wild-type plants and the empty vector control plants clearly showed calcium flux (n= 15 and 10 cells tested respectively, Figure 6.7). These results provide evidence that *LNP* acts upstream of both Nod factor-induced calcium spiking and the calcium influx responses.

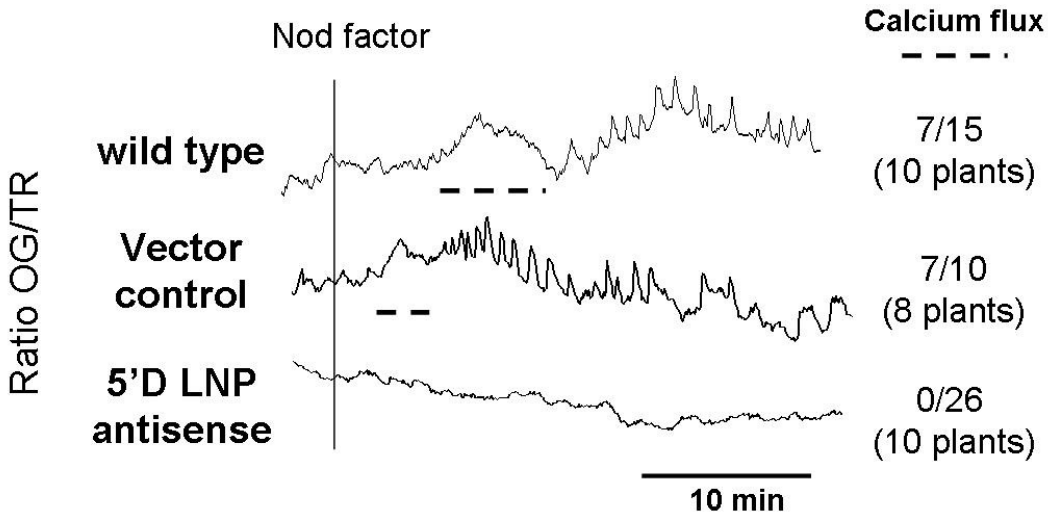


Figure 6.7. Nod factor-induced calcium influx is absent in the *L. japonicus* 5'D LNP antisense line.

Calcium levels were monitored in individual root hairs of wild-type, vector control and 5'D LNP antisense plants after addition of 100 nM Nod factor (black vertical line). Ratios (arbitrary units) of fluorescence of Oregon Green (calcium sensitive dye) and Texas Red (calcium insensitive dye) were calculated every 5 s for > 30 min. Black dashed lines indicate calcium flux. The number of cells showing calcium influx is shown in the inset table as a fraction of the total number of cells analysed (with the total number of plants tested in parentheses).

Root Hair Deformation Is Normal in LNP Antisense Line

The finding that the silencing of LNP resulted in the absence of calcium spiking, calcium influx and *NIN* expression, together with the evidence showing that LNP binds Nod factor (Etzler et al., 1999) suggests that LNP could play a role in the perception of Nod factor, upstream of the common symbiosis signalling pathway in a position shared with *NFR1* and *NFR5*. If this is true and if LNP is, indeed, a Nod factor receptor it would be expected that the antisense line would completely lack all Nod factor responses. Root hair deformation is one of the first observable responses to Nod factor. Most of the Nod-factor signalling mutants show some root hair deformation after addition of Nod factor. The only mutants that do not show any root hair deformation responses to Nod factor are the the Nod factor receptor mutants *nfr-1* and *nfr-5* (Radutoiu et al., 2003). This is why it has been proposed that the induction of root hair deformation is on a separate pathway from induction of early nodulation gene expression and calcium spiking (Miwa et al., 2006b). The LNP antisense line was tested for the root hair deformation response upon *M. loti* inoculation or Nod factor treatment. Surprisingly, root hair deformation was similarly induced in LNP and vector control antisense lines (Figure 6.8). This result indicates that the 5'D LNP antisense line is able to perceive and respond to Nod factor, suggesting that *LNP* acts downstream of the Nod factor receptors *NFR1* and *NFR5*.

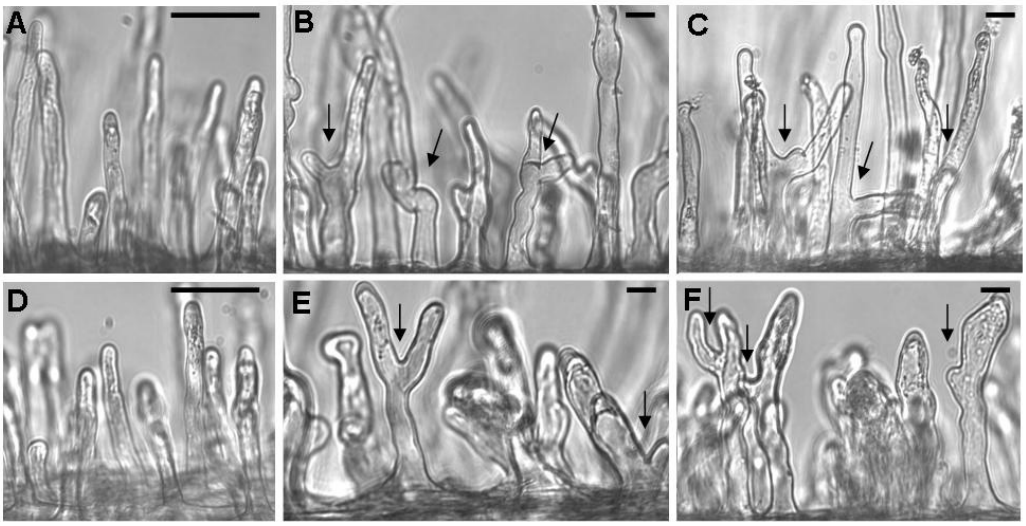


Figure 6.8. Root hair response after Nod factor application or *M. loti* inoculation in Vector control and 5'D antisense line plants.

Vector control root hairs (**[A]**) to **[C]**) of uninoculated plants **[A]**, plants treated with 10 nM Nod factor [**B]** and seedling inoculated with *M. loti* **[C]**. (**[D]** to **[E]**) 5'D LNP antisense root hairs cells of uninoculated plants **[D]**, plants treated with 10 nM Nod-factor **[E]** and seedling inoculated with *M. loti* **[F]**. Arrows indicate root hair deformation. Scale bars: 50 µm (**[A]** and **[D]**), 10 µm (**[B]**, **[C] [E]** and **[F]**).

Discussion

LNP was initially identified based on its Nod factor binding showing preferential binding to Nod factor relative to chitin. The localisation of LNP to legume root hair cells (Etzler et al., 1999), its rhizobial-induced accumulation at root hair tips (Kalsi and Etzler, 2000) and the ability of LNP antiserum to interfere with nodulation (Etzler et al., 1999) all pointed to a function for LNP in nodulation signalling. Subsequent studies in soybean revealed that the *LNP* orthologue *GS52* plays a key role in nodulation. Day et al. (2000) reported that *GS52* mRNA levels increased significantly upon inoculation with *B. japonicus* and that GS52 antiserum blocked soybean nodulation. More recently, knock-down of *GS52* was shown to significantly decrease the formation of mature nodules (Govindarajulu et al., 2009). Moreover, transgenic expression of soybean apyrase *GS52* doubled nodule number and increased root infection by *M. loti* in *L. japonicus* (McAlvin and Stacey, 2005).

If LNP is necessary for nodule formation, what likely roles exist for this protein in nodulation? In this chapter I investigated the role of the *L. japonicus* LNP in the Nod factor signalling pathway necessary for the early stage of nodulation. The results presented in this chapter support the hypothesis that LNP is required for nodulation. Our collaborators have shown that knock-down of *LNP* with antisense technology suppressed completely the formation of nodules and infection by rhizobia (Roberts et al., personal communication) and the phenotype observed with antisense silencing of *Lj*-LNP was much stronger than that induced by *GS52* RNAi resulting in the formation of numerous small empty nodules on soybean. The complete absence of nodule morphogenesis in the 5'D LNP antisense line suggests that in *L. japonicus* cortical responses may have a higher stringency for expression of *LNP* compared to *Glycine soya* or that there may be redundancy in *Glycine* for LNP orthologues. Alternatively, the antisense may have been more effective at preventing the production of LNP in *L. japonicus* than the RNAi on soybean.

During the early stage of nodulation bacterially-derived Nod factor induces oscillation of cytoplasmic and nucleoplasmic calcium which is followed by induction of genes required for nodulation. The results in this chapter show that *LNP* is necessary for activation of calcium spiking and for induction of *NIN*. Previously reported genes of *L. japonicus* required for the induction of calcium spiking are genes that encode for the Nod

factor receptors NFR1 and NFR5, SYMRK located in the plasma membrane, the cation channels CASTOR and POLLUX in the nuclear membrane and the nucleoporin NUP85, NUP133 and NENA (Radutoiu et al., 2003; Imaizumi-Anraku et al., 2005; Kanamori et al., 2006; Miwa et al., 2006b; Saito et al., 2007; Groth et al., 2010). All of these genes are also required for the establishment of the symbiosis with arbuscolar mycorrhizal fungi (Radutoiu et al., 2003; Imaizumi-Anraku et al., 2005; Kanamori et al., 2006; Saito et al., 2007). The commonality of these genes suggests an evolutionary conserved pathway, called the common symbiosis signalling pathway. The results showing that *LNP* is required for induction of calcium spiking and downstream gene induction could be explained by either *LNP* sharing a position in the Nod factor signalling pathway with the *NFR1* and *NFR5*, or by *LNP* being downstream of the *NFR1* and *NFR5* in the common symbiosis signalling pathway.

Mutations in the Nod factor receptors block all Nod factor responses including calcium flux, calcium oscillations and root hair deformation (Radutoiu et al., 2003), whereas mutants *symrk*, *castor*, *pollux*, *nup85* and *nup133* are unable to induce calcium oscillations but are positive for Nod factor-induced calcium influx and root hair deformation (Miwa et al., 2006b). These phenotypes were assessed in the LNP antisense line. The observation that antisense knockdown of LNP abolished induction of calcium influx, together with the previous finding that LNP binds Nod factor support the hypothesis of LNP being a Nod factor receptor. However, the LNP antisense line was positive for root hair deformation, indicating the root hair cells could perceive Nod factor. Kalsi and Etzler (2000) reported that pre-treatment of roots with anti-LNP serum inhibited root hair deformation. I used a system for growing *L. japonicus* plants between filter papers (Miwa et al., 2006b), which allows a very sensitive analysis of root hair deformation. Differences in the sensitivity of the methods used for growing root hairs and testing root hair deformation might explain the different results obtained. Alternatively, it is possible that the presence of a very low amount of LNP in the antisense line which has been detected in the immunoblot (Figure 6.3) is sufficient to trigger root hair deformation but not calcium spiking. If that is the case, a more efficient knock-down of LNP with RNAi might be expected to block this response. The observation that the 5'D LNP antisense line is positive for root hair deformation indicates that LNP plays a role in the Nod factor signalling pathway downstream of NFR1 and NFR5 (Figure 6.8). A possible

experiment to confirm this observation is to test whether the redistribution to root hair tip of LNP induced by Nod factor is observed in the *nfr1* or *nfr5* mutants.

The finding that LNP is a peripheral membrane protein (Kalsi and Etzler, 2000) suggests that if LNP functions also in Nod factor perception it would most likely do so in association with other membrane proteins, perhaps as a part of a receptor complex. *L. japonicus NFR1* and *NFR5* genes are LysM-type serine/threonine receptor kinases (Madsen et al., 2003; Radutoiu et al., 2003) and *SYMRK* gene is a leucine-rich repeat-receptor-like kinase (Stracke et al., 2002). LNP may form a receptor complex with one or more of these gene products and enhance the Nod factor signal required for induction of calcium influx and calcium oscillations.

The results suggest that hydrolysis of extracellular ATP catalysed by LNP may be required for the Nod factor-induced calcium influx, calcium spiking and gene induction (Figure 6.8). To understand how extracellular ATP could play a role in nodulation we should first examine what we know about the biological function of extracellular ATP. In the last two decades reports of ATP in the extracellular matrix of multicellular organisms (Sedaa et al., 1990) and in the extracellular fluid of unicellular organisms (Boyum and Guidotti, 1997) have stimulated research into the role of ATP outside of the cell. In animal cells, extracellular ATP plays an important role in cellular signalling. It binds purinergic receptors and triggers signalling cascades that lead to diverse responses. There are two main families of purinoceptor (P_1 and P_2). P_1 receptors are activated by adenosine (P_2 are not) and are coupled with heterotrimetric G proteins. P_2 receptors are divided into two classes, ligand-gated non selective cation channels (P_2X receptors) and G-protein-coupled receptors (P_2Y receptors). P_2X receptors activate at higher extracellular ATP concentrations (μM) than P_2Y (nM; (Khakh, 2001)). Purinoceptors can contribute directly or indirectly to calcium signal transduction pathways, by functioning through calcium permeability of P_2X channels or via downstream effects of the P_2Y-coupled G-protein. Purinoceptors are involved in a variety of physiological functions ranging from neurotransmission to cell death (for review, see (Ralevic and Burnstock, 1998; Burnstock and Williams, 2000). In animal cells, extracellular ATP is ubiquitously used for cell-to-cell communication. The low concentration of extracellular ATP that exists in an "aura" surrounding resting cells signals the presence of neighbouring living cells. Transient increases in concentration of extracellular ATP can contribute to basic physiological

signalling, specifically in the nervous and vascular systems, whereas larger increases in extracellular ATP concentration are associated with cell death and function as a key "danger" signal in inflammatory processes (Trautmann, 2009).

Plants are able to sense and respond to extracellular nucleotides. In *Arabidopsis*, various nucleoside triphosphates and diphosphates (approximately 1 mM) triggered plasma-membrane depolarisation in root hairs (Lew and Dearnaley, 2000). Extracellular ATP (> 1mM) inhibited root gravitropism, decreased auxin transport, stimulated auxin accumulation in root tips and increased sensitivity of roots to exogenous auxin implying that extracellular ATP inhibits root gravitropism by inhibition of auxin transport (Tang et al., 2003). Hormones like auxin and cytokinin are positive regulators of nodulation (Hirsch et al., 1989; Hirsch and Fang, 1994; Fang and Hirsch, 1998; Mathesius et al., 2000; van Noorden et al., 2007). It has been proposed that accumulation of auxin can modulate flow of cytokinin and this can change the localised levels of cytokinin, which could trigger nodule formation in the cortex (Oldroyd and Downie, 2008). If extracellular ATP inhibits auxin transport, the ATP quenching activity of ecto-apyrase LNP, may indirectly affect nodulation by modulating auxin levels.

Extracellular ATP is one of the primary chemical signals indicating stress conditions. Mechanical stimulation (touch, shear stress and stretch), osmotic shock, and hypoxia can trigger ATP release in a diversity of animals cells (Ostrom et al., 2000; Gerasimovskaya et al., 2002). In plants, touch and osmotic stresses are also able to induce ATP release (Jeter et al., 2004) and are associated with elevated cytoplasmic calcium concentrations (Knight et al., 1991; Knight et al., 1997). Release of ATP could be from damaged cells or as a result of cell death. In this respect, a role in defence signalling could be involved. It has been suggested that, as microbes can release ATP and other purines, extracellular ATP could act as a signalling agent in plant-microbe interactions at the epidermis (Demidchik et al., 2003). Indeed, chitin, a known elicitor of plant defence responses, induces an increase of extracellular ATP that correlates with high ROS activity (Kim et al., 2006). Previous studies indicate that rhizobia might be initially recognised as intruders that somehow evade or overcome the plant defence response and several plant defence responses are induced during root nodule ontogeny (Parniske et al., 1990; Vasse et al., 1993; Gamas et al., 1998). However, it remains unclear how legumes regulate plant defence responses during the symbiosis with rhizobia. The finding that the apyrase LNP is

required during early stage of for nodulation suggest a mechanism for legumes to avoid plant defence responses during symbiosis with rhizobia. Legume specific apyrases, by decreasing the amount of extracellular ATP, could prevent the induction of plant immune responses during nodulation. However, the mechanism behind this remains unclear.

Interestingly, a recent report showed that Nod factor induces a rapid and specific transient increase in intracellular reactive oxygen species (ROS) levels, whereas chitosan (a fungal elicitor) or ATP induced a sustained increase of ROS in the root hair tip (Cardenas et al., 2008). It has been proposed that regulating ROS production at the right time and place, allows rhizobia to enter the host plant without triggering a hypersensitive response (Cardenas et al., 2008). However, it still not fully understood how the precise regulation ROS production occurs. Extracellular ATP has been reported to induce changes in intracellular levels of ROS (Song et al., 2006), and indeed Cardenas et al. (2008) showed that extracellular ATP modulates intracellular levels of ROS. After exposure to Nod factor, LNP redistribution and ROS production both occur at the root hair tip (Cardenas et al., 1998), suggesting that these two processes, may be mechanistically linked. Therefore, possibly, LNP, by controlling the extracellular amount of ATP, might regulate and shape the transient intracellular ROS changes in root hair cells responding to Nod factor. This may be a mechanism for the symbiont to avoid defence reactions that could be triggered by elevated and sustained levels of ROS. The specificity of this transient increase, not observed by chitosan treatment, might be due to the ability of Nod factor to bind LNP and activate his ATP hydrolytic activity. If LNP, by modulating extracellular ATP levels, is indirectly responsible of the transient increase of ROS induced by Nod factor it would be interesting to see whether Nod factor could induce a transient ROS increase in an LNP knockdown plant.

Recently, it has become clear that extracellular ATP also regulates plant viability (Chivasa et al., 2005) and growth (Kim et al., 2006; Roux and Steinebrunner, 2007; Wu et al., 2007a). Extracellular ATP was found in the interstitial spaces between plant epidermal cells (*M. truncatula*), mainly at the regions of actively growing cells, suggesting a role for extracellular ATP during plant cell growth (Kim et al., 2006). Recent evidence has shown that in plants, as in animals, an increase in cytoplasmic calcium is a downstream consequence of extracellular ATP (Demidchik et al., 2003; Hanley et al., 2004; Jeter et al., 2004; Liu et al., 2008; Zeng et al., 2008; Demidchik et al., 2009; Yip et al., 2009). In the

immune system, the activation of T-cell by antigens triggers ATP release which activate the P_2X_7 receptor. As a result, calcium influx is facilitated and this, in turn, induces T-cell activation events through the activation of the nuclear factor of stimulated T cell and interleukin-2 transcription (Yip et al., 2009). In macrophages, ATP triggers oscillations in cytosolic calcium and activate transcription of IL-6 (Hanley et al., 2004). In neurons, membrane depolarisation elicits ATP release, acting through a highly sensitive P_2Y receptor/IP_3-mediated signalling pathway to mediate the propagation of intercellular calcium oscillation responsible for cell-cell communication and cell migration (Liu et al., 2008; Zeng et al., 2008). In plants, Demidchik et al. (2003) first showed that ATP could trigger an increase in intracellular calcium levels using transgenic Arabidopsis expressing aequorin. Jeter et al. (2004) indicated that ATP-induced increases in calcium levels were coupled to downstream gene expression in stress and wound responses. In a process essential for cell elongation and polar root hair growth, calcium-dependent ATP release increased production of reactive oxygen species, which in turn activated calcium influx from extracellular stores (Foreman et al., 2003; Kim et al., 2006; Demidchik et al., 2009). According to our results one could speculate that extracellular ATP may have an inhibitory effect on induction of calcium responses such as calcium influx and calcium spiking induced by Nod factor, although no evidence for this in assays of calcium spiking and calcium influx was observed (Sarah Shailes, G. Oldroyd and A. Downie unpublished data).

These considerations implie that cells have cellular mechanisms to release ATP and sense its presence. In animals, anion channels, ABC transporters or exocytosis contribute to the released of ATP. ATP is released from plant cells by plasma membrane ATP-binding cassette (ABC) proteins (Thomas et al., 2000; Roux and Steinebrunner, 2007), vesicular efflux (Kim et al., 2006) and wounding (Jeter et al., 2004). How plants perceive extracellular ATP remains unknown. There are no equivalents to animal purinoreceptors evident in higher plant genomes (Tang et al., 2003; Kim et al., 2006; Fountain et al., 2008), although inhibitors of purinoreceptors (PPADS and suramin) attenuate the ATP effect on cytosolic calcium concentrations in Arabidopsis (Demidchik et al., 2003). The antagonistic effects of PPADS and suramin suggested the existence of P_2 equivalents in plant cells. In order to elucidate the molecular basis for the perception of ATP and its role

in nodulation, the effects of antagonists of purinergic receptors on Nod factor signalling could be tested.

Addition of exogenous 100 µM ADP to RNAi *GS52* transgenic roots partially rescued nodule formation (Govindarajulu et al., 2009). It would be interesting to know whether addition of ADP to the 5'D LNP antisense line could restore induction of calcium spiking and nodule formation. Our results indicate that the presence of LNP is required for calcium spiking, however it is still unclear whether the ATP hydrolytic activity itself or/and the Nod factor binding property of LNP are necessary for induction of calcium responses. A possible way to test this would be to add exogenous apyrase and see whether the hydrolysis of ATP by the exogenous apyrase could restore induction of calcium spiking.

The LNP antisense lines also lost their ability to associate with mycorrhizal (Myc) fungi (Roberts et al., personal communication). This phenotype defective for mycorrhization (Myc^-) is a characteristic of some of the early Nod^- *L. japonicus* mutants of symbiotic genes like *SYMRK* (Stracke et al., 2002), *CASTOR* and *POLLUX* (Imaizumi-Anraku et al., 2005), *NUP133* (Kanamori et al., 2006), *NUP85* (Saito et al., 2007), *SYM15* (Demchenko et al., 2004) and has been interpreted to suggest that these two symbiotic pathways may share common components that function early in the plant response to these symbionts.

Analyses of seven early Myc^- mutants of *L. japonicus* (Novero et al., 2002; Demchenko et al., 2004; Kistner et al., 2005) identified various steps of fungal colonisation of roots. In all the early Myc^- *L. japonicus* mutants identified so far fungal hyphae have been observed to enter between epidermal cells but the intracellular penetration of epidermal or outer cell layers is blocked (Demchenko et al., 2004). In the *symrk* mutant, fungal hyphae formed appressoria and balloon like deformations but the subsequent fungal entry in cells of epidermis and exodermis was aborted, blocking the intracellular passage (Demchenko et al., 2004; Kistner et al., 2005). Attachment of fungal hyphae and appressoria formation were observed on the root surface of the LNP antisense line but intercellular or intracellular entry in the epidermal cells was not observed. LNP may act upstream of other identified *L. japonicus* symbiotic genes which play a role in the establishment of the mycorrhizal symbiosis. The very late colonisation by *Glomus intraradices* at a very low frequency was observed in the roots of LNP

antisense lines. A similar result was reported with the *L. japonicus* Myc$^-$ mutants in which the mutants had greatly reduced numbers of colonised roots but the subsequent development of the successful mycorrhizae appeared to be normal (Wegel et al., 1998; Kistner et al., 2005). These results suggest that LNP may play a role in events leading to the epidermal cell entry of fungal hyphae but is not required following successful invasion.

Several publications suggested a role for *LNP* in early stages of nodulation, but the mechanism by which *LNP* is involved in such a role was unknown. The results in this chapter provide the missing link by directly showing that *LNP* is a new component in the Nod factor signalling pathway. *LNP* is required for induction of calcium spiking, calcium influx and *NIN* expression, but not for root hair deformation. Taken together these results indicate that *LNP* is in a unique position in the Nod factor signalling pathway, downstream of genes encoding for the Nod factor receptor NFR1 and NFR5 but upstream of SYMRK, cation channels, nucleoporins (Figure 6.9). The finding of *LNP* being a new component in the Nod factor signalling pathway is consistent with earlier reports of Nod signal enhancement of apyrase enzyme activity (Etzler et al., 1999). Therefore, I propose that *LNP* may have a crucial role in determining the amount of extracellular ATP which can trigger calcium influx and calcium oscillations essential for nodulation.

In addition, we showed that *L. japonicus LNP* is also required for symbiotic association with mycorrhizal fungi, indicating that *LNP* is part of the common symbiosis pathway shared by nodulation and mycorrhization (Figure 6.9). Considering the apparent function for *LNP* in Nod factor signalling, I propose an analogous role for *LNP* during induction of calcium oscillations induced by fungal signals. Similarly to nodulation, LNP may be involved in the recognition and signalling of the Myc Factor. This would imply that the Myc factor released by mycorhhizal fungi is structurally similar to the rhizobially-made Nod factor. The data suggest that *LNP* plays an important role in quenching the signalling effect of extracellular ATP. This hypothesis would be consistent with the findings of Jeter et al. (2004), who showed that extracellular ATP is involved in stress-mediated plant responses. Thus, the ability to control extracellular ATP levels by *LNP* could allow fine control of cellular responses both beneficial (e.g. calcium influx and calcium oscillation) and detrimental (e.g. defence responses) to infection by both rhizobial bacteria and mycorrhizal fungi.

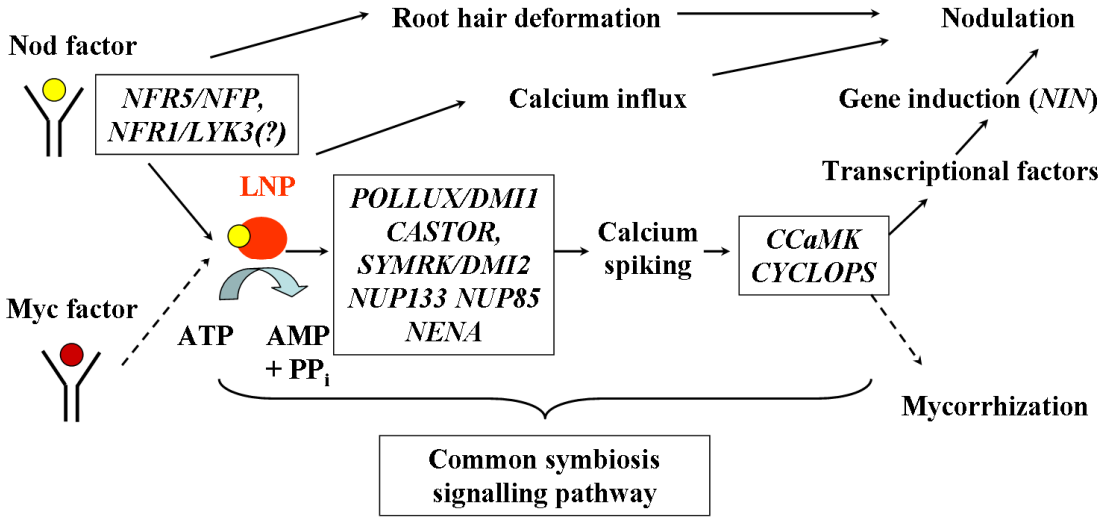


Figure 6.9. Model proposed for Nod factor signalling pathway.

Mutations in *NFR1* and *NFR5/NFP* encoding the predicted Nod factor receptor blocked Nod factor-induced calcium influx, calcium spiking, and root hair deformation. LNP is Nod factor-binding phosphohydrolase that catalyse the hydrolysis of adenosine 5'-triphosphate (ATP) into adenosine 5'-monophosphate (AMP) plus inorganic pirophosphates (PP_i). Knockdown of *LNP* blocked calcium influx, calcium spiking but not root hair deformation. Mutations in *POLLUX/DMI1, CASTOR*, *SYMRK/DMI2, NUP133, NUP85* and *NENA* blocked calcium spiking but not calcium influx; this, together with the observation that calcium spiking can be induced at low levels of Nod-factor but calcium influx cannot, shows that calcium influx is a separate response from calcium spiking. The fact that root hair deformation is induced by low concentrations of Nod factors that do not induce calcium influx shows that the observed calcium influx cannot be required for root-hair deformation. Mutations in *CCAMK, CYCLOPS,* and *NIN* do not block either calcium spiking or calcium influx. The *LNP* antisense line, *pollux/dmi1, castor*, *symRK/dmi2, nup133, nup85* and *ccamk* mutants are defective for early stages of mycorrhization (Kistner et al. 2005) and, therefore, are placed on a common pathway.

Chapter 7

General Discussion and Conclusions

The Nod Factor Signalling Pathway in Nodulation

A complex biological process lasting up to several weeks sits behind the development of a nitrogen-fixing root nodule. Cells from both the root epidermis and root cortex participate at the establishment of this structure, which will eventually enhance the overall plant growth, because nitrogen will be provided to the plant by the nitrogen-fixing bacteroids in symbiosomes within the nodule. Nod factors are essential signalling molecules required for nodulation. Some of the plant responses associated with nodulation can be induced by Nod factors released by rhizobia. Thus, events such as root hair deformation, root hair curling and expression of genes associated with nodule formation, cortical cell divisions and pre-infection thread structures all, remarkably, depend on the plant perception of Nod factor.

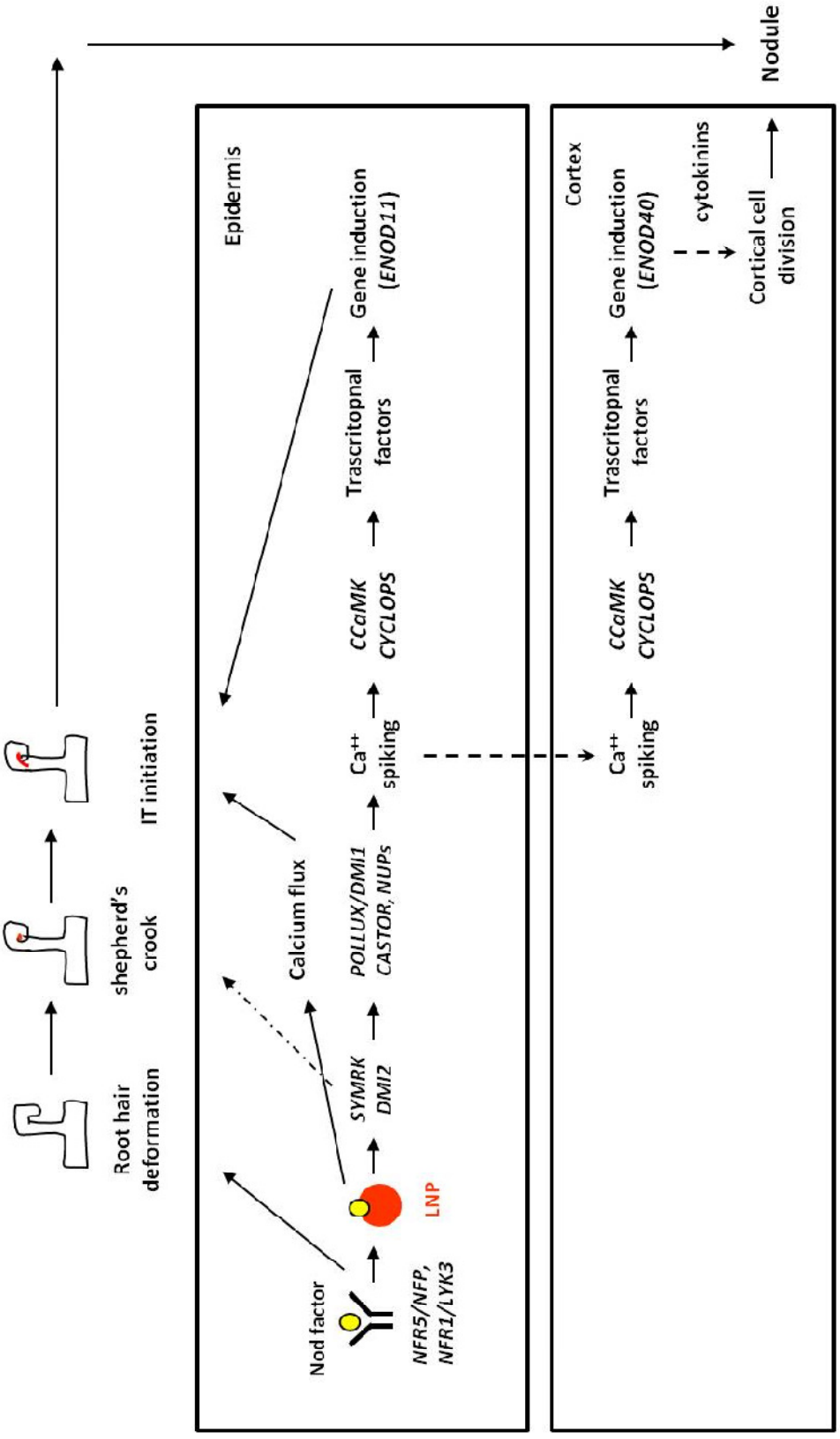
Until a decade ago, very little was known about the molecular components in legume involved in Nod factor signalling. In the last decade, the use of mutants screens, the ongoing genomic sequencing of model legumes *M. truncatula* and *L. japonicus*, and advances in molecular genetic techniques have allowed the identification of several genes responsible for Nod factor responses. The Nod-factor signalling identified so far involves the Nod factor receptors NFP/NFR5 and NFR1, the LRR-receptor-like kinase DMI2/SYMRK, the cation channels DMI1/POLLUX and CASTOR, nucleoporins NUP133, NUP85 and NENA, all required upstream of calcium spiking. Downstream of calcium spiking the calcium calmodulin-dependent kinase CCAMK, IPD3/CYCLOPS and the transcription factors NSP1, NSP2, ERN1, ERN2, ERN3 and NIN are required for nodulin gene expression.

In this thesis, the identification of a new Nod factor signalling component was presented. Antisense suppression of LNP in *L. japonicus* blocked Nod factor-induced calcium spiking, Nod factor-induced calcium flux and Nod factor-induced *NIN* expression without blocking root hair deformation. Thus, LNP is upstream of calcium spiking and calcium flux and sits in a unique position of the Nod factor signalling pathway upstream of

DMI2/SYMRK, DMI1/POLLUX, CASTOR, NUP133, NUP85 and NENA, but downstream the Nod factor receptors NFP/NFR5 and NFR1. LNP may be involved in the formation of a Nod factor receptor complex required for the activation of downstream signalling events such as calcium spiking and gene induction. The observation that legume-mycorrhizal fungal interactions is also blocked in the LNP antisense line suggests that LNP may have a similar function in the mycorhizal and rhizobial symbioses, thus suggesting a structural similarity between Myc and Nod factors. In addition we should not forget that LNP is a phosphohydrolase that can catalyse the hydrolysis of ATP. The finding that both nodulation and mycorrhization are blocked in the LNP antisense line points toward the possibility that ATP plays a role during legume-rhizobia and legume-mycorrhizal fungal interactions. ATP has been shown to be released by plant defence elicitors (Kim et al., 2006), and so it is possible that LNP by reducing ATP, suppresses plant defence response and allows nodulation or mycorrhization to occur (Figure 7.1).

Figure 7.1. Key positional signalling diagram (see next page).

Nod factor is perceived in the epidermis by two receptor-like kinase NFP/NFR5 and LYK3?/NFR1 Nod factor receptors. Subsequently, root hair deformation, including root hair branching and root hair curling are induced. The Nod-factor-binding phosphohydrolase LNP is involved in Nod factor signalling and, through the hydrolysis of ATP, it might suppress defence response in order to promote nodulation. LNP is required for induction of calcium flux which is suggested to play a role, later, during formation of infection threads. The second receptor-like kinase DMI2/SYMRK, containing leu-rich repeat regions, might play multiple roles during Nod factor signalling. SYMRK might have a key role during the touch response that contributes to entrap the bacteria within the shepherd crook structure. At the same time, SYMRK may induce cortical cell division by interacting with HMGR1, involved in the synthesis of cytokinins. Through SYMRK the Nod factor signal is tranduced from the plasma membrane to the nuclear envelope where cation channels DMI1/POLLUX, CASTOR and the nucleoporins, NUP133, NUP85 and NENA, are localized and required for the induction of nuclear calcium spiking. CCAMK, NSP1, NSP2, IPD3/CYCLOPS and ERN are proposed to decode and transduce the calcium signal into expression of gene (ex. ENOD11) necessary for the entrapment of bacteria within the root hair curl and the subsequent initiation of infection. In the cortex activation of CCAMK, possibly by cortical calcium spiking, will lead to the induction of genes required for cortical cell division which is associated with increases in cytokinins.


Nodule
Epidermis
Cortex
Root hair deformation
shepherd's crook
IT initiation
Nod factor
NFR5/NFP, NFR1/LYK3
LNP
SYMRK DMI2
Calcium flux
POLLUX/DMI1 CASTOR, NUPs
Ca++ spiking
CCaMK CYCLOPS
Trascritopnal factors
Gene induction (ENOD11)
Ca++ spiking
CCaMK CYCLOPS
Trascritopnal factors
Gene induction (ENOD40)
cytokinins
Cortical cell division

A Link Between Calcium Spiking and Bacterial Infection

Although several advances have been made in the identification of the molecular components of the Nod factor signalling pathway, the downstream effects of the signalling cascade leading to the nodulin genes expression such as *ENOD11*, are yet not fully understood. The finding that autoactivation of *CCAMK* lead to the formation of spontaneous nodules, which is dependent on *NSP1*, *NSP2*, *ERN1* and *NIN* (Gleason et al., 2006; Marsh et al., 2007; Middleton et al., 2007), highlighted the importance of this pathway for cortical nodule organogenesis. As CCAMK has been proposed to decode calcium spiking, these results led to the idea that calcium spiking is required for nodule formation. Nevertheless, the results reported in this thesis point also toward a role for calcium spiking during bacterial infection. In Chapter 5, I showed that the *symrk14-har1-1* mutant is impaired in Nod factor-induced calcium spiking in root hair cells even though it does form nodules. Interestingly, the phenotype of the *symrk14* mutant was severely compromised for infection thread formation. The impairment of calcium spiking and the lack of infection thread formation suggests a link between calcium spiking and infection thread initiation. Upon inoculation with rhizobia, the root hairs of the *symrk14* mutant curled extensively, often twice around e.g. 720 degrees, but failed to entrap the bacteria within a curl (S. Kosuta, personal communication). This inability to entrap the bacteria, implies that SYMRK is likely to have a specific function in the entrapment of rhizobia within the shepherd's crook. SYMRK could be involved in such a function, directly and/or indirectly, through the activation of the Nod factor signalling pathway involving calcium spiking.

The *dmi1, dmi2* and *dmi3* mutants were originally shown to respond to Nod factor with root hair swelling but not with root hair branching or root hair curling (Catoira et al., 2000; Wais et al., 2000). In contrast Esseling et al. (2004) reported that all the *dmi* mutants showed root hair curling after spot application of Nod factor. Despite the ability to deform in response to Nod factor, none of these mutants can entrap rhizobia within a curl (Catoira et al., 2000). Not only the *dmi* mutants (and the corresponding *Lotus* mutant), but all the *M. truncatula* and *L. japonicus* mutants carrying mutations in the genes involved in the Nod factor signalling pathway, are unable to form the shepherd's crook structure (Schauser et al., 1998; Stracke et al., 2002; Amor et al., 2003; Oldroyd and Long, 2003; Radutoiu et al., 2003; Imaizumi-Anraku et al., 2005; Miwa et al., 2006b; Marsh

et al., 2007; Middleton et al., 2007; Saito et al., 2007). These pieces of evidence support a critical role of the Nod factor signalling pathway for initiation of bacterial infection (Figure 7.1).

In order to envisage the output of this signalling cascade it would be essential to understand the function of the genes induced by this, such as *ENOD11*. *ENOD11*, like *ENOD12* (one of the first identified early nodulin genes), encodes a proline-rich cell wall protein expressed during pre-infection and infection stages of nodulation in root and nodule tissue (Journet et al., 2001). Following inoculation with *S. meliloti*, the *MtENOD11* promoter is first induced after three to six hours in epidermal tissues where successful infections occur one to two days later. At later stages, activation of the *MtENOD11* promoter correlated with presence of infection threads in both roots and nodules (Journet et al., 2001). ENOD11 and ENOD12 are characterised by an overall low tyrosine content, which is normally involved in mechanical strengthening of cell wall (Journet et al., 2001). It has been proposed that the induction *ENOD11* and *ENOD12* results in the presence of significant quantities of ENOD11 and ENOD12 in the root hair cell wall. This could modify the cell wall in such a way as to loosen the cell wall rigidity and, therefore, facilitate entrapment of rhizobia in infection pocket and the subsequent penetration of bacteria, required for the initiation of infection thread (Figure 7.1)(Scheres et al., 1990; Journet et al., 2001). Ultrastructural studies have revealed that infection threads initiate as invaginations of the plant cell wall and occur as a consequence of degradation of the plant cell wall and subsequent extension of the tubular structure (Jordan et al., 1963; Ridge and Rolfe, 1985).

It is noteworthy that, not only *ENOD11* and *ENOD12*, but many of the genes upregulated by Nod factor and rhizobia encode proteins that are targeted to cell wall and extracellular matrix (Brewin, 2004), representing good candidates for the remodelling of cell walls during infection thread initiation and development. This, together with the observation that most of the mutations in the Nod factor signalling pathway affect infection thread growth suggest a link between calcium spiking and bacterial infection. The results showing that the *symrk14-har1-1* mutant forms many nodules but is impaired for calcium spiking and infection thread formation, gave strong support for calcium spiking being essential for initiation of infection thread and perhaps being more specifically involved during the entrapment of bacteria within the shepherd's crook

structure in the epidermis (Figure 7.1). Calcium spiking is also induced during mycorrhization (Kosuta et al., 2008). Interestingly all the components of the common signalling pathway shared by nodulation and mycorrhization are required for fungal infection. It is possible that calcium spiking in both nodulation and mycorrhization is necessary for the entry of the microbes into the root.

In *L. japonicus* the gain-of-function alleles *snf1* and *snf2* encoding the calcium calmodulin-dependent kinase CCaMK, and the cytokinin receptor LHK1, respectively, lead to the development of spontaneous empty nodules. Using double and triple mutants containing the gain-of–function *snf1* and *snf2* mutants recent work has shown that genes upstream of calcium spiking and downstream of Nod factor receptors are required for nodule organogenesis and only indirectly for infection. The *symrk* mutation, for example, did not block the formation of pink infected nodules in the *symrk snf1* double mutant (Madsen et al., 2010), suggesting that *SYMRK* is not directly required for bacterial infection. These results are therefore in contrast the role of SYMRK and calcium spiking during infection. Nevertheless, the same work also showed that inoculation of the double mutant *snf2/ccamk* did not result in the formation of infected nodules (Madsen et al., 2010), thus revealing that activation of CCaMK, which is likely to occur through calcium spiking, does not play only a role in the induction of nodule organogenesis but is also essential for bacterial infection.

A Role for Calcium Flux in Infection Thread Initiation

Calcium flux has been also proposed to be involved in the initiation of infection-thread formation (Miwa et al., 2006b). The results presented in Chapter 3 and 4 support this hypothesis. Inoculation of *M. truncatula* roots with the *nodF nodL* mutant results in the formation of enlarged infection foci where the bacteria accumulate but are not able to initiate the infection thread (Ardourel et al., 1994). The results presented in Chapter 3 show that the LCO, IV, $C_{18:1}$, S Nod factor, structurally equivalent to that made by the *nodF nodL* mutant, was severally impaired for calcium flux induction even when applied at high concentration. These results suggest that the *nodF nodL* mutant cannot initiate infection thread because the Nod factor that is released by this mutant is not able to induce calcium flux. Thus, even if the concentration of this Nod factor increases as a result of the

accumulation of bacteria in the infection foci, calcium flux is not induced and therefore infection can not initiate.

A great reduction in the number of infection threads was also observed after inoculation of *M. truncatula* roots with the *nodL* mutant, although a few infection threads were oberved (Ardourel et al., 1994). The LCO, IV, $C_{16:2}$, S Nod factor, structurally equivalent to that released by the nodL mutant, was also impaired for induction of calcium flux. A 100-fold higher concentration of the *nodL* Nod factor than the wild type Nod factor was required to induce a calcium flux. However when *nodL* Nod factor was added at micromolar concentration calcium flux was normally induced. The few infection threads observed in this mutant could be generated where the bacteria releases high concentration of Nod factor which is able to trigger calcium flux. The different ability of the *nodL* and *nodF nodL* Nod factors to trigger calcium flux is correlated with the different ability of these mutants to form infection threads. In addition, the Nod factor equivalent to that released by the *S. meliloti nodF nodL* mutant that is completely defective for infection threads, was greatly reduced for both calcium flux and calcium spiking. The Nod factor equivalent to that released by the *S. meliloti nodL* mutant that is reduced, but not entirely defective, for infection thread formation, was reduced for induction of calcium flux but normal for calcium spiking. These findings point toward the possibility that both calcium flux and calcium spiking responses are required for infection thread formation. When calcium flux is reduced, but calcium spiking is normal (as observed with *nodL* Nod factor) infection threads can form but the process is reduced, and delayed. However, when both calcium responses are abolished no infection threads are formed, as seen with the *nodF nodL* mutant. It is possible that calcium spiking in epidermal cells below to the infected root hair may be associated with the formation of pre-infection threads and may be required for the progression of the infection in different cells.

Plant mutants defective for infection thread formation, such as *nin*, *bit-1*, and *rit-1*, were somewhat reduced for induction of calcium flux (Chapter 4), providing evidence for a link between calcium flux and infection thread formation. Therefore, based on the results proved in Chapter 3, 4 and 5, I speculate that both calcium flux and calcium spiking play a role during bacterial infection. Calcium spiking might be involved through the induction of *ENOD11* in the formation of root hair shepherd's crook entrapping bacteria within a curl, whereas the calcium flux is likely to be induced by high Nod factor

concentration released by the rhizobia once they are trapped and start replicating within the infection pocket. Thus, calcium flux might play a role during the invagination of the cell wall, required for the initiation of the tubular infection structure (Figure 7.1). Mechanical stimulation by the replicating rhizobia is likely to contribute to such a process. A recent work has shown that mechanical stimulations of roots of *Arabidopsis thaliana*, expressing yellow cameleon 3.6, induces transient cytoplasmic calcium increase which regulates the production of reactive oxygen species and apoplastic and cytoplasmatic pH changes (Monshausen et al., 2009). It is possible that a Nod factor-dependent calcium flux together with a rapid calcium increase induced by mechanical stimulations might stimulate the invagination of cell wall and plasma membrane necessary for the initiation of infection thread.

SYMRK Function: a Branch Point

Several studies have shown that DMI2/SYMRK is required not only for the formation of nodules formed during legume–*Rhizobium* symbiosis but also for the formation of nodules formed during the actinorhiza–*Frankia* association (Endre et al., 2002; Gherbi et al., 2008; Markmann et al., 2008). Moreover, this gene is also necessary for the plant symbiosis with arbuscular mycorrhizal fungi (Stracke et al., 2002). These findings suggest that SYMRK might have a function independent of Nod factor perception. Esseling at al. (2004) have shown that, in *M. truncatula, DMI2* is involved in touch response. Such a function is likely to be essential not only for the legume–*Rhizobium* symbiosis, but also for actinorhiza–*Frankia* association and symbiosis with arbuscular mycorrhizal fungi. Thus, it is possible that SYMRK mediates a Nod factor-independent touch response during these three different symbioses, possibly through the leucine rich repeat (LRR) extracellular domain whose function is still unclear (Figure 7.1).

Nevertheless, it is known that SYMRK is indispensable for the induction of responses triggered by Nod factor, such as calcium spiking, *ENOD11* gene activation and cortical cell division, indicating that SYMRK must also have a Nod factor-dependent function upstream of calcium spiking. Curiously, the *symrk-14* mutation in the extracellular domain just upstream of the LRR, abolished calcium spiking but not cortical cell division, indicating that, initiation of calcium spiking depends on the integrity of the extracellular domain. Although no biochemical evidence has shown the binding of SYMRK

with Nod factor receptors containing LysM domain, it is possible that the extracellular domain of SYMRK could possibly be also involved in binding with NFP/NFR5 or NFR1. Putative alternative interactors of SYMRK are components of the phospholipid pathway, such as G-protein required for activation of PLC and PLD, previously predicted to contribute to Nod factor signalling. Recently, it has been shown that the intracellular kinase domain of DMI2 interacts with HMGR1, an enzyme involved in the mevalonate synthesis pathway leading to the production of different array of isoprenoid compounds, including cytokinins (Kevei et al., 2007). Several lines of evidence have indicated a key role of cytokinins during activation of cortical cell division and nodule organogenesis. Thus, it is possible that SYMRK, through the interaction of HMGR, activates cortical cell divisions. The observation that the *symrk14* mutant is normal for cortical cell division suggests that the point mutation in the extracellular domain does not interfere with this function and further suggests that the SYMRK-dependent cortical cell division is independent of root hair calcium spiking. Hence, SYMRK might play several roles in nodulation. The extracellular domain is likely to be involved in the activation of Nod factor-dependent calcium spiking and, as previously suggested, in Nod factor-independent touch response (Markmann et al., 2008). Together these responses are likely to be essential for initiation of bacterial infection. At the same time, SYMRK through the Nod factor-dependent interaction of the intracellular domain with HMGR1, and possibly through interaction with other proteins, could lead to the activation of cortical cell divisions. Thus, in this model, SYMRK would represent a branching point for epidermal and cortical responses (Figure 7.1).

Activation of Cortical Responses

The observations that autoacitive CCAMK leads to nodule organogenesis in the absence of bacterial infection and that spontaneous nodulation depends on components of the Nod factor signalling pathway such as *NFP*, *DMI1*, *DMI2*, *DMI3 NSP1* and *NSP2* suggest that one of the outputs of the Nod-factor-signalling pathway is nodule organogenesis. Thus, the downstream responses of the Nod factor signalling cascade involving calcium spiking are not only the bacterial infection occurring in the epidermis, but also cortical nodule organogenesis. How can the Nod factor signalling pathway induce such two different outputs? I believe that the activation of the Nod factor signalling

pathway has different effects in distinct cell types and/or in response to different stimuli. Indeed, signalling pathways can induce different downstream effects depending on cell type. For example, in animals, pathologically increased JNK/cJun signalling stimulates apoptosis in neurons, and this can result in neurodegeneration, but triggers proliferation in other cell types, thereby causing cancer (Davis, 2000). Thus, in nodulation, it is possible that, for example, different combination of CCAMK and transcriptional factors such as NSP and ERN proteins might induce *ENOD11*, and other genes required for activation of infection thread, in the epidermis, whereas a different combination of transcriptional factors might induce ENOD40, and other genes required for nodule organogenesis, within the cortex.

An outstanding question that remains to be answered is how the Nod factor signal reaches the cortex from the epidermis. During the early stage of nodulation, cortical cell division is induced by Nod factor before the bacteria have fully penetrated the root cortex (Libbenga and Harkes, 1973). CCAMK is believed to be essential for the activation of cortical cell division, however it is not known how CCAMK is activated within the cortex. Calcium spiking has been reported in root hair cells and in cortical cells below the epidermis (Miwa et al., 2006a). Hence, calcium spiking occurring in cortical cell might activate CCAMK and, thereby, cortical cell division (Figure 7.1). However, it remains to be established whether and how calcium spiking can be induced in deeper cortical cell layers once it is induced in the root hairs. A future challenge will be to understand how the Nod factor signalling pathway can be induced in different cell types during the different stages of nodulation.

Conclusions

Calcium plays an essential role in biological systems. Analysis of calcium responses has the potential to unravel molecular mechanism behind many biological processes. In this thesis, the analysis of calcium responses in the two model legumes *M. truncatula* and *L. japonicus* has given insight into the Nod-factor signalling pathway that leads to the formation of nitrogen-fixing root nodules. In *M. truncatula* calcium flux was found to have the same Nod factor structure stringency required for infection thread formation (Chapter 3). *M. truncatula* mutants compromised for bacterial infection were affected for induction of calcium flux suggesting that calcium flux is required for initiation of bacterial infection (Chapter 4). The different Nod-factor specificity found to be required for calcium flux and not for calcium spiking will open the opportunity to use microarrays to identify genes specifically induced by the calcium flux. Identification of genes downstream of calcium flux will elucidate the function of calcium flux during legume infection. The system developed for the analysis of Nod-factor-induced calcium flux described in this thesis will facilitate further studies on calcium flux using either yellow cameleon transgenic plants or microinjection of calcium sensitive dyes.

Calcium spiking analysis in the *L. japonicus symrk14* mutants has shown a remarkable example where nodule morphogenesis occurs even if only a small number of root hairs is able to spike, and, more interestingly, the absence of infection threads gave strong support to the hypothesis that calcium spiking is essential for bacterial infection (Chapter 5). These results also suggest that the *SYMRK*-dependent calcium spiking involves the extracellular domain of SYMRK providing new insight into SYMRK function. Finally, the work presented in Chapter 6 has added knowledge concerning nodule symbiosis, through the identification of a new component of the Nod factor signalling pathway in *L. japonicus*. The phosphohydrolase LNP act upstream of both calcium spiking and calcium flux, but downstream of the Nod factor receptor, unrevealing a new position of the Nod factor signalling pathway. This gene is also required for the mycorrhization indicating that LNP is part of the common signalling cascade shared by rhizobium-legume symbiosis and mycorrhizal fungus symbiosis. Further analysis on the specific role of LNP will help our understanding on the symbiotic interaction between plant and rhizobia. Revealing the molecular mechanism behind the formation of a nitrogen-fixing nodule will hopefully enable us to improve the sustainability of crop plants in nitrogen-poor soils.

References

Abeijon, C., Yanagisawa, K., Mandon, E.C., Hausler, A., Moremen, K., Hirschberg, C.B., and Robbins, P.W. (1993). Guanosine diphosphatase is required for protein and sphingolipid glycosylation in the golgi lumen of *Saccharomyces-Cerevisiae*. J. Cell Biol. **122:** 307-323.

Akiyama, K., Matsuzaki, K., and Hayashi, H. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature **435:** 824-827.

Allen, G.J., Chu, S.P., Harrington, C.L., Schumacher, K., Hoffmann, T., Tang, Y.Y., Grill, E., and Schroeder, J.I. (2001). A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature **411:** 1053-1057.

Allen, G.J., Chu, S.P., Schumacher, K., Shimazaki, C.T., Vafeados, D., Kemper, A., Hawke, S.D., Tallman, G., Tsien, R.Y., Harper, J.F., Chory, J., and Schroeder, J.I. (2000). Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in *Arabidopsis det3* mutant. Science **289:** 2338-2342.

Allen, N.S., and Bennett, M.N. (1996). Electro-optical imaging of F-actin and endoplasmic reticulum in living and fixed plant cells. Scanning Microsc. Suppl. **10:** 177-186; discussion 186-177.

Amor, B.B., Shaw, S.L., Oldroyd, G.E., Maillet, F., Penmetsa, R.V., Cook, D., Long, S.R., Denarie, J., and Gough, C. (2003). The *NFP* locus of *Medicago truncatula* controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. Plant J. **34:** 495-506.

Andriankaja, A., Boisson-Demier, A., Frances, L., Sauviac, L., Jauneau, A., Barker, D.G., and de Carvalho-Niebel, F. (2007). AP2-ERF transcription factors mediate Nod factor-dependent *Mt ENOD11* activation in root hairs via a novel cis-regulatory motif. Plant Cell **19:** 2866-2885.

Ane, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Oldroyd, G.E., Ayax, C., Levy, J., Debelle, F., Baek, J.M., Kalo, P., Rosenberg, C., Roe, B.A., Long, S.R., Denarie, J., and Cook, D.R. (2004). *Medicago truncatula DMI1* required for bacterial and fungal symbioses in legumes. Science **303:** 1364-1367.

Ardourel, M., Demont, N., Debelle, F., Maillet, F., de Billy, F., Prome, J.C., Denarie, J., and Truchet, G. (1994). *Rhizobium meliloti* lipooligosaccharide nodulation factors: different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. Plant Cell **6:** 1357-1374.

Arrighi, J.F., Barre, A., Ben Amor, B., Bersoult, A., Soriano, L.C., Mirabella, R., de Carvalho-Niebel, F., Journet, E.P., Gherardi, M., Huguet, T., Geurts, R., Denarie, J., Rouge, P., and Gough, C. (2006). The *Medicago truncatula* lysine motif-receptor-like kinase gene family includes *NFP* and new nodule-expressed genes. Plant Physiol. **142:** 265-279.

Bateman, A., and Bycroft, M. (2000). The structure of a LysM domain from *E-coli* membrane-bound lytic murein transglycosylase D (MltD). J. Mol. Biol. **299:** 1113-1119.

Begum, A.A., Leibovitch, S., Migner, P., and Zhang, F. (2001). Specific flavonoids induced nod gene expression and pre-activated *nod* genes of *Rhizobium leguminosarum*

increased pea (*Pisum sativum L.*) and lentil (*Lens culinaris L.*) nodulation in controlled growth chamber environments. J. Exp. Bot. **52:** 1537-1543.

Bek, A.S., Thygesen, M.B., Duus, J., Petersen, B.O., Thirup, S., James, E., Jensen, K.J., Stougaard, J., and Radutoiu, S. (2010). Improved characterization of Nod factors and genetically based variation in LysM receptor domains identify amino acids expendable for Nod factor recognition in *Lotus* spp. Mol. Plant-Microbe Interact. **23:** 58-66.

Berridge, M.J., Bootman, M.D., and Roderick, H.L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. Nat. Rev. Mol. Cell Biol. **4:** 517-529.

Bersoult, A., Camut, S., Perhald, A., Kereszt, A., Kiss, G.B., and Cullimore, J.V. (2005). Expression of the *Medicago truncatula DMI2* gene suggests roles of the symbiotic nodulation receptor kinase in nodules and during early nodule development. Mol. Plant-Microbe Interact. **18:** 869-876.

Besserer, A., Becard, G., Jauneau, A., Roux, C., and Sejalon-Delmas, N. (2008). GR24, a synthetic analog of strigolactones, stimulates the mitosis and growth of the arbuscular mycorrhizal fungus *Gigaspora rosea* by boosting its energy metabolism. Plant Physiol. **148:** 402-413.

Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. **12:** 8711-8721.

Bloemberg, G.V., Thomasoates, J.E., Lugtenberg, B.J.J., and Spaink, H.P. (1994). Nodulation protein NodL of *Rhizobium-leguminosarum O*-acetylates lipo-oligosaccharides, chitin fragments and *N*-acetylglucosamine *in-vitro* Mol. Microbiol. **11:** 793-804.

Bootman, M.D., Fearnley, C., Smyrnias, I., MacDonald, F., and Roderick, H.L. (2009). An update on nuclear calcium signalling. J. Cell Sci. **122:** 2337-2350.

Boyum, R., and Guidotti, G. (1997). Glucose-dependent, cAMP-mediated ATP efflux from *Saccharomyces cerevisiae*. Microbiology-Uk **143:** 1901-1908.

Brewin, N.J. (2004). Plant cell wall remodelling in the rhizobium-legume symbiosis. Crit. Rev. Plant Sci. **23:** 293-316.

Buee, M., Rossignol, M., Jauneau, A., Ranjeva, R., and Becard, G. (2000). The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Mol. Plant-Microbe Interact. **13:** 693-698.

Burnstock, G. (2007). Physiology and pathophysiology of purinergic neurotransmission. Physiol. Rev. **87:** 659-797.

Burnstock, G., and Williams, M. (2000). P2 purinergic receptors: modulation of cell function and therapeutic potential. J. Pharmacol. Exp. Ther. **295:** 862-869.

Capoen, W., and Oldroyd, G. (2008). How CYCLOPS keeps an eye on plant symbiosis. Proc. Natl. Acad. Sci. USA **105:** 20053-20054.

Capoen, W., Goormachtig, S., De Rycke, R., Schroeyers, K., and Holsters, M. (2005). SrSymRK, a plant receptor essential for symbiosome formation. Proc. Natl. Acad. Sci. USA **102:** 10369-10374.

Capoen, W., Den Herder, J., Sun, J.H., Verplancke, C., De Keyser, A., De Rycke, R., Goormachtig, S., Oldroyd, G., and Holsters, M. (2009). Calcium spiking patterns and the role of the calcium/calmodulin-dependent kinase CCaMK in lateral root base nodulation of *Sesbania rostrata*. Plant Cell **21:** 1526-1540.

Cardenas, L., Martinez, A., Sanchez, F., and Quinto, C. (2008). Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs). Plant J. **56:** 802-813.

Cardenas, L., Vidali, L., Domnguez, J., Prez, H., Snchez, F., Hepler, P.K., and Quinto, C. (1998). Rearrangement of actin microfilaments in plant root hairs responding to *Rhizobium etli* nodulation signals. Plant Physiol. **116:** 871-877.

Cardenas, L., Feijo, J.A., Kunkel, J.G., Sanchez, F., Holdaway-Clarke, T., Hepler, P.K., and Quinto, C. (1999). Rhizobium nod factors induce increases in intracellular free calcium and extracellular calcium influxes in bean root hairs. Plant J. **19:** 347-352.

Catoira, R., Timmers, A.C., Maillet, F., Galera, C., Penmetsa, R.V., Cook, D., Denarie, J., and Gough, C. (2001). The *HCL* gene of *Medicago truncatula* controls Rhizobium-induced root hair curling. Development **128:** 1507-1518.

Catoira, R., Galera, C., de Billy, F., Penmetsa, R.V., Journet, E.P., Maillet, F., Rosenberg, C., Cook, D., Gough, C., and Denarie, J. (2000). Four genes of *Medicago truncatula* controlling components of a nod factor transduction pathway. Plant Cell **12:** 1647-1665.

Cavagnaro, T.R., Smith, S.E., and Dickson, S. (2001). Backseat driving? Accessing phosphate beyond the rhizosphere-depletion zone. Trends Plant Sci. **6:** 194-195.

Charpentier, M., Bredemeier, R., Wanner, G., Takeda, N., Schleiff, E., and Parniske, M. (2008). *Lotus japonicus* CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis. Plant Cell **20:** 3467-3479.

Charron, D., Pingret, J.L., Chabaud, M., Journet, E.P., and Barker, D.G. (2004). Pharmacological evidence that multiple phospholipid signaling pathways link rhizobium nodulation factor perception in *Medicago truncatula* root hairs to intracellular responses, including Ca^{2+} spiking and specific *ENOD* gene expression. Plant Physiol. **136:** 3582-3593.

Cohn, J.R., Uhm, T., Ramu, S., Nam, Y.W., Kim, D.J., Penmetsa, R.V., Wood, T.C., Denny, R.L., Young, N.D., Cook, D.R., and Stacey, G. (2001). Differential regulation of a family of apyrase genes from *Medicago truncatula*. Plant Physiol. **125:** 2104-2119.

Cook, D., Dreyer, D., Bonnet, D., Howell, M., Nony, E., and Vandenbosch, K. (1995). Transient induction of a peroxidase gene in *Medicago truncatula* precedes Infection by *Rhizobium meliloti*. Plant Cell **7:** 43-55.

Cooper, J.B., and Long, S.R. (1994). Morphogenetic rescue of *Rhizobium meliloti* nodulation mutants by trans-zeatin secretion. Plant Cell **6:** 215-225.

Cooper, J.E. (2007). Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. J. Appl. Microbiol. **103:** 1355-1365.

Corvera, A., Prome, D., Prome, J.C., Martinez-Romero, E., and Romero, D. (1999). The *nolL* gene from *Rhizobium etli* determines nodulation efficiency by mediating the acetylation of the fucosyl residue in the nodulation factor. Mol. Plant-Microbe Interact. **12:** 236-246.

D'Haeze, W., De Rycke, R., Mathis, R., Goormachtig, S., Pagnotta, S., Verplancke, C., Capoen, W., and Holsters, M. (2003). Reactive oxygen species and ethylene play a positive role in lateral root base nodulation of a semiaquatic legume. Proc. Natl. Acad. Sci. USA **100:** 11789-11794.

Davis, R.J. (2000). Signal transduction by the JNK group of MAP kinases. Cell **103:** 239-252.

Day, R.B., McAlvin, C.B., Loh, J.T., Denny, R.L., Wood, T.C., Young, N.D., and Stacey, G. (2000). Differential expression of two soybean apyrases, one of which is an early nodulin. Mol. Plant-Microbe Interact. **13:** 1053-1070.

de Ruijter, N.C.A., Bisseling, T., and Emons, A.M.C. (1999). *Rhizobium* Nod factors induce an increase in sub-apical fine bundles of actin filaments in *Vicia sativa* root hairs within minutes. Mol. Plant-Microbe Interact. **12:** 829-832.

de Ruijter, N.C.A., Rook, M.B., Bisseling, T., and Emons, A.M.C. (1998). Lipochito-oligosaccharides re-initiate root hair tip growth in *Vicia sativa* with high calcium and spectrin-like antigen at the tip. Plant J. **13:** 341-350.

Debelle, F., Rosenberg, C., Vasse, J., Maillet, F., Martinez, E., Denarie, J., and Truchet, G. (1986). Assignment of symbiotic developmental phenotypes to common and specific nodulation (Nod) genetic-loci of *Rhizobium-Meliloti*. J. Bacteriol. **168:** 1075-1086.

Demchenko, K., Winzer, T., Stougaard, J., Parniske, M., and Pawlowski, K. (2004). Distinct roles of *Lotus japonicus* SYMRK and SYM15 in root colonization and arbuscule formation. New Phytol. **163:** 381-392.

Demidchik, V., Nichols, C., Oliynyk, M., Dark, A., Glover, B.J., and Davies, J.M. (2003). Is ATP a signaling agent in plants? Plant Physiol. **133:** 456-461.

Demidchik, V., Shang, Z.L., Shin, R., Thompson, E., Rubio, L., Laohavisit, A., Mortimer, J.C., Chivasa, S., Slabas, A.R., Glover, B.J., Schachtman, D.P., Shabala, S.N., and Davies, J.M. (2009). Plant extracellular ATP signalling by plasma membrane NADPH oxidase and Ca^{2+} channels. Plant J. **58:** 903-913.

Demont, N., Debelle, F., Aurelle, H., Denarie, J., and Prome, J.C. (1993). Role of the *Rhizobium meliloti nodF* and *nodE* genes in the biosynthesis of lipo-oligosaccharidic nodulation factors. J. Biol. Chem. **268:** 20134-20142.

den Hartog, M., Musgrave, A., and Munnik, T. (2001). Nod factor-induced phosphatidic acid and diacylglycerol pyrophosphate formation: a role for phospholipase C and D in root hair deformation. Plant J. **25:** 55-65.

den Hartog, M., Verhoef, N., and Munnik, T. (2003). Nod factor and elicitors activate different phospholipid signaling pathways in suspension-cultured alfalfa cells. Plant Physiol. **132:** 311-317.

Den Herder, J., Vanhee, C., De Rycke, R., Corich, V., Holsters, M., and Goormachtig, S. (2007). Nod factor perception during infection thread growth fine-tunes nodulation. Mol. Plant-Microbe Interact. **20:** 129-137.

Denarie, J., Debelle, F., and Prome, J.C. (1996). Rhizobium lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. **65:** 503-535.

Diaz, C.L., Gronlund, M., Schlaman, H.R.M., and Spaink, H.P. (2005). Induction of hairy roots for symbiotic gene expression studies. *Lotus Japonicus* Handbook**:** 261-277.

Ding, Y., Kalo, P., Yendrek, C., Sun, J., Liang, Y., Marsh, J.F., Harris, J.M., and Oldroyd, G.E.D. (2008). Abscisic acid coordinates nod factor and cytokinin signaling during the regulation of nodulation in *Medicago truncatula*. Plant Cell **20:** 2681-2695.

Dodd, A.N., Gardner, M.J., Hotta, C.T., Hubbard, K.E., Dalchau, N., Love, J., Assie, J.M., Robertson, F.C., Jakobsen, M.K., Goncalves, J., Sanders, D., and Webb, A.A.R. (2007). The *Arabidopsis* circadian clock incorporates a cADPR-based feedback loop. Science **318:** 1789-1792.

Dolmetsch, R.E., Xu, K., and Lewis, R.S. (1998). Calcium oscillations increase the efficiency and specificity of gene expression. Nature **392:** 933-936.

Downie, J.A., and Walker, S.A. (1999). Plant responses to nodulation factors. Curr. Opin. Plant Biol. **2:** 483-489.

Doyle, J.J. (1998). Phylogenetic perspectives on nodulation: evolving views of plants and symbiotic bacteria. Trends Plant Sci. **3:** 473-478.

Economou, A., Hamilton, W.D., Johnston, A.W., and Downie, J.A. (1990). The *Rhizobium* nodulation gene *nodO* encodes a Ca^{2+}-binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. EMBO J. **9:** 349-354.

Edwards, A., Heckmann, A.B., Yousafzai, F., Duc, G., and Downie, J.A. (2007). Structural implications of mutations in the pea *SYM8* symbiosis gene, the *DMI1* ortholog, encoding a predicted ion channel. Mol. Plant-Microbe Interact. **20:** 1183-1191.

Edwards, F.A., Gibb, A.J., and Colquhoun, D. (1992). ATP receptor-mediated synaptic currents in the central-nervous-system. Nature **359:** 144-147.

Ehrhardt, D.W., Atkinson, E.M., and Long, S.R. (1992). Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science **256:** 998-1000.

Ehrhardt, D.W., Wais, R., and Long, S.R. (1996). Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. Cell **85:** 673-681.

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kalo, P., and Kiss, G.B. (2002). A receptor kinase gene regulating symbiotic nodule development. Nature **417:** 962-966.

Engstrom, E.M., Ehrhardt, D.W., Mitra, R.M., and Long, S.R. (2002). Pharmacological analysis of nod factor-induced calcium spiking in *Medicago truncatula*. Evidence for the requirement of type IIA calcium pumps and phosphoinositide signaling. Plant Physiol. **128:** 1390-1401.

Esseling, J.J., Lhuissier, F.G.P., and Emons, A.M.C. (2003). Nod factor-induced root hair curling: continuous polar growth towards the point of Nod factor application. Plant Physiol. **132:** 1982-1988.

Esseling, J.J., Lhuissier, F.G.P., and Emons, A.M.C. (2004). A nonsymbiotic root hair tip growth phenotype in dulation NORK-mutated legumes: implications for nodulation factor-induced signaling and formation of a multifaceted root hair pocket for bacteria. Plant Cell **16:** 933-944.

Etzler, M.E., Kalsi, G., Ewing, N.N., Roberts, N.J., Day, R.B., and Murphy, J.B. (1999). A Nod factor binding lectin with apyrase activity from legume roots. Proc. Natl. Acad. Sci. USA **96:** 5856-5861.

Evans, N.H., McAinsh, M.R., and Hetherington, A.M. (2001). Calcium oscillations in higher plants. Curr. Opin. Plant Biol. **4:** 415-420.

Fahraeus, G. (1957). The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. **16:** 374-381.

Fang, Y.W., and Hirsch, A.M. (1998). Studying early nodulin gene ENOD40 expression and induction by nodulation factor and cytokinin in transgenic alfalfa. Plant Physiol. **116:** 53-68.

Faucher, C., Camut, S., Denarie, J., and Truchet, G. (1989). The *NodH* and *NodQ* host range genes of *Rhizobium meliloti* behave as avirulence genes in *R. leguminosarum* bv *viciae* and determine changes in the production of plant-specific extracellular signals. Mol. Plant-Microbe Interact. **2:** 291-300.

Felle, H.H., and Hepler, P.K. (1997). The cytosolic Ca^{2+} concentration gradient of sinapis alba root hairs as revealed by Ca^{2+}-selective microelectrode tests and fura-dextran ratio imaging. Plant Physiol. **114:** 39-45.

Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M. (1996). Rapid alkalinization in alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals. Plant J. **10:** 295-301.

Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M. (1998). The role of ion fluxes in Nod factor signalling in *Medicago sativa*. Plant J. **13:** 455-463.

Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M. (1999a). Elevation of the cytosolic free [Ca^{2+}] is indispensable for the transduction of the Nod factor signal in alfalfa. Plant Physiol. **121:** 273-280.

Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M. (1999b). Nod factors modulate the concentration of cytosolic free calcium differently in growing and non-growing root hairs of *Medicago sativa* Planta **209:** 207-212.

Firmin, J.L., Wilson, K.E., Carlson, R.W., Davies, A.E., and Downie, J.A. (1993). Resistance to nodulation of cv Afghanistan peas is overcome by *NodX*, which mediates an O-acetylation of the *Rhizobium leguminosarum* lipo-oligosaccharide nodulation factor. Mol. Microbiol. **10:** 351-360.

Fisher, R.F., and Long, S.R. (1993). Interactions of nodD at the nod box - nodD binds to 2 distinct sites on the same face of the helix and induces a bend in the DNA. J. Mol. Biol. **233:** 336-348.

Foreman, J., Demidchik, V., Bothwell, J.H.F., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D.G., Davies, J.M., and Dolan, L. (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature **422:** 442-446.

Fountain, S.J., Cao, L.S., Young, M.T., and North, R.A. (2008). Permeation properties of a P2X receptor in the green algae *Ostreococcus tauri*. J. Biol. Chem. **283:** 15122-15126.

Fournier, J., Timmers, A.C.J., Sieberer, B.J., Jauneau, A., Chabaud, M., and Barker, D.G. (2008). Mechanism of infection thread elongation in root hairs of *Medicago truncatula* and dynamic interplay with associated rhizobial colonization. Plant Physiol. **148:** 1985-1995.

Franklin-Tong, V.E., Ride, J.P., Read, N.D., Trewavas, A.J., and Franklin, F.C.H. (1993). The self-incompatibility response in *Papaver rhoeas* is mediated by cytosolic-free calcium. Plant J. **4:** 163-177.

Gage, D.J. (2002). Analysis of infection thread development using GFP- and DsRed-expressing *Sinorhizobium meliloti*. J. Bacteriol. **184:** 7042-7046.

Gage, D.J., Bobo, T., and Long, S.R. (1996). Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). J. Bacteriol. **178:** 7159-7166.

Gamas, P., de Billy, F., and Truchet, G. (1998). Symbiosis-specific expression of two *Medicago truncatula* nodulin genes, *MtN1* and *MtN13*, encoding products homologous to plant defense proteins. Mol. Plant-Microbe Interact. **11:** 393-403.

Gamborg, O.L., Miller, R.A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50:** 151-158.

Gao, X.D., Kaigorodov, V., and Jigami, Y. (1999). YND1, a homologue of GDA1, encodes membrane-bound apyrase required for Golgi N- and O-glycosylation in *Saccharomyces cerevisiae*. J. Biol. Chem. **274:** 21450-21456.

Gehring, C.A., Irving, H.R., Kabbara, A.A., Parish, R.W., Boukli, N.M., and Broughton, W.J. (1997). Rapid, plateau-like increases in intracellular free calcium are

associated with Nod-factor-induced root-hair deformation. Mol. Plant-Microbe Interact. **10:** 791-802.

Genre, A., Chabaud, M., Timmers, T., Bonfante, P., and Barker, D.G. (2005). Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. Plant Cell **17:** 3489-3499.

Gerasimovskaya, E.V., Ahmad, S., White, C.W., Jones, P.L., Carpenter, T.C., and Stenmark, K.R. (2002). Extracellular ATP is an autocrine/paracrine regulator of hypoxia-induced adventitial fibroblast growth - Signaling through extracellular signal-regulated kinase-1/2 and the Egr-1 transcription factor. J. Biol. Chem. **277:** 44638-44650.

Geurts, R., Heidstra, R., Hadri, A.E., Downie, J.A., Franssen, H., vanKammen, A., and Bisseling, T. (1997). Sym2 of pea is involved in a nodulation factor-perception mechanism that controls the infection process in the epidermis. Plant Physiol. **115:** 351-359.

Gherbi, H., Markmann, K., Svistoonoff, S., Estevan, J., Autran, D., Giczey, G., Auguy, F., Peret, B., Laplaze, L., Franche, C., Parniske, M., and Bogusz, D. (2008). SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and Frankia bacteria. Proc. Natl. Acad. Sci. USA **105:** 4928-4932.

Gleason, C., Chaudhuri, S., Yang, T.B., Munoz, A., Poovaiah, B.W., and Oldroyd, G.E.D. (2006). Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. Nature **441:** 1149-1152.

Goedhart, J., Hink, M.A., Visser, A., Bisseling, T., and Gadella, T.W.J. (2000). *In vivo* fluorescence correlation microscopy (FCM) reveals accumulation and immobilization of Nod factors in root hair cell walls. Plant J. **21:** 109-119.

Gonzalez-Rizzo, S., Crespi, M., and Frugier, F. (2006). The *Medicago truncatula* CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with *Sinorhizobium meliloti*. Plant Cell **18:** 2680-2693.

Gonzalez, J.E., Reuhs, B.L., and Walker, G.C. (1996). Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*. Proc. Natl. Acad. Sci. USA **93:** 8636-8641.

Goormachtig, S., Capoen, W., James, E.K., and Holsters, M. (2004). Switch from intracellular to intercellular invasion during water stress-tolerant legume nodulation. Proc. Natl. Acad. Sci. USA **101:** 6303-6308.

Govindarajulu, M., Kim, S.Y., Libault, M., Berg, R.H., Tanaka, K., Stacey, G., and Taylor, C.G. (2009). GS52 ecto-apyrase plays a critical role during soybean nodulation. Plant Physiol. **149:** 994-1004.

Groth, M., Takeda, N., Perry, J., Uchida, H., Draxl, S., Brachmann, A., Sato, S., Tabata, S., Kawaguchi, M., Wang, T.L., and Parniske, M. (2010). *NENA,* a *Lotus japonicus* homolog of *Sec13,* is required for rhizodermal infection by arbuscular mycorrhiza fungi and rhizobia but dispensable for cortical endosymbiotic development. Plant Cell **22:** 2509-2526.

Gualtieri, G., and Bisseling, T. (2000). The evolution of nodulation. Plant Mol. Biol. **42:** 181-194.

Hanley, P.J., Mussett, B., Renigunta, V., Limberg, S.H., Dalpke, A.H., Sus, R., Heeg, K.M., Preisig-Muller, R., and Daut, J. (2004). Extracellular ATP induces oscillations of intracellular Ca^{2+} and membrane potential and promotes transcription of IL-6 in macrophages. Proc. Natl. Acad. Sci. USA **101:** 9479-9484.

Harris, J.M., Wais, R., and Long, S.R. (2003). *Rhizobium*-induced calcium spiking in *Lotus japonicus*. Mol. Plant-Microbe Interact. **16:** 335-341.

Harrison, M.J. (1997). The arbuscular mycorrhizal symbiosis: An underground association. Trends Plant Sci. **2:** 54-60.

Harrison, M.J. (1998). Development of the arbuscular mycorrhizal symbiosis. Curr. Opin. Plant Biol. **1:** 360-365.

Harrison, M.J. (2005). Signaling in the arbuscular mycorrhizal symbiosis. Annu. Rev. Microbiol. **59:** 19-42.

Heckmann, A.B., Lombardo, F., Miwa, H., Perry, J.A., Bunnewell, S., Parniske, M., Wang, T.L., and Downie, J.A. (2006). *Lotus japonicus* nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume. Plant Physiol. **142:** 1739-1750.

Heidstra, R., Yang, W.C., Yalcin, Y., Peck, S., Emons, A.M., vanKammen, A., and Bisseling, T. (1997). Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in *Rhizobium*-legume interaction. Development **124:** 1781-1787.

Heine, P., Braun, N., Heilbronn, A., and Zimmermann, H. (1999). Functional characterization of rat ecto-ATPase and ecto-ATP diphosphohydrolase after heterologous expression in CHO cells. Eur. J. Biochem. **262:** 102-107.

Hirsch, A.M., and Fang, Y.W. (1994). Plant hormones and nodulation - What's the connection? Plant Mol. Biol. **26:** 5-9.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc. Natl. Acad. Sci. USA **86:** 1244-1248.

Hirsch, S., Kim, J., Munoz, A., Heckmann, A.B., Downie, J.A., and Oldroyd, G.E.D. (2009). GRAS proteins form a DNA binding complex to induce gene expression during nodulation signaling in *Medicago truncatula*. Plant Cell **21:** 545-557.

HoldawayClarke, T.L., Feijo, J.A., Hackett, G.R., Kunkel, J.G., and Hepler, P.K. (1997). Pollen tube growth and the intracellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. Plant Cell **9:** 1999-2010.

Holford, I.C.R. (1997). Soil phosphorus: its measurement, and its uptake by plants. Aust. J. Soil Res. **35:** 227-239.

Hsieh, H.L., Song, C.J., and Roux, S.J. (2000). Regulation of a recombinant pea nuclear apyrase by calmodulin and casein kinase II. Biochim. Biophys. Acta Gene Struct. Expression **1494:** 248-255.

Hungria, M., Johnston, A.W.B., and Phillips, D.A. (1992). Effects of flavonoids released naturally from bean (*Phaseolus vulgaris*) on NodD-regulated gene-transcription in *Rhizobium leguminosarum* bv *phaseoli*. Mol. Plant-Microbe Interact. **5:** 199-203.

Huse, M., and Kuriyan, J. (2002). The conformational plasticity of protein kinases. Cell **109:** 275-282.

Idzko, M., Hammad, H., van Nimwegen, M., Kool, M., Willart, M.A.M., Muskens, F., Hoogsteden, H.C., Luttmann, W., Ferrari, D., Di Virgilio, F., Virchow, J.C., and Lambrecht, B.N. (2007). Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. Nat. Med. **13:** 913-919.

Imaizumi-Anraku, H., Takeda, N., Charpentier, M., Perry, J., Miwa, H., Umehara, Y., Kouchi, H., Murakami, Y., Mulder, L., Vickers, K., Pike, J., Downie, J.A., Wang, T., Sato, S., Asamizu, E., Tabata, S., Yoshikawa, M., Murooka, Y., Wu, G.J., Kawaguchi, M., Kawasaki, S., Parniske, M., and Hayashi, M. (2005). Plastid

proteins crucial for symbiotic fungal and bacterial entry into plant roots. Nature **433:** 527-531.

Jakobsen, I. (1995). Transport of phosphorus and carbon in VA mycorrhizas. Mycorrhiza: Structure, function, molecular biology and biotechnology**:** 297-324.

Jeter, C.R., Tang, W.Q., Henaff, E., Butterfield, T., and Roux, S.J. (2004). Evidence of a novel cell signaling role for extracellular adenosine triphosphates and diphosphates in *Arabidopsis*. Plant Cell **16:** 2652-2664.

Johnson, C.H., Knight, M.R., Kondo, T., Masson, P., Sedbrook, J., Haley, A., and Trewavas, A. (1995). Circadian oscillations of cytosolic and chloroplastic free calcium in plants. Science **269:** 1863-1865.

Jones, D.A., and Jones, J.D.G. (1997). The role of leucine-rich repeat proteins in plant defences. Adv. Bot. Res. **24:** 89-167.

Jones, K.M., and Walker, G.C. (2008). Responses of the model legume *Medicago truncatula* to the rhizobial exopolysaccharide succinoglycan. Plant Signal. Behav. **3:** 888-890.

Jones, K.M., Sharopova, N., Lohar, D.P., Zhang, J.Q., VandenBosch, K.A., and Walker, G.C. (2008). Differential response of the plant *Medicago truncatula* to its symbiont *Sinorhizobium meliloti* or an exopolysaccharide-deficient mutant. Proc. Natl. Acad. Sci. USA **105:** 704-709.

Jordan, D.C., Grinyer, I., and Coulter, W.H. (1963). Electron microscopy of infection threads and bacteria in young root nodules of *Medicago sativa*. J. Bacteriol. **86:** 125-137.

Journet, E.P., Pichon, M., Dedieu, A., Debilly, F., Truchet, G., and Barker, D.G. (1994). *Rhizobium meliloti* Nod factors elicit cell-specific transcription of the *ENOD12* gene in transgenic alfalfa Plant J. **6:** 241-249.

Journet, E.P., El-Gachtouli, N., Vernoud, V., de Billy, F., Pichon, M., Dedieu, A., Arnould, C., Morandi, D., Barker, D.G., and Gianinazzi-Pearson, V. (2001). *Medicago truncatula* ENOD11: A novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. Mol. Plant-Microbe Interact. **14:** 737-748.

Kalsi, G., and Etzler, M.E. (2000). Localization of a Nod factor-binding protein in legume roots and factors influencing its distribution and expression. Plant Physiol. **124:** 1039-1048.

Kanamori, N., Madsen, L.H., Radutoiu, S., Frantescu, M., Quistgaard, E.M., Miwa, H., Downie, J.A., James, E.K., Felle, H.H., Haaning, L.L., Jensen, T.H., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. (2006). A nucleoporin is required for induction of Ca^{2+} spiking in legume nodule development and essential for rhizobial and fungal symbiosis. Proc. Natl. Acad. Sci. USA **103:** 359-364.

Kawaguchi, M., Imaizumi-Anraku, H., Koiwa, H., Niwa, S., Ikuta, A., Syono, K., and Akao, S. (2002). Root, root hair, and symbiotic mutants of the model legume *Lotus japonicus*. Mol. Plant-Microbe Interact. **15:** 17-26.

Kevei, Z., Lougnon, G., Mergaert, P., Horvath, G.V., Kereszt, A., Jayaraman, D., Zaman, N., Marcel, F., Regulski, K., Kiss, G.B., Kondorosi, A., Endre, G., Kondorosi, E., and Ane, J.M. (2007). 3-hydroxy-3-methylglutaryl coenzyme A reductase1 interacts with NORK and is crucial for nodulation in *Medicago truncatula*. Plant Cell **19:** 3974-3989.

Khakh, B.S. (2001). Molecular physiology of P2X receptors and ATP signalling at synapses. Nat. Rev. Neurosci. **2:** 165-174.

Kim, S.Y., Sivaguru, M., and Stacey, G. (2006). Extracellular ATP in plants. Visualization, localization, and analysis of physiological significance in growth and signaling. Plant Physiol. **142:** 984-992.

Kim, T.H., Bohmer, M., Hu, H.H., Nishimura, N., and Schroeder, J.I. (2010). Guard cell signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. Annu. Rev. Plant Biol. **61:** 561-591.

Kistner, C., Winzer, T., Pitzschke, A., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Webb, K.J., Szczyglowski, K., and Parniske, M. (2005). Seven *Lotus japonicus* genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. Plant Cell **17:** 2217-2229.

Knight, H., Trewavas, A.J., and Knight, M.R. (1997). Calcium signalling in *Arabidopsis* thaliana responding to drought and salinity. Plant J. **12:** 1067-1078.

Knight, M.R., Campbell, A.K., Smith, S.M., and Trewavas, A.J. (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature **352:** 524-526.

Komoszynski, M., and Wojtczak, A. (1996). Apyrases (ATP diphosphohydrolases, EC 3.6.1.5): Function and relationship to ATPases. Biochim. Biophys. Acta. **1310:** 233-241.

Kosuta, S., Chabaud, M., Lougnon, G., Gough, C., Denarie, J., Barker, D.G., and Becard, G. (2003). A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific *MtENOD11* expression in roots of *Medicago truncatula*. Plant Physiol. **131:** 952-962.

Kosuta, S., Hazledine, S., Sun, J., Miwa, H., Morris, R.J., Downie, J.A., and Oldroyd, G.E.D. (2008). Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. Proc. Natl. Acad. Sci. USA **105:** 9823-9828.

Kramadibrata, K., Walker, C., Schwarzott, D., and Schussler, A. (2000). A new species of *Scutellospora* a with a coiled germination shield. Ann. Bot. **86:** 21-27.

Krusell, L., Madsen, L.H., Sato, S., Aubert, G., Genua, A., Szczyglowski, K., Duc, G., Kaneko, T., Tabata, S., de Bruijn, F., Pajuelo, E., Sandal, N., and Stougaard, J. (2002). Shoot control of root development and nodulation is mediated by a receptor-like kinase. Nature **420:** 422-426.

Kukulski, F., and Komoszynski, M. (2003). Purification and characterization of NTPDase1 (ecto-apyrase) and NTPDase2 (ecto-ATPase) from porcine brain cortex synaptosomes. Eur. J. Biochem. **270:** 3447-3454.

Kurkdjian, A.C. (1995). Role of the differentiation of root epidermal-cells in nod factor (from *Rhizobium meliloti*)-induced root-hair depolarization of *Medicago sativa*. Plant Physiol. **107:** 783-790.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J.C., and Denarie, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulfated and acylated glucosamine oligosaccharide signal. Nature **344:** 781-784.

Levy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.P., Ane, J.M., Lauber, E., Bisseling, T., Denarie, J., Rosenberg, C., and Debelle, F. (2004). A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science **303:** 1361-1364.

Lew, R.R., and Dearnaley, J.D.W. (2000). Extracellular nucleotide effects on the electrical properties of growing *Arabidopsis thaliana* root hairs. Plant Sci. **153:** 1-6.

Li, W.H., Llopis, J., Whitney, M., Zlokarnik, G., and Tsien, R.Y. (1998). Cell-permeant caged InsP(3) ester shows that Ca^{2+} spike frequency can optimize gene expression. Nature **392:** 936-941.

Li, Y.H., Sorefan, K., Hemmann, G., and Bevan, M.W. (2004). *Arabidopsis* NAP and PIR regulate actin-based cell morphogenesis and multiple developmental processes. Plant Physiol. **136:** 3616-3627.

Libbenga, K.R., and Harkes, P.A.A. (1973). Initial proliferation of cortical cells in formation of root nodules in *Pisum sativum* Planta **114:** 17-28.

Limpens, E., Franken, C., Smit, P., Willemse, J., Bisseling, T., and Geurts, R. (2003). LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. Science **302:** 630-633.

Liu, X.X., Hashimoto-Torii, K., Torii, M., Haydar, T.F., and Rakic, P. (2008). The role of ATP signaling in the migration of intermediate neuronal progenitors to the neocortical subventricular zone. Proc. Natl. Acad. Sci. USA **105:** 11802-11807.

Lloyd, C.W., Pearce, K.J., Rawlins, D.J., Ridge, R.W., and Shaw, P.J. (1987). Endoplasmic microtubules connect the advancing nucleus to the tip of legume root hairs, but F-actin is involved in basipetal migration Cell Motil. Cytoskeleton **8:** 27-36.

Lohmann, G.V., Shimoda, Y., Nielsen, M.W., Jorgensen, F.G., Grossmann, C., Sandal, N., Sorensen, K., Thirup, S., Madsen, L.H., Tabata, S., Sato, S., Stougaard, J., and Radutoiu, S. (2010). Evolution and regulation of the *Lotus japonicus* LysM receptor gene family. Mol. Plant-Microbe Interact. **23:** 510-521.

Long, S.R. (1996). Rhizobium symbiosis: Nod factors in perspective. Plant Cell **8:** 1885-1898.

Long, S.R. (2001). Genes and signals in the *Rhizobium*-legume symbiosis. Plant Physiol. **125:** 69-72.

Lopez-Lara, I.M., BlokTip, L., Quinto, C., Garcia, M.L., Stacey, G., Bloemberg, G.V., Lamers, G.E.M., Lugtenberg, B.J.J., ThomasOates, J.E., and Spaink, H.P. (1996). NodZ of *Bradyrhizobium* extends the nodulation host range of *Rhizobium* by adding a fucosyl residue to nodulation signals. Mol. Microbiol. **21:** 397-408.

Luan, S. (2009). The CBL-CIPK network in plant calcium signaling. Trends Plant Sci. **14:** 37-42.

Madsen, E.B., Madsen, L.H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczyglowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., and Stougaard, J. (2003). A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature **425:** 637-640.

Malho, R., Read, N.D., Pais, M.S., and Trewavas, A.J. (1994). Role of cytosolic-free calcium in the reorientation of pollen-tube growth. Plant J. **5:** 331-341.

Marcus, A.J., and Safier, L.B. (1993). Thromboregulation - multicellular modulation of platelet reactivity in hemostasis and thrombosis. FASEB J. **7:** 516-522.

Markmann, K., Giczey, G., and Parniske, M. (2008). Functional adaptation of a plant receptor-kinase paved the way for the evolution of intracellular root symbioses with bacteria. PLoS Biol. **6:** 497-506.

Marsh, J.F., Rakocevic, A., Mitra, R.M., Brocard, L., Sun, J., Eschstruth, A., Long, S.R., Schultze, M., Ratet, P., and Oldroyd, G.E. (2007). *Medicago truncatula NIN* is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. Plant Physiol. **144:** 324-335.

Mathesius, U., Charon, C., Rolfe, B.G., Kondorosi, A., and Crespi, M. (2000). Temporal and spatial order of events during the induction of cortical cell divisions in white

clover by Rhizobium leguminosarum bv. trifolii inoculation or localized cytokinin addition. Mol. Plant-Microbe Interact. **13:** 617-628.

McAinsh, M.R., and Pittman, J.K. (2009). Shaping the calcium signature. New Phytol. **181:** 275-294.

McAinsh, M.R., Webb, A.A.R., Taylor, J.E., and Hetherington, A.M. (1995). Stimulus-induced oscillations in guard-cell cytosolic-free calcium. Plant Cell **7:** 1207-1219.

McAlvin, C.B., and Stacey, G. (2005). Transgenic expression of the soybean apyrase in *Lotus japonicus* enhances nodulation. Plant Physiol. **137:** 1456-1462.

Messinese, E., Mun, J.H., Yeun, L.H., Jayaraman, D., Rouge, P., Barre, A., Lougnon, G., Schornack, S., Bono, J.J., Cook, D.R., and Ane, J.M. (2007). A novel nuclear protein interacts with the symbiotic DMI3 calcium- and calmodulin-dependent protein kinase of *Medicago truncatula*. Mol. Plant-Microbe Interact. **20:** 912-921.

Meyer, T., and Stryer, L. (1988). Molecular Model for receptor-stimulated calcium spiking. Proc. Natl. Acad. Sci. USA **85:** 5051-5055.

Middleton, P.H., Jakab, J., Penmetsa, R.V., Starker, C.G., Doll, J., Kalo, P., Prabhu, R., Marsh, J.F., Mitra, R.M., Kereszt, A., Dudas, B., VandenBosch, K., Long, S.R., Cook, D.R., Kiss, G.B., and Oldroyd, G.E. (2007). An ERF transcription factor in *Medicago truncatula* that is essential for Nod factor signal transduction. Plant Cell **19:** 1221-1234.

Miller, D.D., Leferink-ten Klooster, H.B., and Emons, A.M. (2000). Lipochito-oligosaccharide nodulation factors stimulate cytoplasmic polarity with longitudinal endoplasmic reticulum and vesicles at the tip in vetch root hairs. Mol. Plant-Microbe Interact. **13:** 1385-1390.

Mitra, R.M., Shaw, S.L., and Long, S.R. (2004a). Six nonnodulating plant mutants defective for Nod factor-induced transcriptional changes associated with the legume-rhizobia symbiosis. Proc. Natl. Acad. Sci. USA **101:** 10217-10222.

Mitra, R.M., Gleason, C.A., Edwards, A., Hadfield, J., Downie, J.A., Oldroyd, G.E., and Long, S.R. (2004b). A Ca^{2+}/calmodulin-dependent protein kinase required for symbiotic nodule development: gene identification by transcript-based cloning. Proc. Natl. Acad. Sci. USA **101:** 4701-4705.

Miwa, H., Sun, J., Oldroyd, G.E., and Downie, J.A. (2006a). Analysis of calcium spiking using a cameleon calcium sensor reveals that nodulation gene expression is regulated by calcium spike number and the developmental status of the cell. Plant J **48:** 883-894.

Miwa, H., Sun, J., Oldroyd, G.E., and Downie, J.A. (2006b). Analysis of Nod-factor-induced calcium signaling in root hairs of symbiotically defective mutants of *Lotus japonicus*. Mol. Plant-Microbe Interact. **19:** 914-923.

Miyahara, A., Richens, J., Starker, C., Morieri G, Smith, L., Long, S.R., Downie, J.A., and Oldroyd, G.E.D. (2010). Conservation in function of a SCAR/WAVE component during infection thread and root hair growth in *Medicago truncatula*. Mol. Plant-Microbe Interact.

Monahan-Giovanelli, H., Pinedo, C.A., and Gage, D.J. (2006). Architecture of infection thread networks in developing root nodules induced by the symbiotic bacterium *Sinorhizobium meliloti* on *Medicago truncatula*. Plant Physiol. **140:** 661-670.

Monshausen, G.B., Bibikova, T.N., Weisenseel, M.H., and Gilroy, S. (2009). Ca^{2+} regulates reactive oxygen species production and pH during mechanosensing in *Arabidopsis* roots. Plant Cell **21:** 2341-2356.

Mosier, A.R. (2004). Agriculture and nitrogen cycle. (Scientific Commitee on Problems and the Enviroment (SCOPE)).

Muller, J., Staehelin, C., Xie, Z.P., Neuhaus-Url, G., and Boller, T. (2000). Nod factors and chitooligomers elicit an increase in cytosolic calcium in aequorin-expressing soybean cells. Plant Physiol. **124:** 733-739.

Murray, J., Karas, B., Ross, L., Brachmann, A., Wagg, C., Geil, R., Perry, J., Nowakowski, K., MacGillivary, M., Held, M., Stougaard, J., Peterson, L., Parniske, M., and Szczyglowski, K. (2006). Genetic suppressors of the *Lotus japonicus har1-1* hypernodulation phenotype. Mol. Plant-Microbe Interact. **19:** 1082-1091.

Murray, J.D., Karas, B.J., Sato, S., Tabata, S., Amyot, L., and Szczyglowski, K. (2007). A cytokinin perception mutant colonized by *Rhizobium* in the absence of nodule organogenesis. Science **315:** 101-104.

Navarro-Gochicoa, M.T., Camut, S., Niebel, A., and Cullimore, J.V. (2003). Expression of the apyrase-like *APY1* genes in roots of *Medicago truncatula* is induced rapidly and transiently by stress and not by *Sinorhizobium meliloti* or Nod factors. Plant Physiol. **131:** 1124-1136.

Navazio, L., Moscatiello, R., Genre, A., Novero, M., Baldan, B., Bonfante, P., and Mariani, P. (2007). A diffusible signal from arbuscular mycorrhizal fungi elicits a transient cytosolic calcium elevation in host plant cells. Plant Physiol. **144:** 673-681.

Nedeljkovic, N., Banjac, A., Horvat, A., Stojiljkovic, M., and Nikezic, G. (2005). Developmental profile of NTPDase activity in synaptic plasma membranes isolated from rat cerebral cortex. Int. J. Dev. Neurosci. **23:** 45-51.

Newman, E.I., and Reddell, P. (1987). The distribution of mycorrhizas among families of vascular plants. New Phytol. **106:** 745-751.

Nishimura, R., Hayashi, M., Wu, G.J., Kouchi, H., Imaizumi-Anraku, H., Murakami, Y., Kawasaki, S., Akao, S., Ohmori, M., Nagasawa, M., Harada, K., and Kawaguchi, M. (2002). HAR1 mediates systemic regulation of symbiotic organ development. Nature **420:** 426-429.

Novero, M., Faccio, A., Genre, A., Stougaard, J., Webb, K.J., Mulder, L., Parniske, M., and Bonfante, P. (2002). Dual requirement of the *LjSYM4* gene for mycorrhizal development in epidermal and cortical cells of *Lotus japonicus* roots. New Phytol. **154:** 741-749.

Oldroyd, G.E., and Downie, J.A. (2004). Calcium, kinases and nodulation signalling in legumes. Nat. Rev. Mol. Cell Biol. **5:** 566-576.

Oldroyd, G.E., Mitra, R.M., Wais, R.J., and Long, S.R. (2001a). Evidence for structurally specific negative feedback in the Nod factor signal transduction pathway. Plant J **28:** 191-199.

Oldroyd, G.E.D., and Long, S.R. (2003). Identification and characterization of nodulation-signaling pathway 2, a gene of *Medicago truncatula* involved in Nod factor signaling. Plant Physiol. **131:** 1027-1032.

Oldroyd, G.E.D., and Downie, J.A. (2006). Nuclear calcium changes at the core of symbiosis signalling. Curr. Opin. Plant Biol. **9:** 351-357.

Oldroyd, G.E.D., and Downie, J.M. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu. Rev. Plant Biol. **59:** 519-546.

Oldroyd, G.E.D., Engstrom, E.M., and Long, S.R. (2001b). Ethylene inhibits the Nod factor signal transduction pathway of *Medicago truncatula*. Plant Cell **13:** 1835-1849.

Oldroyd, G.E.D., Harrison, M.J., and Paszkowski, U. (2009). Reprogramming plant cells for endosymbiosis. Science **324:** 753-754.

Ooki, Y., Banba, M., Yano, K., Maruya, J., Sato, S., Tabata, S., Saeki, K., Hayashi, M., Kawaguchi, M., Izui, K., and Hata, S. (2005). Characterization of the *Lotus japonicus* symbiotic mutant *lot1* that shows a reduced nodule number and distorted trichomes. Plant Physiol. **137:** 1261-1271.

Ostrom, R.S., Gregorian, C., and Insel, P.A. (2000). Cellular release of and response to ATP as key determinants of the set-point of signal transduction pathways. J. Biol. Chem. **275:** 11735-11739.

Pacios Bras, C., Jorda, M.A., Wijfjes, A.H., Harteveld, M., Stuurman, N., Thomas-Oates, J.E., and Spaink, H.P. (2000). A *Lotus japonicus* nodulation system based on heterologous expression of the fucosyl transferase NodZ and the acetyl transferase NoIL in *Rhizobium leguminosarum*. Mol. Plant-Microbe Interact. **13:** 475-479.

Parniske, M. (2004). Molecular genetics of the arbuscular mycorrhizal symbiosis. Curr. Opin. Plant Biol. **7:** 414-421.

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat. Rev. Microbiol. **6:** 763-775.

Parniske, M., Zimmermann, C., Cregan, P.B., and Werner, D. (1990). Hypersensitive reaction of nodule cells in the glycine sp *Bradyrhizobium japonicum* symbiosis occurs at the genotype-specific level. Bot. Act. **103:** 143-148.

Patil, S., Takezawa, D., and Poovaiah, B.W. (1995). Chimeric plant calcium/calmodulin-dependent protein-kinase gene with a neural visinin-like calcium-binding domain. Proc. Natl. Acad. Sci. USA **92:** 4897-4901.

Pawlowski, K., and Bisseling, T. (1996). Rhizobial and actinorhizal symbioses: What are the shared features? Plant Cell **8:** 1899-1913.

Pawlowski K, N.W. (2008). Comparison between actinorhizal and legume symbiosis. (Dordrecht: Springer Netherlands).

Pearson, J.N., and Jakobsen, I. (1993). Symbiotic exchange of carbon and phosphorus between cucumber and 3 *Arbuscular mycorrhizal* fungi. New Phytol. **124:** 481-488.

Peck, M.C., Fisher, R.F., and Long, S.R. (2006). Diverse flavonoids stimulate NodD1 binding to nod gene promoters in *Sinorhizobium meliloti*. J. Bacteriol. **188:** 5417-5427.

Peiter, E., Sun, J., Heckmann, A.B., Venkateshwaran, M., Riely, B.K., Otegui, M.S., Edwards, A., Freshour, G., Hahn, M.G., Cook, D.R., Sanders, D., Oldroyd, G.E.D., Downie, J.A., and Ane, J.M. (2007). The *Medicago truncatula* DMI1 protein modulates cytosolic calcium signaling. Plant Physiol. **145:** 192-203.

Pellock, B.J., Cheng, H.P., and Walker, G.C. (2000). Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. J. Bacteriol. **182:** 4310-4318.

Penmetsa, R.V., and Cook, D.R. (1997). A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. Science **275:** 527-530.

Penmetsa, R.V., Frugoli, J.A., Smith, L.S., Long, S.R., and Cook, D.R. (2003). Dual genetic pathways controlling nodule number in *Medicago truncatula*. Plant Physiol. **131:** 998-1008.

Perret, X., Staehelin, C., and Broughton, W.J. (2000). Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. **64:** 180-201.

Peters, N.K., Frost, J.W., and Long, S.R. (1986). A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233:** 977-980.

Pingret, J.L., Journet, E.P., and Barker, D.G. (1998). *Rhizobium* nod factor signaling. Evidence for a G protein-mediated transduction mechanism. Plant Cell **10:** 659-672.

Plieth, C. (2001). Plant calcium signaling and monitoring: pros and cons and recent experimental approaches. Protoplasma **218:** 1-23.

Quackenbush, J., Cho, J., Lee, D., Liang, F., Holt, I., Karamycheva, S., Parvizi, B., Pertea, G., Sultana, R., and White, J. (2001). The TIGR Gene Indices: analysis of gene transcript sequences in highly sampled eukaryotic species. Nucleic Acids Res. **29:** 159-164.

Quinn, J.M., and Etzler, M.E. (1987). Isolation and characterization of a lectin from the roots of *Dolichos-Biflorus*. Arch. Biochem. Biophys. **258:** 535-544.

Radutoiu, S., Madsen, L.H., Madsen, E.B., Jurkiewicz, A., Fukai, E., Quistgaard, E.M.H., Albrektsen, A.S., James, E.K., Thirup, S., and Stougaard, J. (2007). LysM domains mediate lipochitin-oligosaccharide recognition and *NFR* genes extend the symbiotic host range. EMBO J. **26:** 3923-3935.

Radutoiu, S., Madsen, L.H., Madsen, E.B., Felle, H.H., Umehara, Y., Gronlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. (2003). Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature **425:** 585-592.

Rae, A.L., Bonfantefasolo, P., and Brewin, N.J. (1992). Structure and growth of infection threads in the legume symbiosis with *Rhizobium leguminosarum*. Plant J. **2:** 385-395.

Ralevic, V., and Burnstock, G. (1998). Receptors for purines and pyrimidines. Pharmacol. Rev. **50:** 413-492.

Ramachandiran, S., Takezawa, D., Wang, W., and Poovaiah, B.W. (1997). Functional domains of plant chimeric calcium/calmodulin-dependent protein kinase: Regulation by autoinhibitory and visinin-like domains. J. Biochem., Tokyo **121:** 984-990.

Ridge, R.W., and Rolfe, B.G. (1985). *Rhizobium* sp degradation of legume root hair cell-wall at the site of infection thread origin. Appl. Environ. Microbiol. **50:** 717-720.

Riely, B.K., Lougnon, G., Ane, J.M., and Cook, D.R. (2007). The symbiotic ion channel homolog DMI1 is localized in the nuclear membrane of *Medicago truncatula* roots. Plant J. **49:** 208-216.

Riewe, D., Grosman, L., Fernie, A.R., Wucke, C., and Geigenberger, P. (2008). The potato-specific apyrase is apoplastically localized and has influence on gene expression, growth, and development. Plant Physiol. **147:** 1092-1109.

Roberts, N.J., Brigham, J., Wu, B., Murphy, J.B., Volpin, H., Phillips, D.A., and Etzler, M.E. (1999). A Nod factor-binding lectin is a member of a distinct class of apyrases that may be unique to the legumes. Mol. Gen. Genet. **262:** 261-267.

Roche, P., Debelle, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Denarie, J., and Prome, J.C. (1991). Molecular-basis of symbiotic host specificity in *Rhizobium-Meliloti* - *NodH* and *NodPQ* genes encode the sulfation of lipo-oligosaccharide signals. Cell **67:** 1131-1143.

Rodpothong, P., Sullivan, J.T., Songsrirote, K., Sumpton, D., Cheung, K., Thomas-Oates, J., Radutoiu, S., Stougaard, J., and Ronson, C.W. (2009). Nodulation gene mutants

of *Mesorhizobium loti* R7A-*nodZ* and *nolL* mutants have host-specific phenotypes on *Lotus* spp. Mol. Plant-Microbe Interact. **22:** 1546-1554.

Rolfe, B.G., and Gresshoff, P.M. (1988). Genetic-analysis of legume nodule initiation. Annu. Rev. Plant Physiol. Plant Mol. Biol. **39:** 297-319.

Roux, S.J., and Steinebrunner, I. (2007). Extracellular ATP: an unexpected role as a signaler in plants. Trends Plant Sci. **12:** 522-527.

Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H., Umehara, Y., Kouchi, H., Murooka, Y., Szczyglowski, K., Downie, J.A., Parniske, M., Hayashi, M., and Kawaguchi, M. (2007). NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in *Lotus japonicus*. Plant Cell **19:** 610-624.

Sanchez, F., Padilla, J.E., Perez, H., and Lara, M. (1991). Control of nodulin genes in root-nodule development and metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42:** 507-528.

Sanders, D., Pelloux, J., Brownlee, C., and Harper, J.F. (2002). Calcium at the crossroads of signaling. Plant Cell **14 Suppl:** S401-417.

Schauser, L., Roussis, A., Stiller, J., and Stougaard, J. (1999). A plant regulator controlling development of symbiotic root nodules. Nature **402:** 191-195.

Schauser, L., Handberg, K., Sandal, N., Stiller, J., Thykjaer, T., Pajuelo, E., Nielsen, A., and Stougaard, J. (1998). Symbiotic mutants deficient in nodule establishment identified after T-DNA transformation of *Lotus japonicus*. Mol. Gen. Genet. **259:** 414-423.

Schenk, P.W., and Snaar-Jagalska, B.E. (1999). Signal perception and transduction: the role of protein kinases. Biochim. Biophys. Acta. **1449:** 1-24.

Scheres, B., Vandewiel, C., Zalensky, A., Horvath, B., Spaink, H., Vaneck, H., Zwartkruis, F., Wolters, A.M., Gloudemans, T., Vankammen, A., and Bisseling, T. (1990). The ENOD12 gene-product is involved in the infection process during the pea rhizobium interaction. Cell **60:** 281-294.

Schultze, M., Quicletsire, B., Kondorosi, E., Virelizier, H., Glushka, J.N., Endre, G., Gero, S.D., and Kondorosi, A. (1992). *Rhizobium-meliloti* produces a family of sulfated lipooligosaccharides exhibiting different degrees of plant host specificity. Proc. Natl. Acad. Sci. USA **89:** 192-196.

Schwedock, J., and Long, S.R. (1990). ATP sulphurylase activity of the Nodp and Nodq gene-products of *Rhizobium-meliloti*. Nature **348:** 644-647.

Sedaa, K.O., Bjur, R.A., Shinozuka, K., and Westfall, D.P. (1990). Nerve and drug-Induced release of adenine nucleosides and nucleotides from rabbit aorta. J. Pharmacol. Exp. Ther. **252:** 1060-1067.

Shaw, S.L., and Long, S.R. (2003). Nod factor elicits two separable calcium responses in *Medicago truncatula* root hair cells. Plant Physiol. **131:** 976-984.

Shibata, K., Abe, S., and Davies, E. (2001). Structure of the coding region and mRNA variants of the apyrase gene from pea (*Pisum sativum*). Acta Physiol. Plant. **23:** 3-13.

Shibata, K., Morita, Y., Abe, S., Stankovic, B., and Davies, E. (1999). Apyrase from pea stems: Isolation, purification, characterization and identification of a NTPase from the cytoskeleton fraction of pea stem tissue. Plant Physiol. Biochem. **37:** 881-888.

Sieberer, B.J., Timmers, A.C., and Emons, A.M. (2005). Nod factors alter the microtubule cytoskeleton in *Medicago truncatula* root hairs to allow root hair reorientation. Mol. Plant-Microbe Interact. **18:** 1195-1204.

Sieberer, B.J., Chabaud, M., Timmers, A.C., Monin, A., Fournier, J., and Barker, D.G. (2009). A nuclear-targeted cameleon demonstrates intranuclear Ca^{2+} spiking in *Medicago truncatula* root hairs in response to rhizobial nodulation factors. Plant Physiol. **151:** 1197-1206.

Smil, V. (1997). Global population and the nitrogen cycle. Sci. Am. **277:** 76-81.

Smit, G., Puvanesarajah, V., Carlson, R.W., Barbour, W.M., and Stacey, G. (1992). *Bradyrhizobium-japonicum*-Nodd1 can be specifically induced by soybean flavonoids that do not induce the nodyabcsuij operon. J. Biol. Chem. **267:** 310-318.

Smit, P., Limpens, E., Geurts, R., Fedorova, E., Dolgikh, E., Gough, C., and Bisseling, T. (2007). *Medicago* LYK3, an entry receptor in rhizobial nodulation factor signaling. Plant Physiol. **145:** 183-191.

Smith, S.E., Smith, F.A., and Jakobsen, I. (2004). Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New Phytol. **162:** 511-524.

Soltis, D.E., Soltis, P.S., Morgan, D.R., Swensen, S.M., Mullin, B.C., Dowd, J.M., and Martin, P.G. (1995). Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen-fixation in angiosperms. Proc. Natl. Acad. Sci. USA **92:** 2647-2651.

Song, C.J., Steinebrunner, I., Wang, X.Z., Stout, S.C., and Roux, S.J. (2006). Extracellular ATP induces the accumulation of superoxide via NADPH oxidases in *Arabidopsis*. Plant Physiol. **140:** 1222-1232.

Spaink, H.P., Sheeley, D.M., van Brussel, A.A., Glushka, J., York, W.S., Tak, T., Geiger, O., Kennedy, E.P., Reinhold, V.N., and Lugtenberg, B.J. (1991). A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature **354:** 125-130.

Steen, A., Buist, G., Leenhouts, K.J., El Khattabi, M., Grijpstra, F., Zomer, A.L., Venema, G., Kuipers, O.P., and Kok, J. (2003). Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. J. Biol. Chem. **278:** 23874-23881.

Stehno-Bittel, L., Luckhoff, A., and Clapham, D.E. (1995). Calcium-release from the nucleus by $InsP_3$ receptor channels. Neuron **14:** 163-167.

Steinebrunner, I., Wu, J., Sun, Y., Corbett, A., and Roux, S.J. (2003). Disruption of apyrases inhibits pollen germination in arabidopsis. Plant Physiol. **131:** 1638-1647.

Stougaard, J. (2000). Regulators and regulation of legume root nodule development. Plant Physiol. **124:** 531-540.

Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Szczyglowski, K., and Parniske, M. (2002). A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature **417:** 959-962.

Subramanian, S., Stacey, G., and Yu, O. (2006). Endogenous isoflavones are essential for the establishment of symbiosis between soybean and *Bradyrhizobium japonicum*. Plant J. **48:** 261-273.

Subramanian, S., Stacey, G., and Yu, O. (2007). Distinct, crucial roles of flavonoids during legume nodulation. Trends Plant Sci. **12:** 282-285.

Sun, J., Miwa, H., Downie, J.A., and Oldroyd, G.E.D. (2007). Mastoparan activates calcium spiking analogous to nod factor-induced responses in *Medicago truncatula* root hair cells. Plant Physiol. **144:** 695-702.

Sun, J.H., Cardoza, V., Mitchell, D.M., Bright, L., Oldroyd, G., and Harris, J.M. (2006). Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation. Plant J. **46:** 961-970.

Suntharalingam, M., and Wente, S.R. (2003). Peering through the pore: Nuclear pore complex structure, assembly, and function. Dev. Cell **4:** 775-789.

Sutton, J.M., Lea, E.J., and Downie, J.A. (1994). The nodulation-signaling protein NodO from *Rhizobium leguminosarum* biovar *viciae* forms ion channels in membranes. Proc. Natl. Acad. Sci. USA **91:** 9990-9994.

Swanson, J.A., Tu, J.K., Ogawa, J., Sanga, R., Fisher, R.F., and Long, S.R. (1987). Extended region of nodulation genes in *Rhizobium meliloti* 1021 .1. Phenotypes of Tn5 insertion mutants. Genetics **117:** 181-189.

Szczyglowski, K., Shaw, R.S., Wopereis, J., Copeland, S., Hamburger, D., Kasiborski, B., Dazzo, F.B., and de Bruijn, F.J. (1998). Nodule organogenesis and symbiotic mutants of the model legume *Lotus japonicus*. Mol. Plant-Microbe Interact. **11:** 684-697.

Takezawa, D., Ramachandiran, S., Paranjape, V., and Poovaiah, B.W. (1996). Dual regulation of a chimeric plant serine/threonine kinase by calcium/calmodulin. Plant Physiol. **111:** 155-155.

Tang, W.Q., Brady, S.R., Sun, Y., Muday, G.K., and Roux, S.J. (2003). Extracellular ATP inhibits root gravitropism at concentrations that inhibit polar auxin transport. Plant Physiol. **131:** 147-154.

Tansengco, M.L., Hayashi, M., Kawaguchi, M., Imaizumi-Anraku, H., and Murooka, Y. (2003). *Crinkle*, a novel symbiotic mutant that affects the infection thread growth and alters the root hair, trichome, and seed development in *Lotus japonicus*. Plant Physiol. **131:** 1054-1063.

Thomas, C., Sun, Y., Naus, K., Lloyd, A., and Roux, S. (1999). Apyrase functions in plant phosphate nutrition and mobilizes phosphate from extracellular ATP. Plant Physiol. **119:** 543-551.

Thomas, C., Rajagopal, A., Windsor, B., Dudler, R., Lloyd, A., and Roux, S.J. (2000). A role for ectophosphatase in xenobiotic resistance. Plant Cell **12:** 519-533.

Tirichine, L., James, E.K., Sandal, N., and Stougaard, J. (2006a). Spontaneous root-nodule formation in the model legume *Lotus japonicus*: A novel class of mutants nodulates in the absence of rhizobia. Mol. Plant-Microbe Interact. **19:** 373-382.

Tirichine, L., Sandal, N., Madsen, L.H., Radutoiu, S., Albrektsen, A.S., Sato, S., Asamizu, E., Tabata, S., and Stougaard, J. (2007). A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. Science **315:** 104-107.

Tirichine, L., Imaizumi-Anraku, H., Yoshida, S., Murakami, Y., Madsen, L.H., Miwa, H., Nakagawa, T., Sandal, N., Albrektsen, A.S., Kawaguchi, M., Downie, A., Sato, S., Tabata, S., Kouchi, H., Parniske, M., Kawasaki, S., and Stougaard, J. (2006b). Deregulation of a Ca^{2+}/calmodulin-dependent kinase leads to spontaneous nodule development. Nature **441:** 1153-1156.

Todorov, L.D., MihaylovaTodorova, S., Westfall, T.D., Sneddon, P., Kennedy, C., Bjur, R.A., and Westfall, D.P. (1997). Neuronal release of soluble nucleotidases and their role in neurotransmitter inactivation. Nature **387:** 76-79.

Trautmann, A. (2009). Extracellular ATP in the immune system: more than just a "danger signal". Sci. Signal. **2:** pe6.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., Debilly, F., Prome, J.C., and Denarie, J. (1991). Sulfated lipo-oligosaccharide signals of *Rhizobium-Meliloti* elicit root nodule organogenesis in alfalfa. Nature **351:** 670-673.

van Brussel, A.A.N., Bakhuizen, R., Vanspronsen, P.C., Spaink, H.P., Tak, T., Lugtenberg, B.J.J., and Kijne, J.W. (1992). Induction of preinfection thread structures in the leguminous host plant by mitogenic lipooligosaccharides of *Rhizobium*. Science **257:** 70-72.

van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., and Sanders, I.R. (1998). Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature **396:** 69-72.

van Noorden, G.E., Kerim, T., Goffard, N., Wiblin, R., Pellerone, F.I., Rolfe, B.G., and Mathesius, U. (2007). Overlap of proteome changes in *Medicago truncatula* in response to auxin and *Sinorhizobium meliloti*. Plant Physiol. **144:** 1115-1131.

Vasse, J., Debilly, F., and Truchet, G. (1993). Abortion of infection during the *Rhizobium meliloti*-alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. Plant J. **4:** 555-566.

Vassileva, V.N., Kouchi, H., and Ridge, R.W. (2005). Microtubule dynamics in living root hairs: transient slowing by lipochitin oligosaccharide nodulation signals. Plant Cell **17:** 1777-1787.

Vernie, T., Moreau, S., de Billy, F., Plet, J., Combier, J.P., Rogers, C., Oldroyd, G., Frugier, F., Niebel, A., and Gamas, P. (2008). EFD is an ERF transcription factor involved in the control of nodule number and differentiation in *Medicago truncatula*. Plant Cell **20:** 2696-2713.

Wais, R.J., Keating, D.H., and Long, S.R. (2002). Structure-function analysis of nod factor-induced root hair calcium spiking in rhizobium-legume symbiosis. Plant Physiol. **129:** 211-224.

Wais, R.J., Galera, C., Oldroyd, G., Catoira, R., Penmetsa, R.V., Cook, D., Gough, C., Denarie, J., and Long, S.R. (2000). Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. Proc. Natl. Acad. Sci. USA **97:** 13407-13412.

Walker, S.A., and Downie, J.A. (2000). Entry of *Rhizobium leguminosarum* bv.*viciae* into root hairs requires minimal Nod factor specificity, but subsequent infection thread growth requires *nodO* or *nodE*. Mol. Plant-Microbe Interact. **13:** 754-762.

Walker, S.A., Viprey, V., and Downie, J.A. (2000). Dissection of nodulation signaling using pea mutants defective for calcium spiking induced by Nod factors and chitin oligomers. Proc. Natl. Acad. Sci. USA **97:** 13413-13418.

Wasson, A.P., Pellerone, F.I., and Mathesius, U. (2006). Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport regulation by rhizobia. Plant Cell **18:** 1617-1629.

Weerasinghe, R.R., Bird, D.M., and Allen, N.S. (2005). Root-knot nematodes and bacterial Nod factors elicit common signal transduction events in *Lotus japonicus*. Proc. Natl. Acad. Sci. USA **102:** 3147-3152.

Weerasinghe, R.R., Collings, D.A., Johannes, E., and Allen, N.S. (2003). The distributional changes and role of microtubules in Nod factor-challenged *Medicago sativa* root hairs. Planta **218:** 276-287.

Wegel, E., Schauser, L., Sandal, N., Stougaard, J., and Parniske, M. (1998). Mycorrhiza mutants of *Lotus japonicus* define genetically independent steps during symbiotic infection. Mol. Plant-Microbe Interact. **11:** 933-936.

White, P.J. (1998). Calcium channels in the plasma membrane of root cells. Ann. Bot. **81:** 173-183.

Wilkinson, D.M. (2001). Mycorrhizal evolution. Trends Ecol. Evol. **16:** 64-65.

Wolf, C., Hennig, M., Romanovicz, D., and Steinebrunner, I. (2007). Developmental defects and seedling lethality in apyrase *AtAPY1* and *AtAPY2* double knockout mutants. Plant Mol. Biol. **64:** 657-672.

Yano, K., Yoshida, S., Muller, J., Singh, S., Banba, M., Vickers, K., Markmann, K., White, C., Schuller, B., Sato, S., Asamizu, E., Tabata, S., Murooka, Y., Perry, J., Wang, T.L., Kawaguchi, M., Imaizumi-Anraku, H., Hayashi, M., and Parniske, M. (2008). CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc. Natl. Acad. Sci. USA **105:** 20540-20545.

Yip, L., Woehrle, T., Corriden, R., Hirsh, M., Chen, Y., Inoue, Y., Ferrari, V., Insel, P.A., and Junger, W.G. (2009). Autocrine regulation of T-cell activation by ATP release and P2X(7) receptors. FASEB J. **23:** 1685-1693.

Yokota, K., Fukai, E., Madsen, L.H., Jurkiewicz, A., Rueda, P., Radutoiu, S., Held, M., Hossain, M.S., Szczyglowski, K., Morieri, G., Oldroyd, G.E., Downie, J.A., Nielsen, M.W., Rusek, A.M., Sato, S., Tabata, S., James, E.K., Oyaizu, H., Sandal, N., and Stougaard, J. (2009). Rearrangement of actin cytoskeleton mediates invasion of *Lotus japonicus* roots by *Mesorhizobium loti*. Plant Cell **21:** 267-284.

Zeng, Y., Lv, X.H., Zeng, S.Q., Tian, S.L., Li, M., and Shi, J. (2008). Sustained depolarization-induced propagation of [Ca2+](i) oscillations in cultured DRG neurons: The involvement of extracellular ATP and P2Y receptor activation. Brain Res. **1239:** 12-23.

Zhang, J., Subramanian, S., Stacey, G., and Yu, O. (2009). Flavones and flavonols play distinct critical roles during nodulation of *Medicago truncatula* by *Sinorhizobium meliloti*. Plant J. **57:** 171-183.