Nod factor signaling in symbiotic nodulation

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CHAPTER ONE

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Abstract

In the rhizobial legume symbiosis, an exchange of signals leads to the specific infection of the host plant by its rhizobial symbiont and the development of specialized organs, the nodules, in which rhizobia fix nitrogen. In response to flavonoids secreted by legume roots, rhizobia notably produce lipochitooligosaccharidic molecules called Nod factors (NFs). Following extensive research, we now understand better the mechanisms of perception and transduction of the NF signal by the legume host. NF signaling results in a wide transcriptional reprogramming that leads to coordinated multistep processes of infection and nodule organogenesis, involving many plant and bacterial molecular components. Recent data suggest that the NF biosynthesis pathway evolved from non rhizobial bacteria and that NF perception/

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signaling in legumes adapted components preexisting in plant signaling pathways controlling in particular the establishment of the more ancient arbuscular mycorrhizal symbiosis.

Introduction

Nitrogen fixing symbioses between rhizobial bacteria and their legume hosts are characterized by the formation on the roots of the host plant of new organs, the nodules, in which bacteria differentiated in bacteroids fix atmospheric dinitrogen. These associations are often specific, a given rhizobium being able to induce nodule formation on a limited number of legume hosts (Dénarié, Debellé, & Rosenberg, 1992). The various rhizobium-legume symbioses exhibit many variations in the mode of infection (through root hairs or intercellular), the type of nodules formed (determinate or indeterminate), or their functioning (Hadri, Spaink, Bisseling, & Brewin, 1998). The symbiotic relationship results from a multi-step process in which the tightly associated partners exchange various signals, making difficult the analysis of the molecular mechanisms involved. However, since the rhizobium-legume symbiosis is not obligatory, non-lethal mutations causing defects in the nodulation process can be obtained in both symbiotic partners, making genetics a powerful approach to dissect the molecular mechanisms controlling nodulation.

1. Identification of Nod factors, rhizobial lipochitooligosaccharidic signals with a major role in nodulation and host range control

1.1 Identification of *nod* genes

The development of molecular genetics of Gram-bacteria in the 1980s was instrumental in our understanding of the molecular mechanisms controlling the nodulation process. The first studies were carried out in model rhizobia such as *Rhizobium leguminosarum* and *Sinorhizobium meliloti*, and made use in particular of the availability of broad host range cloning vectors adapted to rhizobia, and the introduction of methods for random and site-directed mutagenesis by transposons. Early work had shown that genetic determinants of nodulation and host range were located on plasmids (Johnston et al., 1978) and rhizobial mutants affected in nodulation were isolated. This led to the first cloning of a locus controlling nodulation genes by

complementation of a *nod*-mutation (Long, Buikema, & Ausubel, 1982). Further work involving site-directed mutagenesis, physical mapping and sequencing allowed the identification of a series of *nod* genes in various rhizobial species (see reviews Dénarié et al., 1992; Schlaman, Phillips, & Kondorosi, 1998; Downie, 1998). Some of these genes (*nodDABCIJ*) were found in all rhizobium species and were called common *nod* genes. Other *nod* genes, called host specific *nod* genes, were found only in some rhizobial strains. Mutations in the *nodABC* genes usually had a strong effect on rhizobium infection and nodulation, while the effect of mutations in other *nod* genes varied according to the nature of the gene.

1.2 *Nod* gene expression

The use of reporter genes made possible the analysis of *nod* gene expression. The *nodD* gene was found constitutively expressed in rhizobial growth medium while the expression of the other *nod* genes required the addition to the medium of root exudates together with a functional *nodD* gene (Long, 1989). NodD is a transcriptional activator of the LysR family. It can bind to a specific sequence upstream the *nod* gene operons, called the *nod* box, and activate the expression of other *nod* genes in the presence of compounds secreted by roots (Schlaman et al., 1998). The *nod* gene inducing compounds were isolated from exudates of various legume plants. They are generally flavonoids, secondary metabolites biosynthesized by the phenylpropanoid pathway, a metabolic pathway which expanded in legumes leading to a great diversity of molecules. In spite of repeated attempts, no crystal structure of a NodD protein with and without ligands has been obtained and there is little biochemical evidence of flavonoid binding to NodD (Peck, Fisher, Bliss, & Long, 2013). However direct binding of NodD to flavonoids is supported by the existence of *nodD* mutants with altered specificity with regard to flavonoids (Peck et al., 2013; Schlaman et al., 1998). The detailed mechanisms of *nod* gene activation are also poorly understood: NodD could bind to the *nod* box as a dimer or higher order oligomer, and NodD binding to DNA could be stimulated by flavonoids. The way NodD interacts with RNA polymerase to activate *nod* gene expression is not known (Schlaman et al., 1998). This first step of the molecular dialog between the legume host and rhizobium contributes to host range specificity: the NodD protein of a rhizobial species activates *nod* gene expression in the presence of the set of flavonoids produced by compatible host species. Different NodD proteins can have different inducer specificities and a *nodD* mutation in a rhizobial species thus sometimes

cannot be complemented by the *nodD* gene of another rhizobium species (Spaink, Wijffelman, Pees, Okker, & Lugtenberg, 1987). In addition, some rhizobium species such as *S. meliloti* carry several copies of *nodD* that encode proteins with different inducer specificities, or that can activate *nod* gene expression in the absence of flavonoids like NodD3 (Dénarié et al., 1992). Some flavonoids can also act as anti-inducers, inhibiting the activation of *nod* gene expression (Schlaman et al., 1998). Interestingly, different sets of flavonoids can be secreted at different stages of the symbiotic process. In *Medicago truncatula*, luteolin, the first flavonoid identified as a *nod* gene inducer is mostly produced by germinating seeds. In root exudates, another compound with a higher *nod* gene inducing activity than luteolin was identified, methoxychalcone. Synthesis of this compound is controlled by the enzyme chalcone-O-methyl transferase and genes encoding this protein were found induced in root hairs in the presence of rhizobia (Liu & Murray, 2016), suggesting a role for methoxychalcone in the infection process. Recent results on the *Mesorhizobium loti-Lotus japonicus* symbiosis indicate that the two *nodD* genes in *M. loti* might act at different stages in the symbiotic process. NodD2 is mainly involved in induction of *nod* genes in the rhizosphere and within nodules, while *nodD1* acts primarily within root hair infection threads (Kelly et al., 2018). The nature of the Lotus flavonoid compounds involved in *nod* gene activation at these different stages of the symbiotic process is still unknown. However a *Lotus* gene encoding a chalcone isomerase involved in isoflavonoid biosynthesis is induced in the presence of rhizobia or NFs and specifically expressed within root hairs. Mutant analysis indicated that it might be responsible for the production of an inducer specifically recognized by NodD1 in infected root hairs. In addition to induction by flavonoids, *Rhizobium tropici* CIAT899 *nod* gene expression can be induced by salt or osmotic stress (Del Cerro et al., 2019). *R. tropici* CIAT899 is highly resistant to various abiotic stresses, and it contains five *nodD* genes. Among these, *nodD1* is required for *nod* gene induction by flavonoids, while induction by salt or osmotic stress requires *nodD2* (Del Cerro et al., 2019). This might be a strategy to ensure nodulation under abiotic stress conditions.

Other mechanisms contribute to the regulation of *nod* gene expression, which vary from species to species (Schlaman et al., 1998). In *S. meliloti*, another regulator of the LysR family, *syrM*, participates together with *nodD3* in *nod* gene induction. In *Bradyrhizobium diazoefficiens*, a two component regulator *nodVW* controls *nod* gene induction by flavonoids, in parallel to the control exerted by *nodD1*. In this species, quorum sensing also plays a

role in *nod* gene regulation (Calatrava-Morales, McIntosh, & Soto, 2018; Jitacksorn & Sadowsky, 2008). Negative regulators of *nod* gene expression have also been characterized, such as *nolR* in *S. meliloti*, and *nolA* in *B. diazoefficiens* (Schlaman et al., 1998). *nod* gene expression is thus tightly regulated in response to various developmental and environmental conditions by specific mechanisms in different species, which also coordinate *nod* gene expression with that of other symbiosis-related processes (see below).

1.3 *Nod* genes and Nod factors

The biochemical function of the proteins encoded by *nod* genes remained elusive for several years until a link between the genetic determinants of nodulation and the production of secreted molecules was established: it was shown that sterile supernatants of rhizobial cultures grown in the presence of flavonoids could induce root or root hair deformations on homologous hosts and that this activity depended on the common *nodABC* genes (Van Brussel et al., 1986; Faucher et al., 1988). Using an alfalfa root hair deformation assay, active extracellular molecules called Nod factors (NFs) were extracted from cultures of *S. meliloti* overexpressing the *nod* genes. *S. meliloti* NFs consist of a backbone of four to five N-acetyl glucosamine residues, N-acylated at the non-reducing end by various unsaturated C16 fatty acids, O-acetylated at the non-reducing end and sulfated at the reducing end (Fig. 1) (Dénarié, Debellé, & Promé, 1996; Lerouge et al., 1990). NFs have since been purified from other rhizobial species. In most cases, similar molecules have been obtained: mono N-acylated chitooligosaccharides of generally four or five N-acetylglucosamine residues, carrying different substitutions, mainly on the two terminal glucosamine residues (Fig. 1) (see reviews by Dénarié et al., 1996; Perret, Staehelin, & Broughton, 2000; D'Haeze & Holsters, 2002). Rhizobial strains generally produce a mixture of NFs with slightly different structures, whose composition can vary with environmental conditions. No original NF structures have been described in recent years, suggesting that all common NF substitutions have been identified. However, purification and characterization of new LCOs from rhizobial cultures remain tricky particularly when they are produced in small amounts. Identification of new *nod* genes by large-scale genome or metagenome sequencing might help discover new NF molecules.

1.4 Biosynthesis of Nod factors

Since the expression of *nod* genes is required for NF production, *nod* genes were likely to encode the enzymes necessary for NF biosynthesis, with the

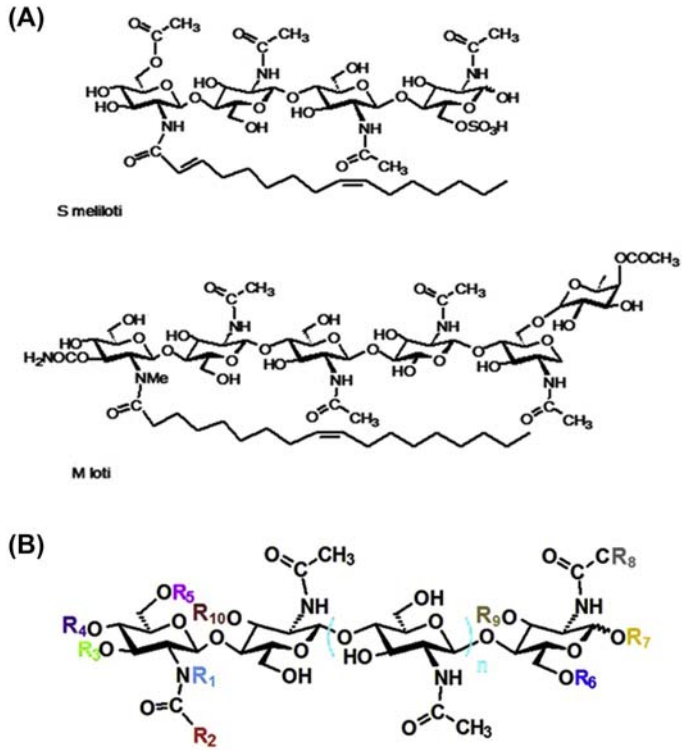


Fig. 1 ***Structure of Nod factors produced by various rhizobia.*** The structure of *S. meliloti* and *M. loti* NFs is represented in (A). In (B) is shown the structure of the chitooligosaccharidic backbone common to all NFs (n = 2 or 3 in most cases) and the positions of the various substitutions (R1 to R9) that have been described in NFs produced by different rhizobial strains. For a detailed list of substitutions and genes controlling them, see Dénarié et al. (1996); Perret et al. (2000); and D'Haeze & Holsters (2002).

common *nodABC* genes directing biosynthesis of the lipooligosaccharidic core common to all NFs, and the host specific *nod* genes controlling the rhizobium species specific substitutions of NFs. The role of each gene was assessed by different approaches: analysis of the structure of NFs produced by *nod* mutants, similarity of Nod proteins to proteins of known function, and direct enzymatic assays (Dénarié et al., 1996; Perret et al., 2000; D'Haeze & Holsters, 2002). For example, *nodH* mutants of *S. meliloti* produced non sulfated NF suggesting a role for *nodH* in NF sulfation; the *NodH* sequence is similar to that of known sulfotransferases; and purified NodH exhibits LCO sulfotransferase activity *in vitro*. The common NodC, NodB and NodA proteins were shown to carry out the synthesis

of the chitooligosacharride backbone, its N-deacetylation at the non-reducing end, and its acylation, respectively. Most of the enzymatic steps leading to the biosynthesis of NFs have been characterized although uncertainties remain about the order in which they are carried out (Broughton, Jabbouri, & Perret, 2000; Poinsot et al., 2016). Little is known about the mechanisms of NF secretion. The *nodIJ* genes, which encode the ATPase and permease of an ABC-type transport system, are found in all NF producing rhizobia, and play a role in NF secretion (Downie, 1998). However other genes are likely involved as well, since *nodIJ* mutants still secrete NFs and have a weak symbiotic phenotype (Downie, 1998).

1.5 Nod factor degradation

NF concentration can be regulated by modulating NF biosynthesis but also NF degradation. Indeed, NFs can be cleaved and inactivated by host enzymes such as Nod Factor hydrolase 1 in *M. truncatula* (MtNFH1) or chitinase 5 in *L. japonicus* (LjCHIT5) (Cai et al., 2018; Malolepszy et al., 2018). Contrary to related chitinases, MtNFH1 hydrolyzes neither chitin nor chitin fragments and seems to have a high cleavage preference for *S. meliloti* LCOs. Upon inoculation by *S. meliloti*, MtNFH1 was found to accumulate in the infection chamber at the curled tip of root hairs and mutants affected in *MtNFH1* exhibited delayed root hair infection, abnormal nodule branching and hypertrophy (Cai et al., 2018). In contrast, in *Ljchit5* mutants, the progression of infection threads in the cortex and of intracellular infections were inhibited, leading to non-functional nodules (Malolepszy et al., 2018). This suggests that in both systems, fine tuning of NF levels is required for proper infection and nodulation of host plants.

1.6 Biological activity of Nod factors and importance for host range specificity

Purified NFs can induce at very low concentrations many symbiotic-like responses specifically on the roots of legume hosts (Hadri & Bisseling, 1998; D'Haeze & Holsters, 2002). These include modifications of root hair growth, rearrangement of cytoplasm in outer cortical cells leading to the formation of preinfection threads, and initiation of cell division in the inner root cortex at the origin of nodule primordium formation. At the cellular level, NFs induce depolarization of the epidermis cytoplasmic membrane, production of reactive oxygen species, and different ion fluxes. Among these, NF induced-calcium spiking in and around the cell nucleus has been extensively used to monitor plant response to LCOs (Ehrhardt,

Wais, & Long, 1996; Kelner, Leitão, Chabaud, Charpentier, & de Carvalho-Niebel, 2018). NFs also induce tissue-specific responses at the transcriptional level originally revealed by the identification of early nodulin (*ENOD*) genes (Hadri & Bisseling, 1998), and then by whole genome transcriptome analyses (Breakspear et al., 2014; Damiani, Drain et al., 2016; Damiani, Pauly, Puppo, Brouquisse, & Boscari, 2016; Jardinaud et al., 2016). LCOs also induce lateral root formation, and might participate in the formation of biofilms (Fujishige et al., 2008; Oláh, Brière, Bécard, Dénarié, & Gough, 2005).

NFs are additionally important determinants of host range. Analysis of NF structure in various rhizobial species generally showed a correlation between the type of NF produced and host range. For example, two symbionts of soybean belonging to different rhizobial genera, *Sinorhizobium fredii* and *B. diazoefficiens*, produce similar NFs. In addition, NF substitutions, which are encoded by host specific nodulation genes, are important for their biological activity. Mutations in these genes or their transfer to another rhizobial strain can result in changes of the host range. Depending on plants, the requirements for NF structure are not always stringent. For example, *Phaseolus* can be nodulated by rhizobia producing NFs with different structures. In addition, NFs are not the only determinants of host range: beside the nature of the plant flavonoid signal, rhizobial surface components or secreted protein effectors interacting with their plant receptor also play a role in partner choice (Wang, Liu, & Zhu, 2018).

Through the regulatory activity of flavonoids and NodD proteins, NF biosynthesis is connected to other biological processes that may play a role in the establishment of the symbiotic interaction. For example, the *S. meliloti nodD3* and *syrM* genes were found to induce the synthesis of exopolysaccharides, rhizobial surface components that play an important role in the infection process. More recently, genomic and transcriptomic analyses performed in different rhizobial species showed that a number of operons regulated by *nodD* and flavonoids through binding to *nod* boxes were not involved in NF biosynthesis. In particular, in the *Sinorhizobium* NGR234 and HH103 strains and in *B. diazoefficiens,* flavonoids induce the expression of the type III secretion system machinery and the synthesis of effector proteins that are injected in the host plant in a process similar to that observed with many plant pathogens (Jimenez-Guerrero et al., 2017). These effectors can have positive or negative effects on the symbiotic process and contribute to host range specificity (Deakin & Broughton, 2009; Miwa & Okazaki, 2017; Nelson & Sadowsky, 2015). In *S. fredii* HH103 and *R. tropici* 899,

genes coding for the biosynthesis of the phytohormone auxin are induced by NodD and flavonoids (Jimenez-Guerrero et al., 2017). Interestingly, auxin and NFs were recently found to have a synergistic effect on lateral root development (Herrbach et al., 2017). Contrary to NF biosynthetic genes that are found in most rhizobia, these NodD/flavonoid responsive genes are found only in some rhizobial species, indicating lineage specific adaptations to NF induced nodulation and infection by rhizobia. In addition, plant flavonoids can also induce responses in rhizobia in a *nodD* independent way, which are likely involved in the rhizospheric life of rhizobia.

2. Nod factor perception and signaling in the host plant

Purified NFs from a rhizobium species were shown to induce, at very low concentrations and in a NF structure-dependent way, symbiotic responses specifically on roots of the legume host. This suggested that these molecules could be perceived by high-affinity receptors, and that a signaling pathway then transduced the NF signal to start the nodulation and rhizobial infection programs. In order to identify components of this signaling pathway, legume mutants affected in the root-nodule symbiosis (RNS) were therefore tested for their responses to purified NFs. Several of these mutants were impaired in their responses to NFs, indicating that the mutations likely affected NF signaling (Catoira et al., 2000). The genes involved in the responses to NFs in legume hosts were then ordered in a putative linear symbiotic signaling pathway. Upstream of the pathway were genes essential for all responses to NF, likely involved in NF perception, such as Nod Factor Perception (*NFP*) in *M. truncatula or* Nod factor Receptor 5 (*NFR5*) in *L. japonicus*. The calcium spiking response to NFs allowed further ordering of downstream signaling genes: genes like *MtNFP (LjNFR5)*, Does not Make Infections 1 (*MtDMI1) (LjCASTOR/POLLUX)*, and *MtDMI2* or SYMBIOSIS RECEPTOR-LIKE KINASE in Lotus *japonicus (LjSYMRK)* were required for calcium spiking while *MtDMI3 or* Calcium/CalModulin-dependent protein Kinase in *L. japonicus (LjCCaMK)* was not. In contrast to the above described genes, Nodulation Signaling Pathway 1 and 2 (*NSP*1 and *NSP2)* seemed dispensable for root hair deformation by NFs, suggesting that they acted downstream of *DMI3*. *L. japonicus* gain of function mutants able to nodulate in the absence of rhizobia also helped to decipher the NF signaling pathway and new actors of the pathway were additionally identified by looking for interactors of known components. Some of the genes

involved, among them *DMI 1, 2,3* were shown to also control the establishment of the symbiosis with endomycorrhizal fungi and thus to define a common symbiotic signaling pathway (CSSP). Genes with similar functions were found in *M. truncatula*, *L. japonicus* and *Pisum sativum,* indicating conservation of the NF signaling pathway in different legumes (Oldroyd, 2013; Oldroyd, Murray, Poole, & Downie, 2011). Map-based cloning of these genes mostly in *M. truncatula* and *L. japonicus* confirmed that they were likely to control a signal perception/transduction pathway, and biochemical and cellular approaches shed light on the molecular mechanisms involved, although many aspects remain poorly understood despite extensive studies. The signaling pathway can be divided in three main steps: perception of microbial signals, transduction of the signals and transcriptional activation of responses leading to the accommodation of rhizobia in the host plant (Fig. 2).

2.1 Perception

In *L. japonicus* and *M. truncatula*, two putative NF receptors, LjNFR5 and MtNFP, were identified as lysin-motif (LysM) receptor-like kinases, with three LysM domains (known to bind N-acetyl glucosamine containing oligosaccharides) constituting the extracellular part of the proteins. The intracellular kinase domain of these proteins is inactive, suggesting that an interaction with other receptors with active kinase domains is required to initiate a signal transduction. Indeed, LysM-RLK with active kinase domains having crucial roles in infection and nodulation were identified in both *L. japonicus* (Nod factor Receptor 1, LjNFR1) and *M. truncatula* (LysM domain-containing receptor-like kinase 3, MtLYK3). Biochemical approaches showed that LjNFR1 and LjNFR5 interact in a heterologous system (Madsen et al., 2011). In *M. truncatula*, NFP and LYK3 localized in the same cell layers in the apical part of the nodule (Moling et al., 2014), where they probably also form a heterodimeric complex (Pietraszewska-Bogiel et al., 2013). However they could not be detected in the root epidermis, probably due to their low levels. NF perception slightly differs between *L. japonicus* and *M. truncatula*: both *NFR1* and *LYK3* are required for infection and nodulation, but contrary to *LjNFR1* mutants, *MtLYK3* mutants still exhibit some responses to NFs (Arrighi et al., 2006; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Smit et al., 2007). This might be due to redundancy of LYK3 with other *M. truncatula* LysM-RLKs. Indeed *M. truncatula* and *L. japonicus* carry genes closely related to *LjNFR1/MtLYK3*. One of these, *LjNFRe*, which has a spatio-temporal

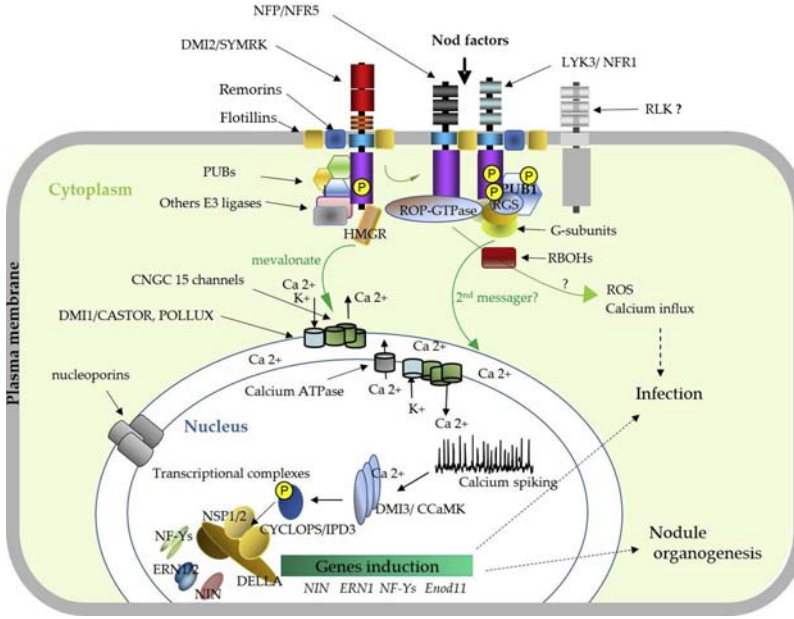


Fig. 2 ***A model for Nod Factor signaling in*** **Medicago truncatula** ***and*** **Lotus japonicus** ***symbiotic nodulation.*** Nod factor (NF) perception is mediated by a set of receptor-like kinases containing a LysM extracellular domain, including NFP/NFR5 and LYK3/NFR1. Other proteins, and among those other LysM-RLKs, are likely contributing to NF perception. During rhizobial infection, ROP-GTPases interact with LysM-RLKs and activate RBOHs, which produce reactive oxygen species and play a positive role in rhizobial infection and nodulation. The DMI2/SYMRK receptor is probably associated to the LysM perception complex within structured micro-domains in the plasma membrane, organized by the scaffolding proteins flotillins and remorins. DMI2/SYMRK could act as a co-receptor leading to the activation of intracellular signaling pathways. DMI2/SYMRK interacts with many partners including several E3 ubiquitin ligase (PUBs, SIE3, SINA4 and LIN/CERBERUS) that could participate in its turnover. The 3-hydroxy-3-methylglutaryl-CoA-reductase (HMGR) enzyme also interacts with DMI2, and could lead to the production of mevalonate as a secondary messenger to transmit the NF signal from the plasma membrane to the cell nucleus. An additional mechanism that is proposed to activate nuclear calcium spiking in *L. japonicus* involves heterotrimeric G-protein complexes that interact with and are phosphorylated by NFR1. This activation would lead to the production of an unknown secondary messenger promoting the calcium spiking. These secondary messengers would activate DMI1/POLLUX and CASTOR cation channels, which interact with three calcium channels from the CNGC15 family. The Ca2+ influx through CNGC15 channels is countered by a DMI1-dependent K+ efflux into the nuclear envelope, resulting in nuclear membrane repolarization to initiate calcium spiking in response to NFs. A calcium ATPase (MCA8) maintains the nuclear calcium concentration, and three nucleoporins identified in *L. japonicus* are also necessary for calcium spiking. In the nucleus, DMI3/CCaMK decodes calcium oscillations, and phosphorylates IPD3/CYCLOPS, a transcriptional activator. The phosphorylated form of IPD3/CYCLOPS would interact with various transcription factors (DELLA, NSP1/2) within a large transcriptional complex, and induce a transcriptional cascade leading to nodule organogenesis and/or rhizobial infection, which involves additional transcription factors (NIN, NF-Ys, and ERN1/2).

expression pattern and signaling properties distinct to those of LjNFR1, was recently shown to contribute also to NF signaling (Murakami et al., 2018).

Transformation of *LjNFR5* and *LjNFR1* into *M. truncatula* led to nodulation of the transformed plants by *M. loti*, the *L. japonicus* symbiont, indicating a role of these genes in host range determination (Radutoiu et al., 2007). The host range might be determined by specific recognition of the NF substitutions by the legume host receptors. Accordingly, high affinity binding of *M. loti* LCOs to both full-length LjNFR1 and LjNFR5 was reported (Broghammer et al., 2012). In addition, NFRe is also able to bind NFs *in vitro* (Murakami et al., 2018). In contrast, in *M. truncatula*, no binding of LCOs to NFP or LYK3 was demonstrated, while high affinity binding was shown for another LysM-RLK with an inactive kinase, MtLYR3, a protein with no evidence of symbiotic role based on mutant analyses (Fliegmann et al., 2013). However, no effect of the rhizobia specific NF substitutions on receptor-ligand affinity was detected in any of these binding experiments. By domain swapping and amino acid substitution, the NFR5/NFP LysM2 domains, and in particular a leucine residue in these domains, were shown as important for NF recognition (Bensmihen, de Billy, & Gough, 2011; Radutoiu et al., 2007). Crystal structures of the receptors in the presence of their ligands are however necessary to decipher the basis of specific LCO recognition. Since biochemical analysis of membrane proteins such as LysM-RLKs is difficult, a currently used approach consists in the characterization of the structure and NF binding properties of the extracellular domain of symbiotic LysM-RLK. However, NF binding *in vivo* could be different from that observed with the extracellular domain of NF receptors: NF receptors are indeed likely part of protein complexes that are located in plasma membrane nanodomains, which probably change along the symbiotic process (Ott, 2017). Interestingly, a *L. japonicus* LysM-RLK with a symbiotic role is involved in the response to modified exopolysaccharides produced by *M. loti*, raising the question of the range of ligands recognized by LysM-RLKs (Kawaharada et al., 2015).

2.2 Transduction

MtDMI2 (*LjSYMRK*) is positioned downstream of the LysM-RLK receptors: in contrast to *nfp* mutants, *dmi2* mutants still exhibit some responses to rhizobia and NFs, such as the early calcium influx (Endre et al., 2002). Over expression of full length *LjSYMRK* or the kinase domain of *MtDMI2* led to nodule induction in the absence of rhizobia, and this effect depended on the *DMI3* (*LjCCaMK*) and *DMI1* (*LjCastor LjPollux*) genes. This

indicates that DMI2 acts upstream of DMI1 and DMI3 (Ried, Antolín-Llovera, & Parniske, 2014; Saha, Dutta, Bhattacharya, & DasGupta, 2014).

In *dmi2/symrk* mutants, initiation of nodule organogenesis is prevented, and neither infection pockets nor infection threads are observed indicating a role of *DMI2/SYMRK* in the early stages of both symbiotic programs (Catoira et al., 2000; Endre et al., 2002; Stracke et al., 2002). In *L. japonicus*, the *symrk-14* mutation (affecting the extracellular domain) uncouples epidermal and cortical symbiotic responses (Kosuta et al., 2011). These phenotypes highlight a major role for *DMI2/SYMRK* in both early events at the root epidermis, where the initial infection occurs, and also in the cortical signaling pathway required for the initiation of nodule primordia and cortical infections. However, how DMI2/SYMRK acts during signal transduction to initiate these symbiotic responses remains unclear.

Many plant developmental programs are controlled by RLKs, which act in protein complexes to perceive an extracellular ligand leading to the activation of intracellular signaling pathways. MtDMI2 (LjSYMRK) could thus act as a coreceptor of LysM-RLK proteins to transduce the NF signal (Osakabe, Yamaguchi-Shinozaki, Shinozaki, & Tran, 2013). DMI2/SYMRK is a receptor-like kinase with an extracellular domain containing three leucine-rich repeats (LRRs) and 2 malectin-like domains (MLDs). MLDs are known to bind oligosaccharide ligands but none have been identified for DMI2/SYMRK. In contrast, LjSYMRK was found by coimmunopurification to bind the NFR5 and NFR1 receptors (Antolín-Llovera, Ried, & Parniske, 2014; Ried et al., 2014). This fits with the report that in *M. truncatula*, the Symbiotic Remorin 1 (MtSYMREM1) interacts with both DMI2 and the symbiotic receptors NFP and LYK3, probably as a scaffolding protein, to regulate spatially the signaling complex along the symbiotic interaction (Lefebvre et al., 2010; Liang et al., 2018).

With the aim to identify targets of symbiotic kinases, quantitative proteomic and phosphoproteomic analyses were performed in *M. truncatula*, which measured rapid changes in the phosphorylation status of about 13 000 sites in 7739 proteins upon NF perception (Grimsrud et al., 2010; Rose, Volkening et al., 2012, Rose, Grimsurd et al., 2012; Valdés-López et al., 2018). Soybean phosphoproteomic analysis of root hair cells identified more than 250 phosphopeptides corresponding to 240 phosphoproteins significantly regulated in response to inoculation with *Bradyrhizobium japonicum* (Nguyen et al., 2012). This approach led to the identification in *M. truncatula* of a new regulator, the Phosphorylated Protein 1 (EPP1) required for nodulation initiation (Valdés-López et al., 2018). However further work is

required to check if any of the proteins characterized by these approaches are direct targets of DMI2 or symbiotic LysM-RLKs.

Several evidences indicate that the amount of the DMI2 (SYMRK) protein is important for proper signaling and is thus tightly regulated (Antolín-Llovera et al., 2014; Pan, Stonoha-Arther, & Wang, 2018). Indeed, RLK signaling not only depends on the perception of the ligand but also needs a precise amount of active membrane-associated RLK. Several studies underline the importance of a fine-tuned regulation of DMI2/SYMRK by its extra cellular domain (Antolín-Llovera et al., 2014; Li et al., 2018; Pan et al., 2018). MLD integrity is required for the DMI2 protein to function properly during infection and early nodule development (Pan et al., 2018). Indeed, MLD stabilizes the full-length DMI2/SYMRK protein (Antolín-Llovera et al., 2014; Pan et al., 2018). On the other side, the conserved GDPC motif connecting LRR and MLD domains is required for MLD cleavage of the SYMRK protein in response to rhizobium (Antolín-Llovera et al., 2014).

Ubiquitination is a major mechanism associated with plasma membrane proteins to regulate the turnover of some receptors (Tian & Xie, 2013). This process occurs through the sequential activation of a cascade of reactions catalyzed by three classes of enzymes referred as E1, E2 and E3 (reviewed in Vierstra, 2009). Symbiotic RLKs were found to interact with different E3 ubiquitin ligases, among which three Plant U-box (PUB) ARMADILLO E3 ubiquitin ligases. MtPUB1 was identified as a negative regulator of nodulation and as a direct interactor of the intracellular kinase domain of the symbiotic RLKs LYK3 (Mbengue et al., 2010) and DMI2 (Vernié et al., 2016). Analyses of a *pub1* mutant affected in the U-box domain revealed that the ubiquitin ligase activity of PUB1 is essential for its negative regulatory function, nevertheless the symbiotic receptors DMI2 and LYK3 do not appear to be the targets of the PUB1 ubiquitination activity. Instead, another PUB ARMADILLO E3 ligase, MtPUB2, mediates MtDMI2 ubiquitination and the associated 26S proteasome-dependent degradation *in vitro* and *in planta* (Liu et al., 2018). MtPUB2 is a positive regulator of nodulation and by a cascade of phosphorylation and ubiquitination regulations, MtDMI2-MtPUB2 forms a negative feedback loop which could participate in the control of nodulation homeostasis (Liu et al., 2018). A third different PUB E3-ubiquitin ligase, LjPUB13, specifically ubiquitinates the kinase domain of NFR5 and has a positive regulatory role during the initial stages of nodulation (Tsikou et al., 2018).

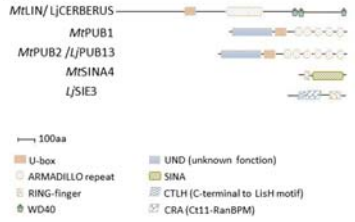
In addition to PUB E3-ubiquitin ligases, the *L. japonicus* SYMRK-INTERACTING E3 ubiquitin ligase (SIE3) was shown to bind and to

ubiquitinate SYMRK to positively regulate nodulation (Yuan et al., 2012). The SEVEN IN ABSENTIA 4 (LjSINA4), another type of E3 ubiquitin ligase, negatively affects the rhizobial infection process and interacts with and destabilizes SYMRK, but ubiquitination of SYMRK was not detected (Den Herder et al., 2008). Finally, Lumpy INfections (*LINs)* and *CERBERUS*, in *M. truncatula* and *L. japonicus* respectively, encode E3 ligases containing U-box, armadillo (ARM) and WD-40 repeats, and control rhizobial infection inside root hairs (Kiss et al., 2009; Yano et al., 2009).

PUB, SIE3, LIN/CERBERUS and SINA4 proteins belong to three structurally very different families of E3 ligases (Fig. 3) and may thus have different modes of action (Hervé, Lefebvre, & Cullimore, 2011). And, to further complicate these regulatory processes, a deubiquitinating enzyme, ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM 1 (AMSH 1) was recently identified as a positive regulator of nodulation (Małolepszy et al., 2015). Currently, it is therefore difficult to get from the literature a clear picture of the role of ubiquitination in nodulation. One of the main issues remains the identification of the E3 ubiquitin ligases targets.

Additional components of the symbiosis signaling pathway downstream of the receptor complex have been identified by looking for interactors of components of this complex. For example, in a yeast two-hybrid screen, MtDMI2 was found to interact with 3-hydroxy-3-methylglutaryl CoA

(A)



(B)

E3 ubiquitin ligase	Receptor	Target	Role in nodulation
*Mt*PUB1	*Mt*LYK3 *Mt*DMI2	?	Negative regulator
*Mt*PUB2	*Mt*DMI2	V	Positive regulator ; proteasome-mediated degradation of target ; nodulation homeostasis
*Lj*PUB13	*Lj*NFR5	V	Positive regulator ; initial stages of nodulation
*Lj*SIE3	*Lj*SYMRK	V	Positive regulator
*Lj*SINA4	*Lj*SYMRK	?	Negative regulator of infection
*Mt*LIN	?	?	Infection in root hairs
*Lj*CERBERUS	?	?	Infection in root hairs, growth, differentiation of nodules

Fig. 3 ***E3 ubiquitin ligases involved in*** **Medicago truncatula** ***and*** **Lotus japonicus** ***symbiotic nodulation.*** (A) Schematic representation of E3 ubiquitin ligases (adapted from Hervé et al., 2011). (B) Summary of different types of E3 ubiquitin ligases and their role in nodulation. The column « receptor » indicates the symbiotic receptor like-kinases which interact with each E3 ubiquitin ligase, and in the next column, « V » indicates that the ubiquitination of these RLKs by the corresponding E3 ubiquitin ligase was validated.

Reductase 1 (HMGR1), an enzyme involved in the biosynthesis of mevalonate, a precursor of isoprenoid secondary metabolites (Kevei et al., 2007). RNAi silencing of HMGR1 reduced nodulation and responses to NFs, such as calcium spiking and *ENOD11* marker gene expression (Venkateshwaran et al., 2015), indicating a role of HMGR1 in NF signaling. In addition, exogenous application of mevalonate induced calcium spiking in *M. truncatula* root epidermal cells, and this effect depended on DMI1 but not DMI2, indicating that the mevalonate signal acts downstream of DMI2 but upstream of DMI1. Finally, the application of mevalonate to kidney cells expressing the nuclear cation channel DMI1 (see below) induced calcium spiking, suggesting an interaction between mevalonate and DMI1 (Venkateshwaran et al., 2015). Thus, mevalonate or products of its metabolism could act as secondary messengers to transmit the NF signal from the plasma membrane to the cell nucleus.

More recently in *L. japonicus*, a receptor-like cytoplasmic kinase interacting with LjNFR5 was identified by a coimmunoprecipitation-based proteomic approach (Wong et al., 2019). This protein named NFR5-interacting cytoplasmic kinase 4 (NiCK4), was able to bind and phosphorylate LjNFR5 and LjNFR1 *in vitro*. The corresponding gene has an expression profile similar to that of LjNFR5 and its mutation resulted in decreased nodulation. Interestingly, this protein was found to relocate to the nucleus upon NF treatment in an *NFR1/NFR5* dependent manner indicating that it could contribute directly to the transmission of the NF signal from the plasma membrane, where receptors are located, to the nucleus where transcriptional activation leading to nodulation occurs (Wong et al., 2019).

Another mechanism could contribute to the transmission of the NF signal from the plasma membrane to the nucleus. Pharmacological approaches indicated a role in NF signaling of heterotrimeric G-protein complexes comprising Gα, Gβ, and Gγ subunits and the Regulator of G-protein Signaling (RGS) protein. Mastoparan 7 (Mas7), a G-protein agonist was shown to induce early nodulin gene expression and calcium spiking in *M. truncatula* roots in the absence of rhizobia (Pingret, Journet, & Barker, 1998; Sun, Miwa, Downie, & Oldroyd, 2007). This required DMI3 but not NFP, DMI1 or DMI2. More recently in soybean, RGS, Gβ, and Gγ were shown to positively regulate nodule formation, while Gα appears to be a negative regulator (Choudhury & Pandey, 2013, 2015). In addition, RGS interacts with and is phosphorylated by the LysM-RLK NFR1. It is thus proposed that active, phosphorylated RGS maintains Gα proteins in their trimeric inactive conformation, and initiates the signaling that leads

to calcium spiking. However the role of putative second messengers and other signaling components acting downstream the G-protein complex remains unknown.

Three nucleoporins were also identified in *L. japonicus* as necessary for various steps of the root nodule symbiosis including calcium spiking (Groth et al., 2010; Kanamori et al., 2006; Saito et al., 2007). Nucleoporins are components of the nuclear pore complexes that control the exchange of macromolecules across the nuclear membranes and play a role in many different physiological processes. However, the symbiotic Lotus mutants affected in nucleoporins have only slight growth or developmental phenotypes and it is not clear how the mutations inhibit calcium spiking. In addition, mutations in genes encoding such proteins were so far not identified in screens of *M. truncatula* symbiotic mutants, suggesting different mechanisms and/or levels of redundancy between the two model legumes.

It took more than 10 years to identify the nuclear-localized ion channels and calcium pump required to generate the nucleo-cytoplasmic calcium spiking observed in response to NFs (Charpentier, 2018). DMI1 in *M. truncatula* and CASTOR and POLLUX in *L. japonicus* were first characterized as putative cation channels likely to control ion transport upstream of calcium spiking (Ané et al., 2004; Charpentier et al., 2008; Venkateshwaran et al., 2012). In addition, Cyclic Nucleotide-Gated Channels (CNGC15a, CNGC15b, and CNGC15c) were identified as calcium channels located on the nuclear membrane, which interact with DMI1 and mediate nuclear calcium oscillations in response to symbiotic LCOs (Charpentier et al., 2016). A model proposed that the influx of calcium through CNGC15 channels is countered by a DMI1-dependent potassium efflux into the nuclear envelope, resulting in nuclear membrane repolarization to enable a sustained calcium spiking (Charpentier et al., 2016). This model may however need revision due to the recent study of Kim et al. (2019) indicating that CASTOR/DMI1 is more likely a calcium channel. Finally, the nuclear calcium concentration is maintained by the action of the M. truncatula calcium ATPase MCA8 (Capoen et al., 2011). Overall, through these ion fluxes, the NF signal perceived at the plasma membrane is transmitted into the nucleus to generate calcium oscillations that are decoded by DMI3 and associated proteins to initiate transcriptional regulations (see below; Charpentier, 2018).

In addition, Morieri and collaborators proposed a model in which NFs, during the rhizobial infection process, would activate two pathways, one

involving calcium spiking, as described above, and another one acting independently of calcium spiking (Morieri et al., 2013). This second pathway includes small Rho-Of-Plants (ROP) GTPases, which bind to and can be activated by the NFP and LYK3 components of the symbiotic receptor complex (Ke et al., 2012). During rhizobial infection, ROP-GTPases activate NADPH oxidases (Kiirika et al., 2012), also named Respiratory Burst Oxidase Homologs (RBOHs), which produce reactive oxygen species and play a positive role in rhizobial infection and nodulation (Marino et al., 2011).

2.3 Transcriptional activation

MtDMI3 (*LjCCaMK*) is a central regulator of NF-dependent early signaling. *DMI3* mutants are affected in infection and nodulation, but not in calcium spiking, indicating that this gene acts downstream of calcium spiking in NF signaling. DMI3 was identified as a calcium and calmodulin dependent protein kinase (CCaMK) (Lévy et al., 2004; Mitra et al., 2004), and was shown to have a nuclear localization (Smit et al., 2005). This confirmed the importance of calcium signaling in nodulation and made DMI3 a good candidate to decode oscillations of calcium concentration in the nucleus. Autoactive forms of DMI3 can induce spontaneous nodulation in the absence of rhizobia (Gleason et al., 2006; Takeda, Maekawa, & Hayashi, 2012; Tirichine et al., 2006) and can rescue calcium-spiking deficient mutants. This underlines the central role of DMI3 and suggests that the primary function of NF signaling components acting upstream of the calcium spiking (described above) is the activation of DMI3. Contrary to more common calcium-dependent protein kinases (CDPKs), DMI3 can bind calcium both directly through three EF-hand domains, and indirectly through calmodulin calcium-binding domains. Although the structure of the DMI3 protein is not known, genetic and modeling analyses allowed to propose a putative mechanism for the activation of DMI3 by calcium (Miller et al., 2013). At basal calcium concentrations, binding of calcium to the EF-hands inhibits the activity of DMI3 by inducing an autophosphorylation of the protein, which promotes bond formation between the kinase and the calmodulin-binding domains. When the calcium concentration increases, calcium/calmodulin binds to DMI3, which blocks its autophosphorylation and promotes substrate phosphorylation.

In order to understand how DMI3 controls the expression of genes leading to nodulation, it was important to identify target substrates. Interacting

Protein of DMI3 (IPD3) was identified as a DMI3 interactor in a yeast two hybrid screen (Messinese et al., 2007). IPD3 localizes to the nucleus and can be phosphorylated by DMI3. Mutants in *IPD3* and its *L. japonicus* ortholog *CYCLOPS* are impaired in rhizobial colonization but exhibit less severe phenotypes than *dmi3* mutants, suggesting a partial functional redundancy. CYCLOPS was shown to act as a transcriptional activator that binds DNA in a phosphorylation-dependent manner (Singh, Katzer, Lambert, Cerri, & Parniske, 2014). Two phosphorylated serine residues of CYCLOPS are necessary for its activity and phosphomimetic versions of CYCLOPS are able to trigger root nodule organogenesis in the absence of rhizobia and CCaMK (DMI3). Upon phosphorylation by CCaMK, CYCLOPS can bind to the promoter of the Nodule Inception (NIN) transcription factor, and induces a transcriptional cascade leading to root nodule development. IPD3/CYCLOPS acts within a larger transcriptional complex that includes several GRAS-domain containing transcription factors such as Nodulation Signaling Pathway 1 and 2 (NSP1/2) and DELLA proteins that are required for rhizobial infection (Floss, Levy, Lévesque-Tremblay, Pumplin, & Harrison, 2013; Fonouni-Farde et al., 2016; Jin et al., 2016; Pimprikar et al., 2016). DELLA proteins are required for gibberellic acid (GA) signaling, and then likely participate in the coordination of early symbiotic signaling with plant development. The NIN transcription factor acts as a master coordinator of responses leading to nodule organogenesis and infection, together with the Ethylene Response Factor Required for Nodulation 1 (ERN1) and NF-YA transcription factors (Liu, Rutten et al., 2019, Liu, Breakspear, 2019; Schauser, Roussis, Stiller, & Stougaard, 1999). A number of studies shed light on the hierarchical relationships between these transcriptional regulators, mainly by monitoring symbiotic gene expression in various lines overexpressing or mutated for these TFs (Cerri et al., 2012; Fonouni-Farde et al., 2016; Laloum et al., 2014; Liu, Rutten et al., 2019, Liu, Breakspear, 2019; Soyano, Kouchi, Hirota, & Hayashi, 2013). However the interpretation of these analyses is difficult due to partial redundancy, feedback regulations and subtle spatio-temporal expression patterns of these genes.

Beside GA signaling, other hormone signaling pathways, involving notably cytokinins and auxins, interact with NF signaling to ensure the coordination between symbiotic responses induced in the root epidermis, pericycle and cortex tissues, which are associated respectively with infection by rhizobia and cell divisions leading to nodule primordium formation (Boivin, Fonouni-Farde, & Frugier, 2016; Gamas, Brault, Jardinaud, &

Frugier, 2017, Buhian & Bensmihen, 2018; see also Chapter 12: Hormonal interactions in the regulation of the nitrogen-fixing legume Rhizobium symbiosis, by Mathesius, 2019).

3. Origin and evolution of Nod factor signaling

3.1 Origin and evolution of rhizobial nod genes

For many years the common *nod* genes were considered as indispensable to establish RNS on legumes as well as on *Parasponia*, the only known non-legume plant able to nodulate with rhizobia. The presence of these genes was the main criterium to identify a bacterial strain as a rhizobium. However in 2007, it was shown that photosynthetic *Bradyrhizobium* strains that efficiently fix nitrogen on some *Aeschynomene* plant species lack *nodABC* genes (Giraud et al., 2007). The mechanism inducing nodule formation in this biological system remains not known, as in spite of an extensive mutagenesis, no strict non-nodulating bacterial mutant was obtained (Bonaldi et al., 2010). In addition, there was no evidence of the production by photosynthetic *Bradyrhizobium* of a diffusible bacterial signal able to induce symbiotic responses. More recently, a *nodC* mutant of *Bradyrhizobium elkanii*, unable to produce NFs, was reported to induce nodule formation on soybean, although with a lower efficiency than the WT strain (Okazaki et al., 2016; Okazaki, Kaneko, Sato, & Saeki, 2013). This nodule induction pathway depended on an intact type 3 secretion system suggesting that this mutant could bypass NF perception/signaling likely through the action of effectors secreted through this secretion machinery.

Soil Actinobacteria of the genus *Frankia* can induce nodulation and establish symbiotic nitrogen fixing symbioses with a diverse group of plants in the orders Cucurbitales, Fagales and Rosales, which belong to the same clade of plants able to establish nitrogen fixing symbioses as the Legume family-containing Fabales order (Soltis et al., 1995). Many studies aimed at identifying putative NF-like molecules produced by *Frankia* and involved in actinorhizal plant nodulation. These efforts are hampered by the difficulty to genetically manipulate *Frankia* strains, and even to grow them in the absence of the host plant. Sequencing of the genome of a number of *Frankia* strains able to nodulate *Alnus* or *Casuarina* plants (strains that belong to *Frankia* cluster I) showed that close homologs of *nodABC* genes were absent in these *Frankia* strains, indicating that other mechanisms than LCO signaling are likely involved in nodulation (Normand et al., 2007). Indeed,

molecules secreted by *Frankia* and potentially involved in early symbiotic signaling have been described, based on biological tests such as root hair deformation, the induction of the *Casuarina NIN* symbiotic gene, or the induction of calcium spiking (Chabaud et al., 2016; Cissoko et al., 2018). These molecules have biochemical properties different from that of LCOs (more hydrophilic and resistant to chitinases), but their exact nature remains to be determined. Interestingly, canonical *nodABC* genes were more recently identified in genomes of uncultured *Frankia* strains Dg1 and Dg2 (strains that belong to *Frankia* cluster II) whose sequence was obtained by metagenome analysis of samples isolated from nodules of an actinorhizal plant, *Datisca glomerata* (Nguyen et al., 2016; Persson et al., 2015). The Dg1 *nodABC* genes are expressed in nodules and the *nodC* gene could partially complement a mutation of the *R. leguminosarum nodC* gene, and rescue the nodulation on its legume host, *P. sativum*. The Dg1 and Dg2 genomes also contain distant homologs of *nodIJ*, and the Dg2 genome contains two copies of the *nodH* sulfotransferase gene. However, since these *Frankia* strains cannot be cultured, the production of LCOs could not be validated. More recently, the genome of a *Frankia* isolate from cluster III was sequenced and found to contain the *nodABCIJH* genes (Ktari et al., 2017). This strain can be cultivated, so that it should be possible to test its LCO production capacity. However, since none of the *Frankia* strains can be genetically manipulated, the importance of *nodABC* and LCO production in the nodulation of actinorhizal plants will remain difficult to establish. In addition, no *nodD*-like gene was identified in the *nodABC* containing *Frankia* genomes, raising the question of how *nodABC* gene expression is regulated.

The identification of canonical *nodABC* genes in *Frankia* strains suggests a role for LCO signaling in the nodulation of some actinorhizal plants. It also sheds light on a possible origin and evolution of LCO signaling in bacteria. The phylogenetic analysis of *nodA* is particularly informative. For a long time, this gene encoding an acyl transferase was considered as specific to rhizobia, as it could not be identified in non-rhizobial members of the alpha- and beta-Proteobacteria, even in the form of distantly related paralogs. In contrast, more recent studies revealed that NodA homologs can be detected across the whole Actinobacteria phylum, including non-nodulating genera, and exhibit a higher diversity than the rhizobial NodA proteins (Persson et al., 2015). In a NodA phylogenetic tree, the *Frankia* proteins cluster with the rhizobial proteins and are basal to the rhizobial NodA orthologs. This suggests that the *nodA* gene responsible for the acyl transfer step in

LCO biosynthesis originated in Actinobacteria, and was later transmitted to Proteobacteria by lateral transfer. Similarly, the *nodBC* genes are likely to have originated in Actinobacteria before being transferred to Proteobacteria, although the analysis of phylogenetic trees is more complex since these genes encoding a deacetylase and a glycosyl transferase, respectively, are members of multigene families. The presence of *nodABC* genes in Actinobacteria that are not known to nodulate raises the question of a non-symbiotic role of these genes. In contrast to *nodABC,* the *nodIJ* genes that are also common to all rhizobia are likely to have a different origin. Although in *Frankia* genes similar to *nodIJ* are found in the vicinity of *nodBC* genes, their similarity with rhizobial *nodIJ* genes is low and they probably originate from a different lineage. Thus, the *nodIJ* genes likely evolved independently in Actinobacteria and Proteobacteria (Aoki, Ito, & Iwasaki, 2013). With respect to NodH, the actinobacterial sequences form a sister group of rhizobial NodH sequences, suggesting that a gene transfer occurred between rhizobia and *Frankia*, or that both groups acquired the gene from a common ancestor.

Phylogenetic analyses also indicate that the *Frankia* cluster II, in which the *nodABC* were first identified, is in basal position relative to other *Frankia* groups. This suggests that the ancestor of symbiotic *Frankia* contained the canonical *nod* genes, which were transferred to Proteobacteria and have been lost in some strains of *Frankia* clusters I and III. Within the Proteobacteria, the *nodABC* genes spread to various branches of the Rhizobiales (alpha-Proteobacteria) and Burkholderiales (beta-Proteobacteria) orders by horizontal gene transfer. The high frequency of *nod* gene horizontal transfer can be detected through comparisons of phylogenetic trees of *nod* genes with those of 16S rRNA or other non-symbiotic genes (Andrews et al., 2018). The frequent incongruence detected between housekeeping and symbiosis gene trees fits with the older observation that *nod* genes and genes involved in nitrogen fixation are located on plasmids or chromosome symbiotic islands that are transferable from strain to strain. Accordingly, a transfer of *nod* genes has been observed in a few cases (Sullivan, Patrick, Lowther, Scott, & Ronson, 1995; Nandasena, O'Hara, Tiwari, & Howieson, 2006; Nandasena, O'Hara, Tiwari, Sezmiş, & Howieson, 2007), and interestingly, such transfer of *nod* genes was recently found to be induced by plant flavonoids similar to those inducing *nod* gene expression (Ling et al., 2016). This induction of gene transfer was however not dependent on NodD but on a transcriptional regulator of the same family.

Horizontal transfer of symbiotic genes seems frequent within species or among related species (Andrews et al., 2018; Remigi, Zhu, Young, & Masson-Boivin, 2016; Rogel, Ormeno-Orrillo, & Martinez-Romero, 2011). It is often detected when analyzing rhizobia isolated from nodules of a given legume species: for example, rhizobia isolated from pea nodules generally belong to several different species related to *R. leguminosarum* and contain very similar *nod* genes acquired by horizontal transfer between these species, which are designated as the symbiovar *viciae*. The *nod* genes in different species of the symbiovar *viciae* can be more similar than those found in *R. leguminosarum* strains which nodulate other hosts like *Trifolium* or *Phaseolus*. Transfer of the *nod* genes from one symbiovar to another one in the same species can result in a change of host range. Transfer of *nod* genes between genera seems less frequent but does occur: the *Sesbania* symbiont *Agrobacterium* IRBG74 has *nod* genes very similar to those of *Sinorhizobium* strains nodulating the same legume, indicating transfer of *nod* genes between the *Sinorhizobium* and *Agrobacterium* genera. In contrast, the *Bradyrhizobium* and *Sinorhizobium* strains nodulating soybean carry divergent *nod* genes. There is also evidence of lateral transfer of *nod* genes from alpha-Proteobacteria to beta-Proteobacteria: the *nod* genes of *Burkholderia* species nodulating South African legumes seem related to those of *Mesorhizobium* nodulating the same legumes (Andrews et al., 2018).

A recent large scale analysis of Rhizobiales bacteria found in soil and root-associated microbiomes reinforced these evolutionary hypotheses (Garrido-Oter et al., 2018). Rhizobiales were found in a high relative abundance in soil, root, and rhizosphere samples indicating a good adaptation to the root environment. Nearly 1000 members of this order were isolated and had their genome sequenced. A minority of them (less than 0.3 % of bacteria isolated from roots) had *nod* genes and thus the potential to nodulate. Analysis of all available Rhizobiales genome sequences allowed a large scale phylogenetic reconstruction of ancestral characters. This confirmed that the most common ancestor of Rhizobiales likely did not have canonical *nod* genes, and that the ability to nodulate was acquired multiple times after the divergence of the major groups of Rhizobiales, probably through multiple horizontal gene transfers after acquisition from a non-Rhizobiales bacterium. By testing representative members of the different Rhizobiales groups isolated from roots, it was shown that the majority had a root growth promoting activity, indicating a plant commensal lifestyle that likely predated and perhaps favored the acquisition of *nod* genes and their biological success. Similar processes might have occurred in the Burkholderiales

order of the beta-Proteobacteria which contains nodulating rhizobia as well as plant growth promoting bacteria. Although there are a few unconfirmed reports of the presence of nodulating rhizobia outside these two groups (Martínez-Hidalgo & Hirsch, 2017), further analysis of the nodule and rhizophere microbiome of various legumes with genome and metagenome sequencing is required to get a more detailed understanding. This would also help to assess the spread of *nod* genes to other groups of bacteria abundant in the soil and in the rhizosphere, which might have been limited by a lesser pre-adaptation of these taxa to symbiosis, the difficulty of genetic transfer across large phylogenetic distances, and/or various environmental factors.

The analysis of the Rhizobiales genomes also showed that some clades were richer in nodulating strains (*Mesorhizobium*, *Bradyrhizobium*) than other ones (*Agrobacterium*), and that after an initial acquisition of *nod* genes in a lineage, some strains in that lineage could lose the ability to nodulate. Comparison of nodulating with non-nodulating bacteria did not provide evidence of whole-genome level signatures of adaptation (Garrido-Oter et al., 2018). However, nodulation and infection of legumes by rhizobia require many genes in addition to the *nodABC* genes, and these have been likely recruited from the genome of the *nod* gene acquiring strain. These genes can be ubiquitous or lineage-specific genes. For example, the *S. meliloti* genes involved in the synthesis of succinoglycan exopolysaccharides are required for plant infection, and are found in *Sinorhizobium* species but not in other rhizobial lineages (Tian et al., 2012). Similarly lineage-specific genes involved in the translocation of effectors through type 3 or type 4 secretion systems also play a role in modulating infection and host range. Accommodation of acquired symbiotic functions likely required adaptations in these genes together with the coordination between the expression of plasmid-located symbiotic genes and chromosome encoded genes necessary for successful symbiosis (Jiao et al., 2018; Remigi et al., 2016). Such modifications in the recipient genome after the acquisition of *nod* genes have been detected in experimental evolution experiments carried out on rhizobia (Capela et al., 2017; Remigi, Masson-Boivin, & Rocha, 2019).

3.2 A single origin for nodulation in plants?

Root nodule nitrogen fixing symbioses occur in several orders of Angiosperms that all also include non-nodulating plants. In addition, a polyphyletic group of bacteria in Proteobacteria and Actinobacteria induces a symbiotic process which exhibits a great diversity in the modes of infection,

types, and functioning of nodules. It was therefore important to assess whether common mechanisms controlled these different symbiotic processes, and in particular to establish the role of the NF signaling pathway. In *Parasponia*, a member of the *Cannabaceae* family nodulated by rhizobia, NFs were shown to induce calcium spiking, and an autoactive form of CCaMK was able to induce nodule formation on *Parasponia* roots in the absence of rhizobia (Granqvist et al., 2015; Op den Camp et al., 2011). This indicated that components of the NF signaling pathway play a role in rhizobial nodulation on hosts outside of the *Fabaceae* family.

In *Aeschynomene evenia,* a legume nodulated by photosynthetic *Bradyrhizobium* strains lacking the *nodABC* genes required for NF biosynthesis, RNAi inactivation of the closest homologs of *DMI2* and *DMI3* impaired nodule development, and expression of a deregulated form of *AeDMI3* led to nodule formation in the absence of rhizobia (Fabre et al., 2015). This showed that at least some components of the NF signaling pathway are required for this NF-independent nodulation. In contrast, orthologs of the *NFP* and *LYK3* genes were not consistently found in the *Aeschynomene* hosts of the *nod*-independent *Bradyrhizobium*, suggesting that they are likely to be dispensable for nodulation in these species. Screening and characterization of plant symbiotic mutants should help to understand how *A. evenia* recognizes its rhizobial symbiont (Arrighi et al., 2012).

In the case of the *Frankia*-actinorhizal plant symbiosis, genes in the NF signaling pathway, orthologous of *DMI2, DMI3*, and *NIN*, are required for nodule induction in *Casuarina* (Gherbi et al., 2008; Svistoonoff et al., 2013). The putative role of LysM-RLKs in actinorhizal plant symbioses with *Frankia* containing or not *nod* genes however remains to be assessed.

There is therefore strong evidence that, although each type of symbiosis presents specificities, common mechanisms are involved in the establishment of RNS in these different plant families. In addition, all nodulating plants belong to a monophyletic group of Angiosperms including the orders Fabales, Fagales, Cucurbitales, and Rosales, the so-called nitrogen-fixing clade (Soltis et al., 1995). This suggests a single origin of nodulation in an ancestor of this clade. To explain that members of this clade are not able to nodulate, one has to assume that, once acquired, the ability to nodulate was lost in some lineages. Indeed, the comparison of genomes from nodulating and non-nodulating plants in the nitrogen-fixing clade revealed that non-nodulating plants had lost genes such as *NIN* or *NFP/NFR5* that are required for RNS (Griesmann et al., 2018; van Veltzen et al., 2018; van Velzen, Doyle, & Geurts, 2018). A single origin of nodulation would also

imply that there are genes or regulatory elements specific to the nitrogen fixation clade and present in all nodulating species. Finding these would help to understand the origin of nodulation.

Early in the analysis of legume genetic determinants of nodulation, it was observed that legume mutants affected in the RNS were also defective for the establishment of the arbuscular endomycorrhizal symbiosis (AMS) (Duc, Trouvelot, Gianinazzi-Pearson, & Gianinazzi, 1989), and later on several components of the NF signaling pathway were found to also control the AMS, constituting the so-called common symbiotic signaling pathway (see above). Since the AMS is much more ancient than the RNS (450 million years vs. 80 million years), the hypothesis was that the RNS had coopted key components of the AMS (Parniske, 2008). Mutants in genes controlling NF perception (MtNFP/LYK3 and LjNFR5/NFR1) were not originally described as altered in AMS, raising doubts about the role of chitin-like molecules in AMS establishment. However Myc-LCOs, with a structure similar to that of NFs (Maillet et al., 2011), as well as short-chain chitin oligomers (Myc-COs), were identified in AM fungi spore exudates and found to induce calcium spiking in roots of *M. truncatula* as well as other legume and non-legumes plants (Genre et al., 2013; Sun et al., 2015). The implication of these chitin-like molecules reinforces that LysM-RLK proteins are involved in their perception and thus in mycorrhization. Indeed, silencing of NFP/NFR5 homologs in tomato led to a strong reduction in mycorrhization (Buendia, Wang, Girardin, & Lefebvre, 2016). In legumes, inactivation of the *MtLYK3* gene decreases mycorrhization (Zhang et al., 2015). In addition, two LysM-RLKs, homologous to the Arabidopsis chitin receptors AtCERK1 and AtLYK5, MtLYK9 and MtLYR4, have been identified as candidate CO receptors (Bozsoki et al., 2017). However, the precise role of LCOs and COs in the AM symbiosis remains poorly understood. No gene similar to the canonical *nodABC* genes has been yet identified in AM fungi sequenced genomes. The biosynthetic pathways leading to LCO and CO production in AMF are therefore not known, although a number of chitin synthases involved in fungal cell wall synthesis could be involved. Recent work indicates that LCOs and LCO signaling pathways also play a role in the establishment of some ectomycorrhizal symbioses (Cope et al., 2019). Moreover, COs and LCOs seem to be produced by a large range of fungi, symbiotic or not (Plett, 2018). A diverse range of organisms carry genes likely to control the synthesis of COs, which could thus have a wide role as signaling molecules in various biological processes beyond symbiosis (Dénarié et al., 1996). In particular, recognition of

chitin-like molecules plays an important role in plant-pathogen interactions. Understanding how plants distinguish CO and LCO producing rhizobia, AMF and pathogenic fungi to induce appropriate responses is a challenge for future research (Zipfel & Oldroyd, 2017).

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