Early Signaling in the Establishment of Beneficial Plant-Microbe Symbioses

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ABSTRACT

Due to the technologies of the Green Revolution, yields of rice, corn and wheat have tripled over the second half of the twentieth century. Much of that yield increase was due to the availability of synthetic nitrogen fertilizer, however, their widespread use is no longer economically or environmentally sustainable. Beneficial plant-microbe symbioses can reduce the need for fertilizer. Rhizobia are bacteria that provide fixed atmospheric nitrogen to their legume hosts through root nodules. Arbuscular mycorrhizal fungi can colonize the roots of ~80% of land plants and improve plant nutrient acquisition. The work presented here sought to improve understanding of these beneficial associations so that they might be better used in agriculture as an alternative to fertilizer.

The formation of these symbioses is facilitated by the production of lipo-chito-oligosaccharide molecules called Nod factors in rhizobia and Myc factors in arbuscular mycorrhizal fungi, as well as the production of chitin oligomers by arbuscular mycorrhizal fungi. Perception of Nod factors by the host plant root requires nuclear ion channels and specific nucleoporin proteins. Nucleoporins are part of the nuclear pore complex, the gateway into and out of the nucleus. Using transmission electron microscopy and immuno-localization, I found that these nucleoporins are required for proper localization of the symbiotic nuclear ion channels.

These results suggest a critical role for nuclear trafficking in the establishment of symbiosis with rhizobia and mycorrhizal fungi.

Due to the activity of rhizobia, legume crops such as soybean and peas do not require application of nitrogen fertilizer. While cereals cannot form nitrogen fixing associations with rhizobia, their interaction with mycorrhizal fungi suggests that they may be able to perceive and respond to Nod factors. Some rhizobia, such as *Rhizobium* sp. IRBG74, are capable of colonizing cereal crops. By inoculating plants with *Rhizobium* sp. IRBG74 containing mutations in genes associated with Nod factor production, we revealed that chitin oligomers are important for proper root surface attachment and internal root colonization in a host dependent manner. Combined with experiments in rice which demonstrated that symbiotic processes and lateral root growth are stimulated by chitin oligomers, Myc factors, and *Rhizobium* sp. IRBG74 Nod factors, this suggests that these signaling molecules facilitate symbiotic interactions in cereals.

Manipulation of mycorrhizal and rhizobial interactions in cereals for enhanced nutrient uptake will require knowledge of the genetic pathways involved in perception of these microbes by the host plant. Using transcriptomic analysis of rice treated with arbuscular mycorrhizal spore exudates, chitin oligomers, Myc factors, and *Rhizobium* sp. IRBG74 Nod factors, I found that genetic regulation induced by these signals is distinct. *Rhizobium* sp. IRBG74 Nod factors were most similar to arbuscular mycorrhizal spore exudates in gene expression responses, suggesting that this rhizobium is capable of co-opting the mycorrhizal signaling pathway. Taken together, these studies clarify the relative role that Nod and Myc factors, as well as chitin oligomers, play in mediating associations of plants with rhizobia and mycorrhizal fungi, and reveal the specific function of nucleoporin proteins in Nod factor perception.

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CHAPTER 1: INTRODUCTION

Nitrogen is an essential nutrient for life and a limiting factor for producing high yields in both commercial and subsistence agriculture. Unfortunately, its extensive use has proven detrimental to the economic stability and environmental sustainability of food and biofuel production. Nitrogen fertilizers are produced using the Haber-Bosch process, which annually uses 1% of the world's non-renewable energy (1). Additionally, their overuse results in the pollution of marine and freshwater ecosystems due to runoff, and contributes to global warming via the production of nitrous oxide, a potent greenhouse gas (2). To alleviate their dependency on nitrogen fertilizer, farmers can maintain soil fertility by practicing crop rotation with legumes such as soybean and peas (3). This is due to the activity of nitrogen-fixing bacteria referred to as rhizobia. Legume species interact with a specific set of rhizobia, which are housed in root-derived structures called nodules. There the bacteria fix atmospheric nitrogen in return for plant host supplied carbon.

Legume nodulation is established via a molecular dialog that has been well characterized in two model legumes, *Lotus japonicus* (Lotus) and *Medicago truncatula* (Medicago) (4-6). When rhizobia detect the presence of flavonoids and isoflavonoids released by the host, expression of bacterial *nodulation (nod)* genes is induced and lipo-chito-oligosaccharidic (LCO) molecules known as Nod factors are released (**Fig 1**) (7-9). The LCO structure is composed of a chitin backbone formed by three, four or five β-1, 4 linked N-acetyl-glucosamine residues that are N-acylated at the non-reducing terminal residue by a fatty acid (10). This basic structure is frequently decorated with sugars (e.g. fucose, mannose) or other modifications such as sulfate groups along the chitin backbone (**Fig 2**). Each species of rhizobia produces a unique mixture of LCO structures and this is part of what gives specificity to the legume-rhizobia symbiosis. The legume host preferentially responds to certain LCO structures even at very low concentrations (10^{-9} to 10^{-12} M) (10), resulting in nodule development and colonization of the host by the rhizobia.

The legume detects the presence of Nod factors via plasma membrane receptor-like kinases (RLKs) containing LysM chitin recognition extracellular domains. Two LysM-domain-containing LRKs known to perceive Nod factors are NFP and LYK3 in Medicago and NFR1 and NFR5 in Lotus (11). Another plasma membrane protein, a leucine-rich repeat receptor kinase (DMI2 in Medicago and SymRK in Lotus), is involved in downstream signal transduction most likely responsible for transmitting the signal from the plasma membrane to the nucleus (**Fig 3**) (12-14). When the signal reaches the nucleus, root cells undergo perinuclear "calcium spiking" (**Fig 3**). This refers to the oscillation of calcium concentration both within and around the nucleus (15, 16). Nuclear envelope-localized ion channels, DMI1 in Medicago, and CASTOR and POLLUX in Lotus, are thought to act as counter ion channels during this response, initiating and maintaining the calcium spiking via hyperpolarization of the nuclear membrane (4, 15, 17-20). A nuclear envelope-localized calcium pump, MCA8, has been identified in Medicago and is thought to move calcium back to the inter-membrane space during spiking (21).

Calcium spikes are perceived by a calcium and calmodulin-dependent protein kinase, DMI3 in Medicago and CCaMK in Lotus, which then interacts with IPD3/CYCLOPS to induce transcription of early nodulin (*ENOD*) genes (22). Perception of Nod factors is followed by the root hair curling around the rhizobia, and formation of a tubular structure derived from the plant plasma membrane called the infection thread, whereby the bacteria enter the cell (23). *ENOD* and transcription of other late symbiotic genes allows the symbiosis to proceed to nodule organogenesis and finally nitrogen fixation.

The plant nucleus regulates symbiosis

Nucleoporins, NUP133, NUP85 and NENA, are required for calcium spiking and nodulation in Lotus but their precise role is not understood (**Fig 3**) (24-26). The nuclear pore complex (NPC) is a large multi-protein assembly that spans the nuclear envelope and facilitates the trafficking of macromolecules between the cytoplasm and the nucleus (27). The general structure of the NPC consists of a nucleoplasmic basket, cytoplasmic filaments and a core scaffold within the nuclear envelope that connects the nucleoplasmic and cytoplasmic elements, and also forms the central channel (**Fig 4**) (28). The NPC is made up of approximately 30 different individual nucleoporins or NUPs which can be divided into functional groups: phenylalanine-glycine (FG), linker, inner ring and outer ring (29). The inner and outer ring NUPs form the core scaffold of the NPC (**Fig 4**). LjNUP133, LjNUP85 and LjNENA are members of the NUP107-160 sub-complex which forms the NPC outer ring of the core scaffold (27, 30).

The central channel of the NPC does not inhibit movement of small molecules between the cytoplasm and nucleus but transport of larger molecules over 40kDa in size is typically regulated (31). Proteins to be transported into the nucleus or to the inner nuclear membrane typically contain a Nuclear Localization Signal (NLS) which is a non-sequence specific, short stretch of basic amino acids (31). The NLS allows the protein to be recognized by transport molecules called karyopherins, also referred to as Importins (**Fig 4**) (32). The nuclear import cycle of soluble proteins is well characterized in yeast and mammalian cells, and is known to be primarily regulated by two karyopherins, Importin α and β.

Transport to the nucleus is initiated when the NLS signal on the cargo is recognized and bound by Importin α which is then in turn bound by Importin β. The cargo/Importin α/Importin

β complex then traverses the NPC into the nucleus while interacting with NUPs (**Fig 4**). Once the transport complex reaches the nucleus, binding of RanGTP to Importin β causes it to disassociate from Importin α, triggering the release of the cargo (**Fig 4**) (31). A similar transport model for the movement of integral membrane proteins from the Outer Nuclear Membrane (ONM) to the Inner Nuclear Membrane (INM) has recently been elucidated in yeast via domain deletion and localization studies of the Heh2 INM protein (**Fig 5A**) (33). Transport of Heh2 requires an unstructured "linker" region of a certain length between a soluble domain containing an NLS sequence and the trans-membrane region of the protein. The work with Heh2 demonstrated that transport to the INM is similar to the transport of soluble proteins to the nucleoplasm, in that Importins α and β bind the NLS sequence and guide this region through the central channel (**Fig 5A**) (34). Unique to nuclear import of membrane-bound proteins is this unstructured linker region that stretches across the scaffold of the NPC, allowing the trans-membrane portion to stay within the nuclear membrane.

As the scaffold of the NPC may play an important role in the transport of membrane proteins to the INM and ions such as calcium should travel freely through the central channel, the symbiotic phenotype observed in LjNUP85, LjNUP133 and LjNENA may be due to the mis-localization of nuclear proteins required for Nod factor-induced calcium spiking. Considering NPC structure is highly conserved, one would expect NUPs to regulate symbiosis in other legumes as well. DMI1 and MCA8 have both been localized to the INM in Medicago and both contain an NLS sequence, but when mutated only *dmi1* has a striking nodulation defect similar to that observed in the Lotus *nup* mutants (**Fig 5B & Fig 6**) (21). While CASTOR and POLLUX have not been precisely localized in this way, they are homologs to DMI1. Thus, CASTOR and POLLUX are good candidates for mis-localization in the Lotus *nup* mutant background.

Symbiotic signaling in cereals

Clearly, much has been learned about the formation of rhizobia-legume interactions. Nodulation is primarily restricted to legumes, however, and the most important crops in terms of caloric intake are cereals such as rice and corn (35). If we are to address the problem posed by overuse of nitrogen fertilizers, we must consider nitrogen use in the context of non-legumes. One alternative could be to increase nitrogen use efficiency in cereals but there are many variables that control this trait and measurement of nitrogen use efficiency is not always straightforward (36). Another approach would be to improve nitrogen-fixing associations in cereals, which may require engineering of both the host plant and the symbiotic microbe to allow for internal colonization and efficient transfer of fixed nitrogen. Research on another root endosymbiosis, arbuscular mycorrhization (AM), has demonstrated that this second option might be much simpler than originally believed.

AM is present in approximately 80% of land plants, including agronomically important cereals (37). During this symbiosis, Glomeromycete fungi colonize the root cortex cells where they develop a specialized branched hyphal structure called the arbuscule, and use this interface to transfer soil nutrients to the plant in exchange for carbon. Investigation into AM in Lotus and Medicago has revealed that many of the early signaling mechanisms required for legume nodulation are also involved in mycorrhization, including DMI2/SYMRK, CASTOR, POLLUX/DMI1, NUP133, NUP85, NENA, DMI3/CCaMK and IPD3/CYCLOPS (38). While the Myc factor receptor has not yet been identified in Lotus and Medicago, it is likely also a LysM-domain RLK as the sole non-legume to form nodules with rhizobia, *Parasponia andersonii*, requires the NFP homolog for both nodulation and mycorrhization (39). Due to the requirement for these proteins in both AM and legume nodulation, they are referred to as the "Common Symbiosis Pathway" (CSP) (**Fig 3**) (40). Another striking similarity of AM to legume nodulation

is that AM fungi also produce LCO signals (Myc factors) as part of AM spore exudates and that these "Myc-LCOs" trigger calcium spiking in Medicago (41, 42).

In addition to LCOs, AM fungi also produce short chain chitin oligomers (COs), which trigger calcium spiking in excised Medicago roots grown in media as root organ cultures (43). Thus it appears that AM fungi use both LCOs and COs as symbiotic signaling molecules. Before our recent publication (44), however, the role of LCOs and COs during AM symbiosis in rice was unknown.

Applying knowledge of the CSP to engineering N fixing cereals

Several species of rhizobia have been described as colonizing cereal roots including *Azorhizobium caulinodans*, *Rhizobium leguminosarum*, *Rhizobium etli*, and *Mesorhizobium mediterraneum* (45-50). While some of these interactions result in the enhanced growth of the host, there is no reliable evidence for transfer of fixed nitrogen to the host plant (45, 47, 49). In a single experiment using measurement of ^{15}N dilution as a means to assess fixation, Van Nieuwenhove et al. (2001) claimed to have demonstrated transfer of fixed nitrogen from *A. caulinodans* to the host. These experiments were not done under sterile conditions, however, and so this claim remains dubious (51). The likely reason why N fixation in cereals remains elusive is that the nitrogenase enzyme is extremely sensitive to oxygen and the reaction is energy intensive (52, 53). Unlike the specialized environment of the nodule, the conditions in the root rhizosphere would therefore likely not support the levels of N fixation that would make a significant difference in the nitrogen metabolism of cereal crops. Finally, not only must fixation take place but efficient transfer to the plant host must also occur.

The nodule provides a habitat for rhizobia which is suitable for nitrogen fixation. It establishes an efficient means of supplying energy in the form of host-fixed carbon, and plant leghemoglobin binds oxygen and thus protects the nitrogenase enzyme from oxygen induced degradation (52, 54). It also establishes an efficient mechanism of transferring fixed nitrogen to the host plant. Initiation of nodule organogenesis requires components of the CSP, in addition to components involved in regulation of lateral root development, and these genes are already present in cereal hosts due to the widespread occurrence of AM symbiosis (55, 56). Therefore, a more thorough understanding of cereal-rhizobia interactions might help identify missing signaling components that are required for nodule organogenesis but not mycorrhization and are thus good candidates for engineering efficient nitrogen fixing rhizobial interactions into cereals. Detailed analysis of the colonization of cereals by rhizobia has only really been conducted on the interaction of *Azorhizobium caulinodans* with rice and wheat, where it was found that colonization occurs via crack entry at the lateral root junction, followed by colonization of the epidermal and cortical cells of the root. Furthermore, this interaction is not dependent upon Nod factor production establishing that *A. caulinodans* does not require the CSP to colonize rice (45, 57). We cannot rule out the fact that the CSP may be involved in the interactions of cereals with other rhizobia, however.

Azorhizobium caulinodans forms the classical legume-rhizobium symbiosis with the tropical legume *Sesbania rostrata* (58). *Rhizobium* sp. IRBG74 also forms nodules on species of *Sesbania* and like *A. caulinodans*, can also colonize rice (59). The inoculation of rice with *Rhizobium* sp. IRBG74 enhances rice growth; however, this is likely the result of growth stimulation through plant hormone production and not nitrogen fixation (59). We have shown that *Rhizobium* sp. IRBG74 colonization of rice is dependent upon the CSP, as colonization is reduced in *pollux* and *castor* mutants (**Fig 7**). These results suggest that unlike *A. caulinodans*, *Rhizobium*

sp. IRBG74 uses Nod factors and the CSP to facilitate its colonization of cereal host. While *Rhizobium* sp. IRBG74 does not fix nitrogen on cereal hosts as it does in the nodule environment; it seems possible to engineer this capability. The first step in this process is to understand how *Rhizobium* sp. IRBG74 colonizes cereals and which plant and bacterial genes are involved.

RESEARCH OBJECTIVES

Despite what has been learned, further studies into the signaling pathway are required to completely understand how the LCO signals are perceived and symbiosis established in both legumes and cereal crops. This research sought to advance basic knowledge of symbiosis as well as develop tools to improve nitrogen-fixing associations in cereals. My first research objective is to characterize the role of the NUP85, NUP133 and NENA in symbiosis. This analysis will also advance our understanding of the nuclear transport and the function of specific NUPs in plants. My next objective is to identify whether LCOs, COs, or both, function as signaling molecules during arbuscular mycorrhization in rice. My final objective is to characterize the colonization of rice and the C4 model plant *Setaria viridis* by *Rhizobium* sp. IRBG74 and understand the role of the Nod factors in these interactions.

The following specific objectives were chosen for my doctoral thesis research:

1. Obtain and test symbiotic phenotypes in Medicago *nup* mutants.

2. Perform precise localization of CASTOR and POLLUX proteins at the nuclear membrane.

3. Assess the role of NUP85, NUP133 and NENA in trafficking proteins required for Nod factor-induced calcium spiking.

4. Investigate perception of mycorrhizal LCOs and COs in rice.

5. Characterize the colonization of rice and *Setaria viridis* by *Rhizobium* sp. IRBG74.

6. Investigate the role of Nod factors in the cereal-*Rhizobium* sp. IRBG74 interaction in comparison to legume hosts

Each of the three chapters of this dissertation represent either accepted, in progress or planned publications. Chapter 2 has been prepared for submission to Plant Cell, Chapter 3 contains material that has been published in Plant Cell as part of a collaboration, and Chapter 4 constitutes at least one planned publication.

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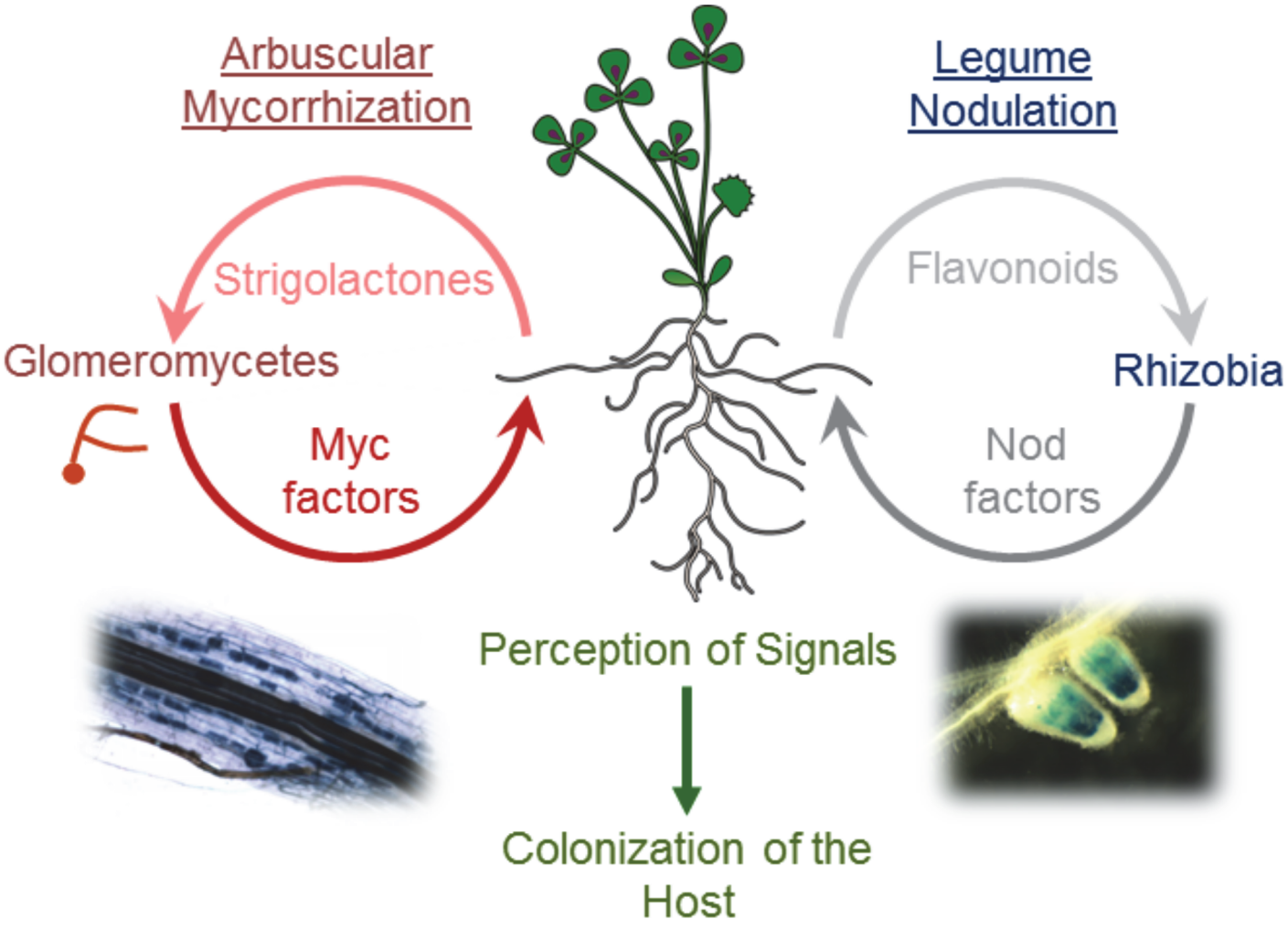

Arbuscular Mycorrhization
Legume Nodulation
Strigolactones
Flavonoids
Glomeromycetes
Rhizobia
Myc factors
Nod factors
Perception of Signals
Colonization of the Host

Figure 1. Plant-microbe communication during arbuscular mycorrhization and legume nodulation

Plant roots secrete strigolactones and flavonoids which are perceived by AM fungi and rhizobia respectively. This triggers production of Myc and Nod factors. These signals communicate to the host the presence of the symbiont and allows the symbiosis to proceed. Picture on the lower left is a root colonized by arbuscular mycorrhizal fungi. Image on the lower right is a root nodule colonized by rhizobia expressing a *nifH*:GUS fusion which turns nitrogen fixing rhizobia blue in the presence of the stain.

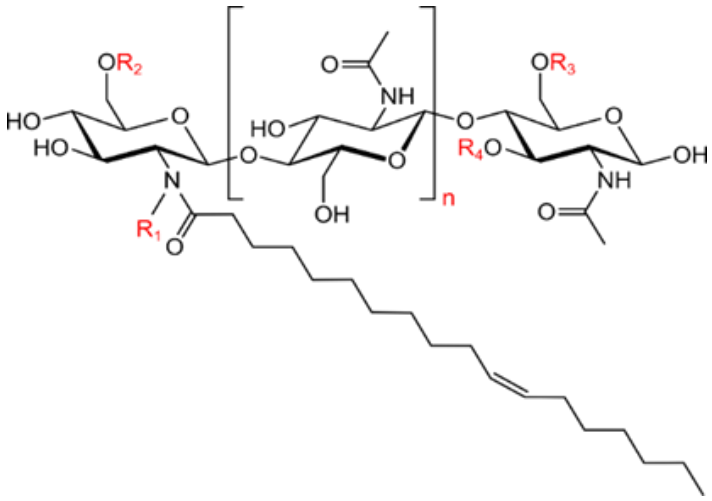

OR2
HO
HO
O
N
R1
O
O
O
HO
O
NH
O
OH
n
OR3
O
R4O
NH
O
OH

Figure 2. General lipo-chito-oligossacharide structure

Nod and Myc factors contain a chitin backbone typically 4-5 residues in length (n = 1-2). The lipid chain varies in degrees of unsaturation and length. R groups indicate positions of resides where substitutions vary based on species. Examples include methyl, arabinosyl, fucosyl, carbamoyl etc. as well as hydrogen when no substitution is present.

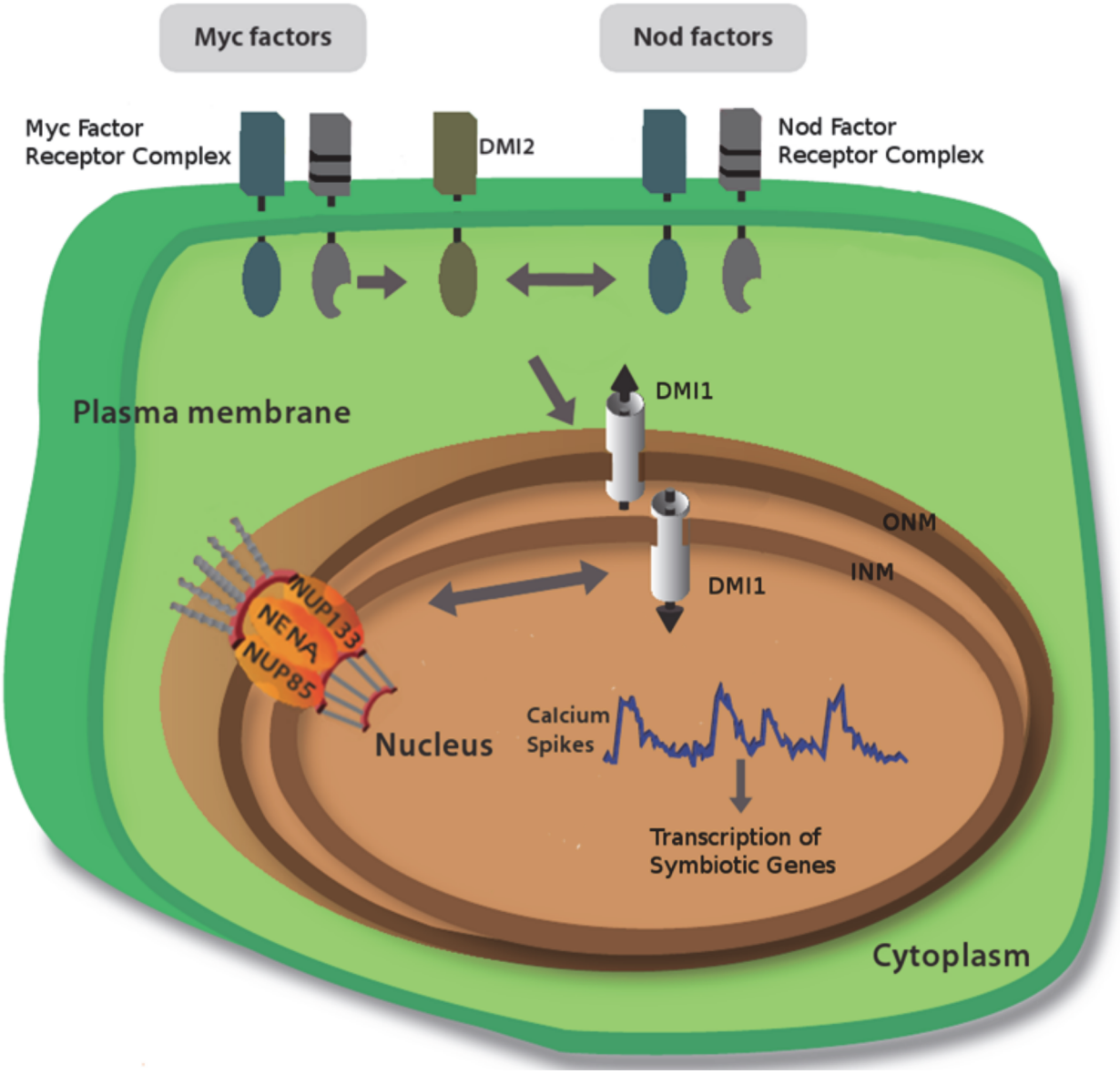

Myc factors
Nod factors
Myc Factor
Receptor Complex
DMI2
Nod Factor
Receptor Complex
Plasma membrane
DMI1
ONM
INM
DMI1
NUP133
NENA
NUP85
Nucleus
Calcium
Spikes
Transcription of
Symbiotic Genes
Cytoplasm

Figure 3. Model for the LCO signaling pathway in *M. truncatula*

Nod and Myc factors are perceived at the plasma membrane and activate a signaling cascade including MtDMI2 and an unknown secondary messenger. Putative nucleoporins at the nuclear envelope MtNUP133, MtNUP85 and MtNENA and counter ion channel MtDMI1 are required for calcium spiking. Calcium spiking results in early nodulin (*ENOD*) gene expression. Adapted from Venkateshwaran and Ané (15).

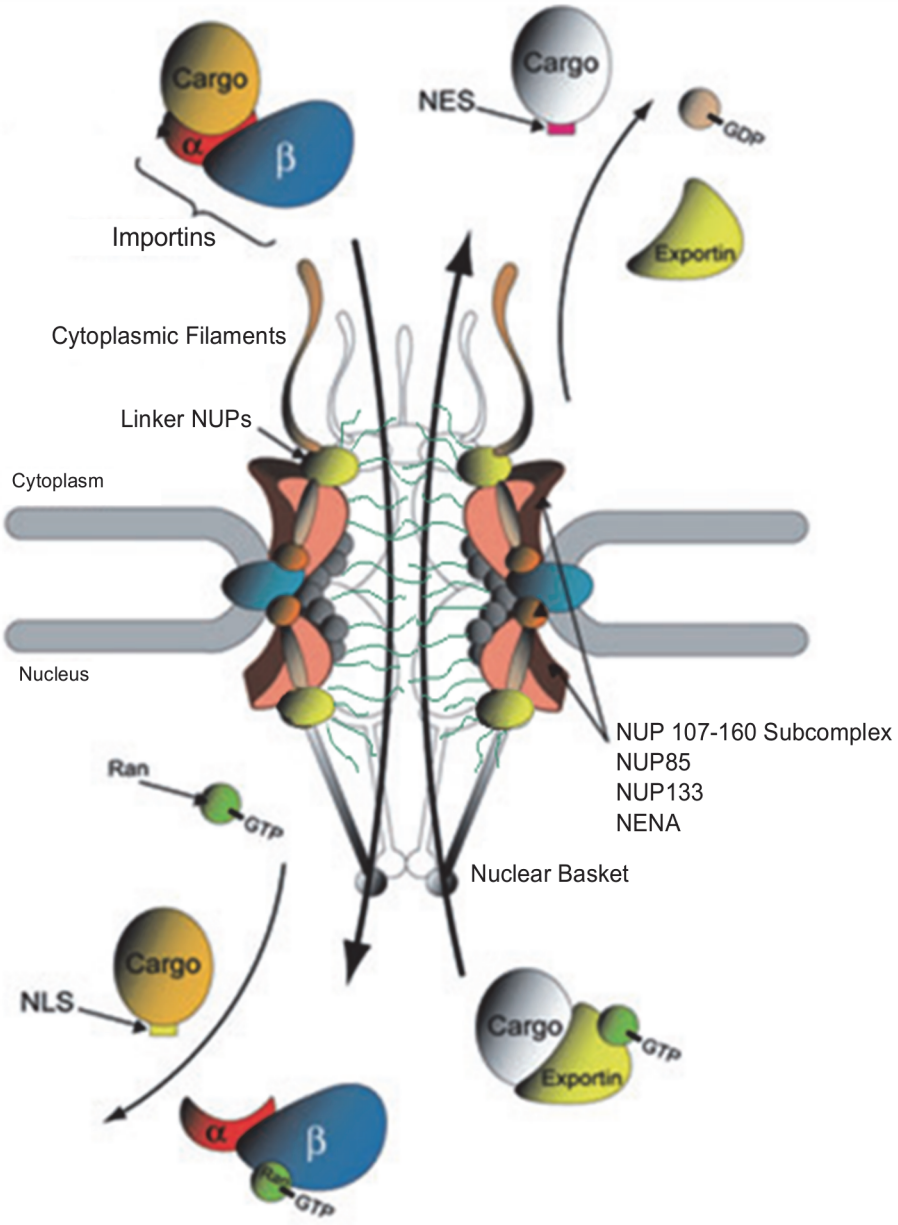
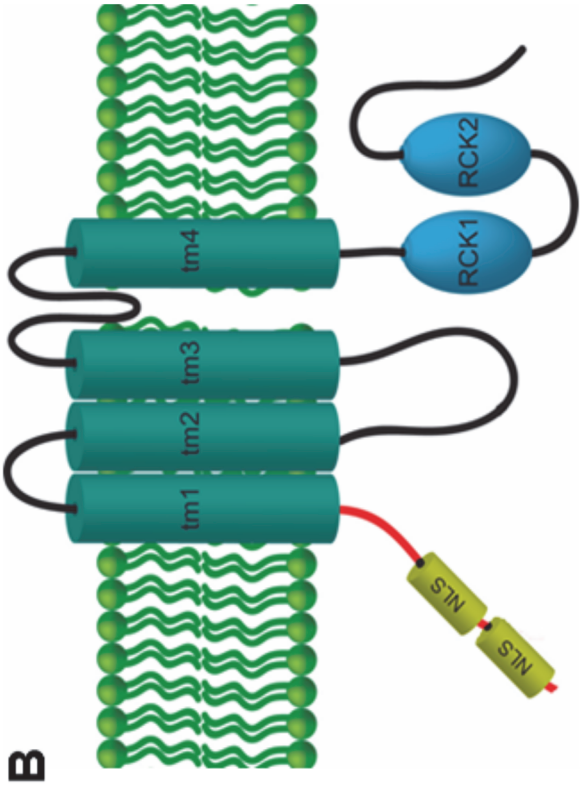

Cargo
α
β
Importins
Cytoplasmic Filaments
Linker NUPs
Cytoplasm
Nucleus
NES
Cargo
GDP
Exportin
NUP 107-160 Subcomplex
NUP85
NUP133
NENA
Nuclear Basket
Ran
GTP
NLS
Cargo
Cargo
Exportin
GTP
α
β
GTP

Figure 4. Nucleo-cytoplasmic transport of soluble proteins

Importin α and β bind proteins containing NLS sequences and shuttle them into the nucleus. Binding of Ran-GTP to Importin β causes dissociation of the import complex from the nucleoplasmic protein. Likewise, GTP bound Exportin proteins ferry cargo marked by an NES sequence to the cytoplasm where GTP hydrolysis releases the cargo from the export machinery. The NUP107-160 complex containing LjNUP133, LjNUP85 and LjNENA is part of the core scaffold of the NPC. Transport cargo interacts with FG NUPs (green) which span the central channel, and extend into both the cytoplasm and the nucleus. Linker nucleoprins connect FG NUPs to the core scaffold. (Adapted from Wiermer *et al.* 2007)


B
tm1
tm2
tm3
tm4
RCK1
RCK2
NLS
NLS

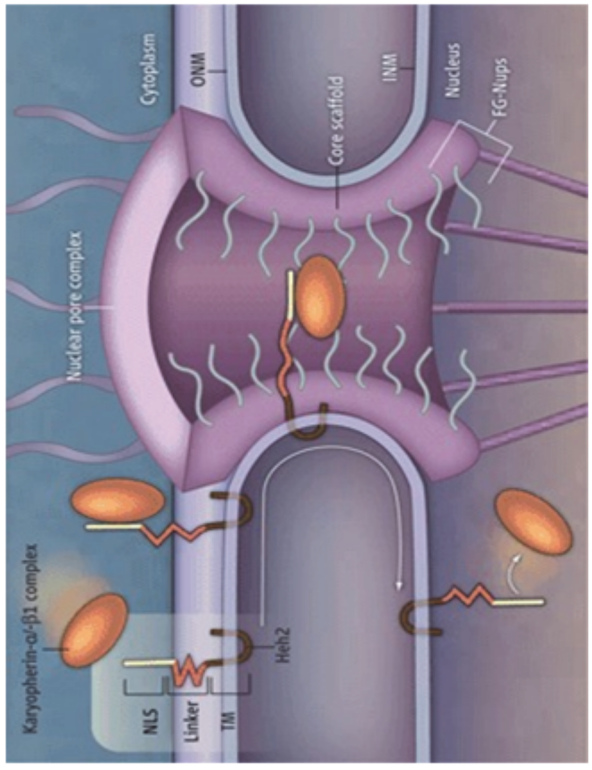

A
Cytoplasm
ONM
Nuclear pore complex
Core scaffold
INM
Nucleus
FG-Nups
Karyopherin-α/-β1 complex
NLS
Linker
TM
Heh2

Figure 5. Structural requirements for nuclear import of a membrane proteins

(A) Importin α and β binds the NLS (white) labeled protein. The soluble portion travels through the NPC connected to the trans-membrane portion by an unstructured linker region (pink) (Strauch, Science, 2011) (B) Structure of the DMI1, POLLUX and CASTOR proteins with predicted NLS (yellow) in the N-terminus (red). RCK (blue) domains are predicted calcium binding sites (Jean-Michel Ané).

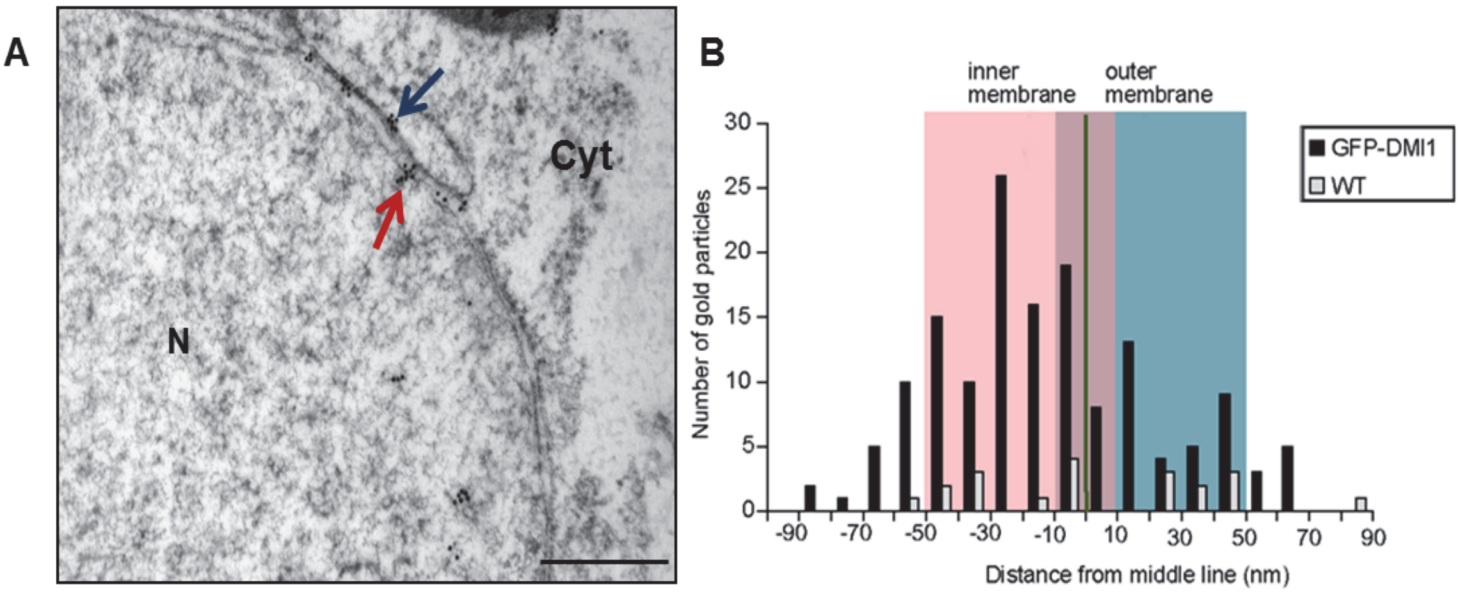

A
Cyt
N
B
inner membrane
outer membrane
GFP-DMI1
WT
Number of gold particles
0
5
10
15
20
25
30
-90
-70
-50
-30
-10
10
30
50
70
90
Distance from middle line (nm)

Figure 6. Sub-cellular localization of DMI1 on the nuclear envelope

(A) Immuno-gold labeling of 35S:DMI1:GFP-expressing transgenic root cells using anti-GFP antibody. Signals were detected on both INM and ONM. N = nucleus; Cyt = cytoplasm (Scale bar = 500 nm). (B) Quantitative analysis of the relative abundance of DMI1 in INM and ONM. DMI1 preferentially localizes to the INM. Adapted from Capoen *et al*. 2011.

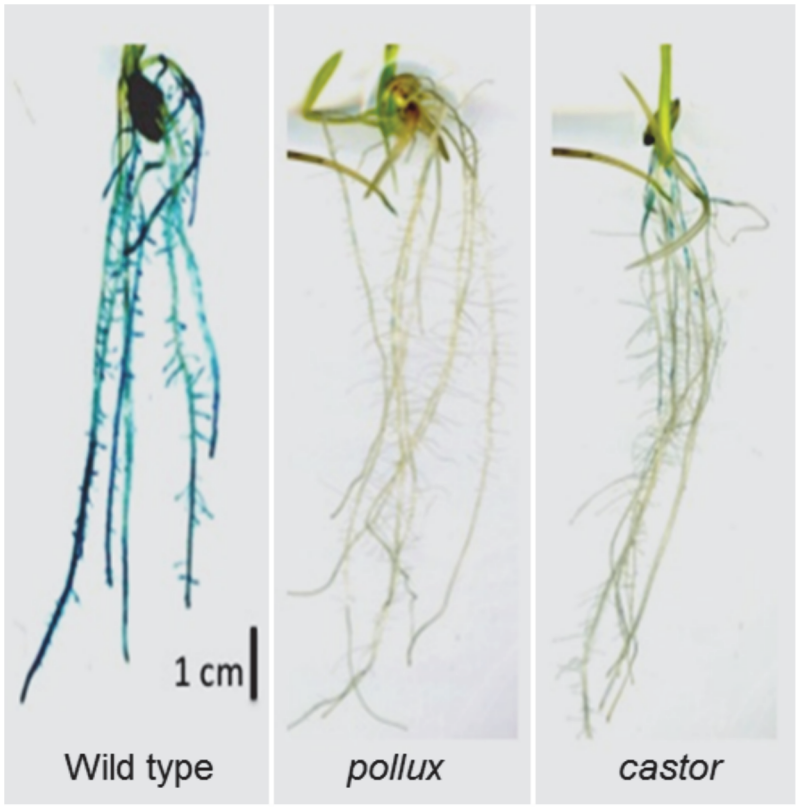

1 cm
Wild type
pollux
castor

Figure 7. CSP dependent colonization of rice by *Rhizobium* sp. IRBG74

Rice inoculated with *Rhizobium* sp. IRBG74 expressing a GUS marker. Blue coloration indicates *Rhizobium* sp. IRBG74 colonization of wild-type rice compared to rice containing knock out mutations in *pollux* or *castor*. Data provided by Dr. Arijit Mukherjee

CHAPTER 2: NUP107-160 NUCLEAR PORE SUB-COMPLEX MEMBERS ARE REQUIRED FOR THE PROPER LOCALIZATION OF SYMBIOTIC ION CHANNELS IN *LOTUS JAPONICUS* AND *MEDICAGO TRUNCATULA*

This chapter is a version of the following proposed manuscript:

Audrey Wiley-Kalil, Andreas Binder, Lu Han, Weiqiang Li, Muthusubramanian Venkateshwaran, Junko Maeda, Pierre-Marc Delaux, Kirankumar S. Mysore, Jiangqi Wen, Haruko Imaizumi-Anraku, Martin Parniske, Marisa S. Otegui and Jean-Michel Ané. NUP107-160 nuclear pore sub-complex members are required for the proper localization of symbiotic ion channels in *Lotus japonicus* and *Medicago truncatula.*

Contribution: A.W-K. wrote the manuscript, designed figures, and conducted the localization work and most of the *Mtnup133* work. *Mtnup133* calcium spiking analysis was done by M.V. Quantitative RT-PCR in *Mtnup133* was done by J.M. *NUP133* phylogenetic analysis was done by P.M. LORE1 data was provided by A.B. and rescue analysis was completed by A.B., L.H. and W.L.

ABSTRACT

Restriction of the movement of proteins into and out of the nucleus by the nuclear pore complex allows the cell to regulate gene expression and thus how an organism will react to changes in its environment. Components of the NPC, such as the NUP107-160 sub-complex, are known to be critical for the ability of plants to regulate plant-microbe interactions but the means of regulation was not fully understood. A reverse genetics approach revealed that *NUP107* is required for legume nodulation, solidifying the role of this sub-complex during symbiosis. Furthermore, the requirement for *NUP133* during symbiosis is conserved in Medicago. Nuclear ion channels *DMI1* and *POLLUX* are mis-localized in NUP107-160 sub-complex mutants and complementation experiments suggest that the proper localization of *POLLUX* to the inner nuclear membrane is essential for Nod factor induced calcium spiking. Unstructured "linker" regions at the N-terminus of *POLLUX* and *DMI1*, along with nuclear localization signal domains, indicate the involvement of the classical import machinery in the localization of these proteins to the inner nuclear membrane.

INTRODUCTION

The evolution of the nucleus as a separate compartment of the cell marked a critical transition in the history of life on Earth. The Nuclear Pore Complex (NPC) is a large multi-protein assembly that spans the nuclear envelope and facilitates the trafficking of macromolecules between the cytoplasm and the nucleus (1). The general structure of the NPC is highly conserved among eukaryotes. It consists of a nucleoplasmic basket, cytoplasmic filaments and a core scaffold within the nuclear envelope that connects the nucleoplasmic and cytoplasmic elements, and also forms the central channel (2). The NPC is made up of approximately 30 unique proteins referred to as nucleoporins or NUPs (3). Studies on NUPs have identified several functional groups: phenylalanine-glycine (FG), linker, inner ring and outer ring NUPs (4). The inner and outer rings form the core scaffold of the NPC; the inner rings consisting of multiple copies of the NUP107-160 sub-complex. This sub-complex contains eight NUPs in *Arabidopsis thaliana*; NUP43, NUP85, NUP96, NUP107, NUP133, NUP160, SEH, and SEC13 (5).

The vast majority of research on the NPC, nucleocytoplasmic transport and NUPs has been conducted in yeast and mammalian cells, and while the NPC structure appears to be well-conserved across eukaryotes, relatively little is known about plant NPCs. Reverse genetics in *Arabidopsis thaliana* has identified *nup* mutants that, as one would expect, are deficient in mRNA export, protein trafficking, and exhibit growth defects (6-11). Contrary to this, NUPs of the NUP107-160 sub-complex have been shown to have a function in plant-microbe symbiosis and plant defense against bacterial pathogens (12-15). In the model legume *Lotus japonicus* (Lotus), *nup85, nup133* and *nena (seh1)* mutants exhibit specific, temperature-dependent defects in root symbiosis. While there was a reduction in seed yield in *Ljnup133* and *Ljnup85*, no other growth defects were reported. Interestingly, all three mutants are also deficient in nuclear calcium

spiking, that is, oscillations of calcium concentration in the nucleus that occur shortly after host perception of rhizobia and arbuscular mycorrhizal fungi. This phenotype indicates a defect in the molecular processes occurring between perception of the symbiont and calcium spiking, suggesting a specific role of the NUP107-160 sub-complex in facilitating symbiotic signal transduction. One might thus expect that other NUPs in this sub-complex may similarly be involved in symbiotic interactions.

Rhizobium-legume and arbuscular mycorrhizal symbioses are initiated when lipo-chito-oligosaccharide molecules produced by the microbe, called Nod or Myc factors respectively, are perceived by the plant host by the symbiosis signaling pathway. In Lotus, recognition of rhizobial Nod factors and early symbiotic responses are dependent on the lysin motif (LysM) domain receptors NFR1 and NFR5 (16, 17). Symbiotic signal transduction further depends on a plasma membrane localized leucine-rich repeat receptor kinase (SYMRK), which interacts with NFR5 upon cleavage of its ectodomain (18-21). A secondary messenger is thought to transduce the signal from the epidermis to the nucleus where calcium spiking induces Nod- and Myc-factor dependent gene expression (22, 23). Nuclear envelope-localized ion channels, CASTOR and POLLUX in Lotus and DMI1 in the model legume *Medicago truncatula* (Medicago), are required for calcium spiking to occur (22, 24-29). They are thought to act as counter-ion channels transmitting cations to the perinuclear space during calcium spiking (30). POLLUX and DMI1 are orthologs but differ in the filter region which determines ion selectivity and conductance of the channel (31). Previously we demonstrated that the serine to alanine substitution at the third amino acid position in the filter region which occurs in DMI1 but not POLLUX, allows the DMI1 channel to stay open longer. Work in HEK-293 cells has shown that the presence of DMI1 is sufficient to allow for calcium spiking, most likely using endogenous calcium channels and pumps (31). While

in Lotus, POLLUX and CASTOR are unique ion channels which are both required for symbiosis, in Medicago, CASTOR is completely dispensable (31).

The classical model for transport of proteins to the nucleus is initiated by the recognition of a Nuclear Localization Signal (NLS), a non-sequence specific, short stretch of basic amino acids, by nuclear karyopherin import proteins (importins) (32-34). These importins then guide the cargo through the NPC and into the nucleus. While this model is best described for soluble proteins, the involvement of the nuclear import machinery and the NPC in the transport of the yeast Heh2 integral membrane protein from the outer nuclear membrane (ONM) to the inner nuclear membrane (INM) has recently been elucidated (35, 36). Transport of Heh2 requires an unstructured "linker" region of a certain length between a soluble domain containing an NLS sequence and the trans-membrane region of the protein.

DMI1 has been previously localized to both ONM and INM and thus must pass through the NPC to reach the INM (37). POLLUX, the DMI1 homolog in Lotus, contains three predicted NLS sequences so most likely also localizes to the INM (26). We propose that the phenotype observed in the *nup85*, *nup133* and *nena* mutants of Lotus is due to mis-localization of POLLUX and potentially also CASTOR on the nuclear envelope. As DMI1 is the POLLUX homolog in Medicago, we hypothesized DMI1 would similarly be mis-localized in a *nup* mutant background. Finally, the involvement of three NUP107-160 sub-complex members in root symbiosis in the absence of other obvious morphological or physiological defects suggests that other members of this sub-complex may also be required for nodulation or mycorrhization.

Our investigation of NUP107-160 sub-complex members in Lotus and Medicago revealed the specific role of these proteins in establishing the proper distribution of DMI1 and POLLUX on

the nuclear envelope. This suggests that depletion of one member of this sub-complex may be sufficient to alter nuclear trafficking of specific membrane bound INM proteins.

MATERIALS AND METHODS

Plant growth

Medicago truncatula R108 and *Lotus japonicus* Gifu or MG20 seeds were scarified in sulfuric acid for 8 minutes followed by sterilization in an 8.25% sodium hypochlorite solution for 2 minutes. Medicago seeds were imbibed before plating on 1.5% agar plates containing 1 µM gibberellic acid. Plates were incubated at 4°C for 2-4 days before germination. Medicago seeds were germinated overnight before transfer to Fahräeus medium. For rescue experiments, *Lotus japonicus* Gifu and MG20 seeds were surface scarified with sand paper, followed by sterilization in a 2% of sodium hypochlorite solution for 10 minutes. Lotus seeds were germinated for 5 days before plating on water agar, nitrogen free Fahräeus medium, or Gamborg B5 medium supplemented with 1% sucrose respectively (51, 52). Plants were grown in growth chambers at 24°C unless indicated otherwise. Plant root transformation was carried out by using *Agrobacterium rhizogenes* MSU440 or LBA1334. Freshly germinated Medicago and Lotus roots were cut using sterile razor blades and dipped or submerged into *A. rhizogenes* cultures plated on Luria-Bertani agar plates containing selective antibiotics (53, 54). These plants were transferred to Fahräeus medium agar plates (Medicago), or Gamborg's B5 medium with 1% sucrose agar plates (Lotus). After 10 days Lotus plants were transferred to Fahräeus medium agar plates before further experimentation.

Analysis of the Medicago NUP133 *Tnt1* insertion line

A NUP133 Medicago R108 *Tnt1* retrotransposon insertion line was obtained from the Medicago mutant retrotransposon database (http://medicago-mutant.noble.org/mutant/). DNA was extracted from Medicago leaf tissue for genotyping of the *Mtnup133* mutant line using the Epoch GenCatch plant genomic DNA extraction kit. PCR was performed using MtNUP133 F1 and R1 primers spanning the insertion, as well as the LTR6 primer on the T-DNA (Supplementary Table 1) (55). To isolate RNA, root tissue from 4-5 week old Medicago seedlings in micro-centrifuge tubes was frozen in liquid nitrogen. Glass beads were added to the tubes and the tissue was ground using a mixer mill at top speed for 2 minutes. To extract RNA, 1 ml of TRIzol was added to each sample followed by 0.2 ml of chloroform and this was incubated at room temperature before centrifugation. RNA was located in the upper aqueous phase which was isolated and precipitated with isopropyl alcohol. Samples were centrifuged to collect RNA which was washed with ethanol and then dried before suspension in diethylpyrocarbonate (DEPC) treated water. RNA was treated with DNase using the DNA free kit from Ambion and cDNA was synthesized using the Fermentas RevertAid First Strand cDNA synthesis kit. To confirm knock-out, semi-quantitative RT-PCR was conducted on cDNA samples from *Medicago truncatula* R108 wild-type and *Mtnup133* mutant using the MtNUP133 F1 and R1 primers and the Actin F/R primer pair as the control (Supplementary Table 1) (56).

MtNUP133 cross species rescue assay

We were unable to amplify the full length coding sequence of MtNUP133 so we sent the full length sequence with and without the stop codon with restriction sites added to both ends to Biomatik (http://www.biomatik.com/) for gene synthesis. The gene was cloned into pENTR1A

and then cloned into the destination vector (pK7FWG2 or pK7WGF2) by LR recombination. To assess functionality of the MtNUP133 protein, the full length coding sequence was expressed under the CaMV 35S promoter in *Ljnup133-2* (roots using the pK7FWG2 construct with DsRed1 serving as a visual reporter). Lotus *nup133* and wild-type transformed with an empty vector pK7GWIWG2(II) construct also containing a DsRed1 visual reporter served as controls. Lotus plants with transgenic roots plated on Fahräeus medium agar plates were inoculated with 50ml of OD_{600} of 0.01 *Mesorhizobium loti* NZP2235 for 5 minutes before inoculum was poured off. Plants were checked for nodules after 3 weeks. Transgenic Medicago plants were potted and inoculated for nodulation assays as described below.

***Mtnup133* nodulation and arbuscular mycorrhization assays**

Three week old Medicago seedlings were planted in bleach sterilized pots containing sterilized sand and Turface®, and inoculated with 10 ml per pot of *Sinorhizobium meliloti* expressing *nifH:GUS* directly at the base of the plant. Plants were grown under continuous light. Medicago was harvested after 3-4 weeks to count nodules. We assessed *nifH* expression by vacuum infiltration of GUS staining solution, followed by incubation for 12 hours at 37°C. The stain contained 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 50 mM sodium phosphate (pH 7.2), 1 mM potassium ferrocyanide, and 1 mM potassium ferricyanide and 5 mM Na-EDTA, as described in Journet *et al.* (57).

For mycorrhization experiments, Medicago seedlings were planted in rinsed non-sterile Turface® containing 200 spores of *Glomus intraradices* (Mycorise® ASP, PremierTech Biotechnologies) per pot. After planting the seedlings were further inoculated with 1 ml of water containing 200 spores/ml. Plants were fertilized with Long Ashton low phosphorous solution and

grown under greenhouse conditions for 8 weeks before harvesting (58). Rinsed roots were stored in 60% ethanol before analyzing colonization. Quantification of AM colonization was via the gridline intersect method (59). Briefly, roots were cut into 1 cm pieces and stained using Sheaffer ink stain (5 % Sheaffer black ink and 5% acetic acid in water). Roots were cleared by submerging in 10% KOH and incubating at 95°C for 8 minutes, rinsed and then incubated at 95°C for 10 minutes in the staining solution. Roots were again rinsed before visualization and quantification.

Calcium imaging

A Förster Resonance Energy Transfer (FRET)-based assay was used to analyze Nod factor-induced calcium spiking in Medicago root hair cells. *Agrobacterium rhizogenes*-mediated root transformation was performed on the seedlings of Medicago wild-type and *Mtnup133* mutant lines to express calcium sensor Yellow Cameleon 3.6 (YC3.6) (23). Two weeks after transformation transgenic roots were identified using the YC3.6 fluorescence. Plants with transgenic roots selected and mounted onto custom-built slide holders with a capacity of 10 ml of Fahräeus agar medium. After mounting, plants were incubated in a growth chamber for at least 24 hours to establish normal root and root hair growth. *Sinorhizobium meliloti* Nod factors (10^{-8} M), purified following a previously described protocol (60) were suspended in Fahräeus medium broth and applied directly to roots to detect nuclear-associated calcium spiking. A Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Inc., Germany) was used to collect data on the intensity of the YFP and FRET fluorescent signals every 3 seconds for 30 minutes. Variations in the nuclear and perinuclear calcium concentrations are indicated by FRET-based changes in the YFP/CFP ratio.

Quantitative RT-PCR

Medicago wild-type and *Mtnup133* were grown on Fahräeus medium plates supplemented with 100nM AVG (aminoethoxyvinylglycine) ethylene inhibitor for 7 days before treatment with Nod factors isolated from *Sinorhizobium meliloti* (10^{-8} M) or water (control) for 1 hour. RNA was extracted from roots using GenCatch Total RNA Extraction Kit (Epoch). DNA contaminants were removed and cDNA was synthesized from 1µg of RNA. Marker genes used in this study (Medtr5g083030 and Medtr8g091720) were identified in a large scale proteomic screen of Medicago plants treated with Nod factors for 1 hour (61). Expression of Medtr5g083030 and Medtr8g091720 was normalized to PRS, PDF2, and/or SecAgent. Primers used are listed in Table S1 (62-64). Results are the combined data from four replicated experiments, with at least three biological replicates per treatment.

Transmission electron and confocal microscopy

Plant material used was Lotus wild-type, *Ljnup133-2* and *Ljnup85-2* roots expressing POLLUX:GFP under the CaMV 35S promoter in the pK7FWG2 construct pre-selected using the DsRed1 visual reporter. Similarly, Medicago wild-type and *Mtnup133* expressing GFP:DMI1 under the CaMV 35S promoter in the pK7WGF2 construct were selected. Untransformed Medicago and Lotus served as controls. The confocal microscopic analysis of these constructs was conducted on the Zeiss LSM510 described above or the Zeiss ELYRA microscope. To localize POLLUX and DMI1 on the nuclear envelope using transmission electron microscopy, we used previously published methods for freezing and embedding tissue, preparation of grids and immunolocalization (37). At least two roots were sectioned per treatment. Analysis of gold particle distribution on the nuclear envelope was conducted in the ImageJ program

(http://rsb.info.nih.gov/ij/) using methods previously described (37). As the primary and secondary antibodies together are approximately 15 nm in size, those gold particles falling between -15 nm and +15 nm of the middle line cannot be distinguished from locating to the INM or the ONM. Gold particles outside of this range, but within 50 nm of the midline are considered as localizing to the INM or the ONM.

Lotus nucleoporin rescue assays

Lotus roots were transformed via *Agrobacterium rhizogenes* LBA1334 or AR1193 (65) carrying the constructs *$35S_{pro}$:GFP* (as an empty vector; EV), *$35S_{pro}$:GFP-$35S_{pro}$:NUP133*, *$35S_{pro}$:GFP-$35S_{pro}$:NUP85*, *$35S_{pro}$:GFP-$35S_{pro}$:DMI1*, *$35S_{pro}$:GFP-35S:CASTOR*, *$35S_{pro}$:GFP-$35S_{pro}$:POLLUX*, *UB_{pro}:CASTOR*, *UB_{pro}:POLLUX* and *UB_{pro}:CASTOR+POLLUX* respectively. The Lotus seedlings were grown on 0.8% bacto agar plates for 3 days in the dark and 4 days in the light at 24 °C prior to transformation as described previously (52). The plants were co-cultivated in the dark at approximately 20 °C for 2 days, then incubated for 3 days in 16 h light/8 h dark condition at 24°C in a growth chamber. Successively, the plants were transferred to B5 plates containing cefotaxime (333 µg/ml) to kill off the *Agrobacterium*. The plants were then cultivated at 24 °C in 16 h light/8 h dark conditions until emergence of transformed roots (2 to 3 weeks).

To analyze nodulation, the Lotus plants with transformed roots were transferred from the B5 agar plates into closed sterile glass jars (WECK) containing 300 g of sand/vermiculite (1:1) supplemented with 60 ml Fahräeus medium (66) (15-20 plants per jar). The plants were grown for two weeks at 24°C, then inoculated evenly with 30 ml of *M. loti* MAFF303099 dsRED (OD_{600} of 0.02) (67). Nodulation was investigated four weeks after inoculation. Transformed roots were

either identified by the GFP fluorescence of the transformation marker or by the fluorescence of YFP tagged POLLUX and mCherry tagged CASTOR (UB_{pro}:CASTOR+POLLUX).

Analysis of NUP107 LORE1 Lotus insertion line

NUP107 Lotus MG20 LORE1 retrotransposon insertion lines were obtained from Lotus Base (http://users-mb.au.dk/pmgrp/) (38, 39). Segregating R4 (fourth generation of tissue regenerated plants) plant populations derived from heterozygous R3 parents were genotyped and phenotyped in parallel. The plants were germinated and grown on 0.8% agar (w/v) plates for 1 week at 18°C then transferred to pots, watered with 10 ml Fahräeus medium each and grown for five weeks at 18°C. Each plant was inoculated with 5 ml of *M. loti* MAFF303099 dsRED diluted in Fahräeus medium (OD_{600} of 0.02) and grown again at 18°C. Nodulation was quantified four weeks after inoculation. Genomic DNA was extracted from 3 small leaves of each plant as described previously (68). Genotyping was performed by PCR using 10-50 ng of genomic DNA as a template. Primers flanking the LORE1 insertion point (forward primer P225 and reverse primer P226) were used to identify the wild type allele, a second PCR using the forward primer P225 together a reverse primer located in the LORE1 transposon (P252) identified the *Ljnup107-1* mutant allele (LORE1 insertion).

Molecular cloning

CASTOR and *POLLUX* constructs expressed under the ubiquitin promoter for *nup* mutant rescue were assembled using a Golden Gate cloning system (for modules Gx) (69). For primers mentioned below see Table 1. The *CASTOR* and *POLLUX* genomic sequences were amplified from *L. japonicus* Gifu B-129 DNA as multiple fragments in order to remove BsaI and BpiI restriction sites by introduction of silent mutations. Additionally, fragments that could not be

amplified were further subdivided into smaller pieces. In total, *CASTOR* was split into 7 fragments, which were amplified with the following primers: 1) P122/P123; 2) P132/P151; 3) P152/P133; 4) P134/P154; 5) P153/P135; 6) P128/P129; 7) P130/P131. *POLLUX* was split into 6 fragments and amplified with the following primers: 1) P93/P94; 2) P95/P96; 3) P97/P98; 4) P99/P100; 5) P101/P102; 6) P103/P104. The fragments were assembled by BpiI cut-ligation into the LI+BpiI backbone, resulting in the LI constructs *LI C-D CASTOR and LI C-D POLLUX*. For generation of expression constructs *UB_{pro}CASTOR* and *UB_{pro}POLLUX*, Level I modules *LI C-D CASTOR* and *LI C-D POLLUX* were first assembled individually into LII F 1-2 backbones by BsaI cut-ligation. The LII constructs combined sequences of *CASTOR* or *POLLUX* with the *L. japonicus* polyubiquitin promoter element (G7) and a nos terminator sequence (G6). The final LIII constructs *UB_{pro}:CASTOR* and *UB_{pro}:POLLUX* were assembled from *CASTOR* and *POLLUX* LII F 1-2 synthetic genes together with a LII R 3-4 element containing a GFP transformation marker under the control of a *35S* promoter. For assembly of the binary plasmid *UB_{pro}:CASTOR+POLLUX*, LI C-D POLLUX was first combined with the polyubiquitin promoter (G7), C-terminal YFP tag (G12) and nos terminator (G6) into a LII F 1-2 construct, while CASTOR was combined with polyubiquitin promoter (G7), C-terminal mCherry tag (G25) and HSP terminator (G45) into a LII R 3-4 construct. The resulting *LII CASTOR* and *POLLUX* plasmids were then assembled into the LIII binary plasmid (a modified version of pICH50505 from Icon Genetics http://www.icongenetics.com/) by BpiI cut-ligation resulting in the final *UB_{pro}:CASTOR+POLLUX* vector.

NUP85 cDNA was amplified by PCR and cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA). *NUP133* cDNA was amplified by PCR and digested with *Sac*II and *Asc*I, then inserted into the *Sac*II-*Asc*I site of pENTR/D-TOPO. For expression controlled by the 35S

promoter, the entry clones constructed in this study and entry clones of *CASTOR*, *POLLUX* and *DMI1* were recombined into the destination vector *$35S_{pro}$:GW-$35S_{pro}$:GFP* (70).

DMI, CASTOR and POLLUX structural analysis

The presence of unfolded linker regions in DMI1, POLLUX and CASTOR was predicted using the FoldIndex (http://bip.weizmann.ac.il/fldbin/findex) as described in Meinema *et al*. (35). The FoldIndex value of a residue is calculated based on the hydrophobicity of the amino acids and the proteins net charge (71, 72). NLS domains were predicted using cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (33, 73).

Phylogenetic analysis

To collect sequences corresponding to potential NUP133 orthologs, LjNUP133 amino acid sequence was searched in publicly available genomes using BLASTp. Hits with an e-value greater than 10-90 were selected for the phylogenetic analysis. Collected sequences were aligned using MAFFT (http://mafft.cbrc.jp/alignment/server/) and the alignment manually curated with Bioedit. Phylogenetic trees were generated by Maximum-likelihood in MEGA6. Partial gap deletion (90%) was used together with the JTT substitution model. Bootstrap values were calculated using 100 replicates.

Synteny analysis

A ~200kb sized region around the NUP133 orthologs from *Populus trichocarpa*, *Glycine max*, *Medicago truncatula*, *Phaseolus vulgaris*, *Solanum tunerosum* and *Sorghum bicolor* were compared using CoGe Gevo (https://genomevolution.org/CoGe/GEvo.pl) as described in Delaux et al. 2014 (74). Lotus was not included given that its genome is not available in CoGe.

RESULTS

NUP107 is required for nodulation in Lotus

Lotus LORE1 retrotransposon lines (38, 39) were screened for insertions in genes coding for putative NUP107-160 sub-complex members besides NUP85, NUP133 and NENA, to study if they also function in nodulation. A line containing a LORE1 insertion in the Lotus homolog of NUP107 was identified and designated as *nup107-1* (Lotus Base Plant ID: 30054066; http://users-mb.au.dk/pmgrp/). The *LjNUP107* gene is located on chromosome 3 and has a predicted size of 18273 bases containing 23 exons with a coding sequence of 3228 bases (chr3.CM0416.660.r2.m). The LORE1 retrotransposon is inserted into the first predicted exon between bases 12 and 13 (**Fig 1A**). A segregating *nup107-1* R4 (fourth generation of tissue culture regenerated plants) population was genotyped and tested for nodulation, revealing a nodulation defect that clearly co-segregated with homozygous *nup107-1* individuals. In total, 83 plants were genotyped, of which 31 were homozygous wild type, 41 heterozygous and 11 homozygous *Ljnup107-1* individuals. The wild type plants harbored an average of 10.04 nodules, while *Ljnup107-1* mutants did not produce any nodules or nodule primordia (**Fig 1B & C**). All heterozygous individuals also carried nodules (not quantified). The distribution of wild type, heterozygous, and mutant plants was different from the expected segregation ratio of 1:2:1 ($p < 0.01$; X^2 test), suggesting that gametophyte (pollen/embryo sac) or seed viability could be affected in *Ljnup107-1*. Other pleiotropic phenotypes were not observed.

Identification of Medicago NUP133

Based on the similarities between Medicago and Lotus in the symbiotis signaling pathway, we hypothesized that NUP133 would also be required for legume nodulation and arbuscular

mycorrhization in Medicago. We identified a single NUP133 ortholog in the *Medicago truncatula* genome, Medtr5g097260.1, which has 81% amino acid identity with LjNUP133 (**Fig 2A & Fig S1**). We found that *MtNUP133* was expressed in all tissues tested, similar to the expression pattern observed from *LjNUP133* (**Fig 2B**) (15). To assess the identity of MtNUP133 as a nucleoporin, the protein was localized to the nuclear envelope by confocal microscopy using a Green Fluorescent Protein (GFP) fusion protein (**Fig 2C & D**). *MtNUP133* was expressed in *Ljnup133* roots and was able to restore nodulation, although not to wild-type levels (**Fig 2E**), thereby confirming that the genes are homologous and perform the same function.

Medicago *NUP133* is not essential for nodulation

To assess the function of MtNUP133, a homozygous mutant containing a *Tnt1* insertion 15 bp after the start codon in the first exon was obtained (*Mtnup133*). The lack of expression of the full length *MtNUP133* in the mutant plants was confirmed by RT-PCR using primers spanning the insertion site **(Fig 2F).** Analysis of the mutant revealed that nodulation was reduced by approximately 50%, which is a much milder phenotype than the *Ljnup133* mutant where nodulation is almost completely abolished **(Fig 2G)** (15). The reduction in nodulation we observed in *Mtnup133* was not temperature dependent, another departure from the *Ljnup133* phenotype. Nitrogen fixation seemed to proceed normally in the *Mtnup133* mutant, based on the expression of the *nifH*:*GUS* reporter in *S. meliloti*-colonized nodules **(Fig 2H).** We were not able to detect any reduction in arbuscular mycorrhization rates in *Mtnup133* **(Fig 2I).** We also assessed Nod factor-induced calcium spiking in *Mtnup133* and found that, unlike the *Lotus nup133* counterpart, calcium spiking occurred normally in *Mtnup133*, although the number of cells spiking out of the total was lower in the mutant background **(Fig 2J).** We found a significant reduction in Nod factor

induced gene expression of one gene tested, whereas another appeared indistinguishable from wild type suggesting a variation in sensitivity of some genes to this mutation **(Fig 2K).**

DMI1, CASTOR and POLLUX contain N-terminal unfolded "linker" regions

Previously it was demonstrated that the presence of an intrinsically disordered linker approximately 120 amino acid (aa) residues in length is required for the localization of an NLS carrying membrane protein to the INM (35). We identified the presence of unfolded linker regions in DMI1, POLLUX and CASTOR using the FoldIndex software **(Fig 3).** Thus, it is plausible that these ion channels are using the same classical import pathway for localization to the INM that was observed in the Heh2 yeast protein. Interestingly, the unstructured region in CASTOR and DMI1 is shorter than that in POLLUX. The unstructured region of CASTOR (~80 aa) is much shorter than the 120 aa criterion for translocation through the NPC in yeast, while DMI1 is just under this limit at approximately 110 aa.

POLLUX and DMI1 are mis-localized in NUP107-160 sub-complex mutants

DMI1 has previously been localized to both ONM and INM using immuno-gold labeling and transmission electron microscopy (TEM) (37). We investigated whether members of NUP107-160 sub-complex play a role in the INM localization of DMI1 and its homolog POLLUX. Localization of POLLUX and DMI1 proteins fused to GFP using confocal microscopy established that both in the wild-type and *nup* mutants these ion channels appear to be localized to the nuclear envelope **(Fig 4).** Similar methods used to localize CASTOR were unsuccessful. As confocal microscopy cannot provide sufficient resolution to localize DMI1 and POLLUX at the nuclear membrane level, we used immuno-gold labeling and TEM to test for mis-localization of the ion channels in the *nup* mutants. We found that POLLUX localized to both the INM and ONM in the

wild type background **(Fig 4C & Fig. 5A).** In *Ljnup85* and *Ljnup133*, however, a significant reduction of gold particle accumulation on the INM, and an increase outside the ONM, likely on the endoplasmic reticulum, was observed **(Fig 4F & I).** Thus, it appears that while INM localization of POLLUX is not fully disrupted in either *Ljnup85* or *Ljnup133* mutants, there is a significant shift in localization towards the ONM that may be sufficient to disrupt function during calcium spiking **(Fig 5B).**

Localization of DMI1 in the wild-type background was similar to that previously observed (37), with a slight preference for the INM (**Fig 4L & Fig 5C).** In the *Mtnup133* mutant, however, the localization of DMI1 is very clearly shifted to the ONM **(Fig 4O & Fig 5C).** Given the symbiotic phenotype of this mutant, however, this seems insufficient to abolish the function of DMI1 during symbiosis.

Lotus *nup* mutants can be rescued by DMI1, or simultaneous over-expression of POLLUX and CASTOR

TEM analysis demonstrated a mis-localization of POLLUX in the Lotus *nup* mutant background. We hypothesized that the symbiotic phenotypes observed in these mutants are the result of a loss of POLLUX and potentially CASTOR ion channel functionality during Nod factor-induced calcium spiking, perhaps resulting from reduced import of the proteins into the INM.

To evaluate this, we introduced DMI1, POLLUX and/or CASTOR into Lotus *nup* mutants. Previously, we reported that expression of either *POLLUX* or *CASTOR* alone is not sufficient to restore nodulation in a *castor/pollux* double mutant, whereas *DMI1* over-expression is, indicating that DMI1 alone can substitute for both POLLUX and CASTOR (31). When over-expressed under the control of the 35S promoter (**Fig 6, Fig S2**) or the ubiquitin promoter respectively (**Fig S3**),

DMI1 alone or both *POLLUX* and *CASTOR* together were able to restore nodulation in *Ljnup133, Ljnup85, and Ljnena* mutants. However, over-expression of either *POLLUX* or *CASTOR* alone was not sufficient to restore nodulation at the levels observed in *nup/ox-DMI1* or *nup/ox-POLLUX+CASTOR* (**Fig 6**, **Fig S2, Fig S3**).

The over-expression of *DMI1* in Lotus only partially complemented the nodulation phenotype in Lotus *nup* mutants as the proportion of mature nodules formed on the roots of *Ljnup133/35S$_{pro}$:DMI1* was lower than that of *Ljnup133/35S$_{pro}$:NUP133* (**Fig 6G**). Even so, DMI1 rescued more fully than over-expression of either *POLLUX* or *CASTOR*, which resulted in even fewer mature nodules. (**Fig 6G**). While average nodule numbers per plants were comparable between *Ljnup133/35S$_{pro}$:DMI1 and Ljnup133/35S$_{pro}$:NUP133* (**Fig 6H)**, mean nodule size was significantly lower (**Fig 6I**).

DISCUSSION

NUP107-160 sub-complex members required for symbiosis

In this study we investigated the role of the NUP107-160 sub-complex during Nod factor induced signaling. We found another member of this sub-complex in Lotus, NUP107, is also required for nodulation. As now half of the eight members of this sub-complex have been specifically implicated in symbiotic signaling, it would suggest that mutations in this NPC component lead to a highly targeted disruption in nuclear transport. Given the general role of the NPC in nuclear trafficking, this finding is surprising. However, there is precedent for specificity in the requirement of some NUPs over others in the translocation of membrane proteins into the nucleus (40). As the NUP107-160 sub-complex is part of the NPC scaffold, the disruption of this structure may hinder INM protein transport (1). INM accumulation of proteins actively translocated through the NPC by Importins can be achieved by faster import combined with slower

passive diffusion back through the NPC to the ONM (35, 41). During active import of transmembrane proteins, part of the protein has to pass through both the NPC central channel and the NPC scaffold (35). As this implies a remodeling of nucleoporin connections in order to create on opening, structural defects in *nup* mutants could impair localization of nuclear envelope membrane proteins by affecting either their active import or their passive export. In line with this hypothesis, Heh2 ER accumulation and INM depletion was observed in deletion strains of NUP170, another NPC scaffold protein (36).

Medicago and Lotus are two model organisms used to study plant-microbe symbiosis and their Nod factor perception pathways are largely conserved. However, we found that unlike in Lotus, NUP133 is required but not essential in Medicago for nodulation and dispensable for mycorrhization. Despite the occurrence of calcium spiking in *Mtnup133* plants, Nod factor-induced gene expression of one out of two genes tested was reduced compared to wild-type (**Fig 2G**). This suggests that calcium spiking is disrupted in some root epidermal cells but not all, explaining an overall reduction in nodulation, and that some LCO-induced genes may be more sensitive to this global reduction in calcium spiking than others (**Fig 2J**).

INM localization in the function of DMI1 and POLLUX

We hypothesized that the reduction in nodulation and/or mycorrhization observed in *Ljnup85, Ljnup133, Ljnena* and *Mtnup133* was due to the mis-localization of POLLUX, CASTOR or DMI1. The differences in the symbiotic phenotypes between Lotus and Medicago *nup133* lines may be due to the different behavior of POLLUX and DMI1 as ion channels during Nod factor induced calcium spiking. DMI1 has a longer open time and lower potassium conductance than CASTOR, and theoretically POLLUX (31). Over-expression of *DMI1* in *Ljnup85, Ljnup133* and *Ljnena* roots

rescued the nodulation defects. In contrast, over-expression of *POLLUX* or *CASTOR* alone in these same mutants only partially rescues nodulation. It is possible that due to its channel properties, DMI1 is able to trigger calcium spiking despite reduced accumulation on the INM. On the other hand, the over-expression of DMI1 is not as efficient as the over-expression of NUP133 in rescuing nodulation defects (**Fig 6K** and **6L**), suggesting that there may be other components affected by the *nup133* mutation, which are required for proper development and maturation of the nodule.

Localization of DMI1 and POLLUX in their respective *nup* mutant backgrounds (*Mtnup133, Ljnup85, Ljnup133*) demonstrated a partial shift in the localization of these ion channels from INM to the ONM. Although we did not conduct this analysis in the *Ljnena* mutant, given the very similar phenotypes among the Lotus nucleoporin mutants, we propose that a similar mis-localization may occur in that mutant background as well. Taken together with our rescue data, this indicates that a slight reduction of POLLUX on the INM is sufficient to abolish calcium spiking, whereas this is not the case for DMI1. Given that simultaneous over-expression of both CASTOR and POLLUX is needed to complement the nodulation defect in the *Ljnena, Ljnup85* and *Ljnup133* mutants, INM localization of CASTOR may also be a requirement for calcium spiking.

INM localization of DMI1 and POLLUX was only slightly reduced in the case of *Mtnup133,* or *Ljnup85* and *Ljnup133* respectively, suggesting that the NUP107-160 sub-complex is still mostly intact in the single *nup* mutants. Mis-localization of POLLUX seemed more pronounced in the *Ljnup85* background. This is consistent with previous studies showing that LjNUP133 protein content is reduced in the *Ljnup85* mutant whereas the *Ljnup133* mutation does not affect the abundance of the LjNUP85 protein (42). A likely interpretation of the rescue data is that despite the import defect, over-expression provides sufficient amounts of the ion channels in

the INM for calcium spiking to take place. It is also possible that the NUP107-160 sub-complex may be required for transport of other INM proteins such as MCA8, a calcium pump localized to both the INM and ONM in Medicago and involved in LCO-induced calcium spiking (37). Given that over-expression of both CASTOR and POLLUX together as well as DMI1 alone was sufficient to rescue the NUP107-160 mutants, however, it seems that the localization of other ion channels may not rely upon this sub-complex or that a potential reduction in their INM import is not sufficient to disrupt symbiosis.

Nuclear trafficking of symbiotic ion channels

Different mechanisms have been reported for trafficking of integral membrane proteins into the INM (43). According to the diffusion/retention model some proteins with a mass of less than 60 kDa can reach the INM by passive diffusion through small peripheral channels (~10 nm) between the central pore of the NPC and the nuclear membrane, where they are retained on the INM via tethering to nuclear components such as lamina (44-48). Other, in particular large, membrane proteins that carry a NLS are actively transported by utilizing the central channel (~39nm) via binding to Importins (35, 49, 50). We propose that DMI1 and POLLUX can traverse the nuclear pore complex via a method similar to that described in Meinema *et al.* (2011), where the transmembrane region stays within the nuclear membrane, and the presence of an unstructured linker region allows the soluble portion of the protein containing the NLS domain to pass through the central channel bound to Importin proteins (**Fig 7**). In line with this theory, DMI1 missing the N-terminal region containing most of the unstructured linker region domain and the NLS sequences (t-DMI1) cannot rescue the nodulation phenotype of a *dmi1* mutant and seems to accumulate in the endoplasmic reticulum (27).

The predicted disordered linker region of CASTOR is shorter than that of POLLUX and falls far under the minimum of 120 aa that was determined for NLS mediated INM import in yeast (35). In addition, the confidence score for a putative NLS at the N-terminus of CASTOR is very low (score = 2.3; http://nls-mapper.iab.keio.ac.jp/). CASTOR may reside primarily on the ONM, or utilize a non-canonical nuclear import mechanism to reach the INM. Studies on CASTOR localization in both wild-type and *nup* mutant plants will be needed to discern these alternatives (**Fig 7**).

Concluding remarks

Our work highlights the importance of understanding transport of nuclear membrane-localized proteins for discerning their function. While our data combined with previous analysis of DMI1 is consistent with a model proposed for the translocation of INM proteins in yeast, there is still much left to be understood about trafficking of INM proteins in plants. While we cannot rule out that other processes may be affected, our data suggest that symbiotic signaling is sensitive to slight alterations in INM protein accumulation. This provides us with a tool to understand the specific role of NUP107-160 sub-complex members in nodulation and arbuscular mycorrhization, and may serve as an excellent system to further characterize ER-nuclear trafficking across the NPC.

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Table S1. Primers used in this study for *Lotus japonicus* and *Medicago truncatula*

Primer	Sequence (5' - 3')	Source or Reference
MtNUP133 F1	ACCCTTCCATAAACCCTAATT	This manuscript
MtNUP133 R1	CGTTGCAGAGACGAGTTTCAT	This manuscript
LTR6	TTGACTTGGTTTGGTTGGTAGC	(55)
Actin F	GCAGATGCTGAGGATATTAACCC	(56)
Actin R	CCTCTCCCTCTATGCAAGTGGTCG	(56)
Medtr5g083030-F	TTGAGTATTGTTAAAAGTGGAGCAG	This manuscript
Medtr5g083030-R	CTTTATCTCTTCCAACCCCTCAG	This manuscript
Medtr8g091720-F	ACATCAGGAAACATCTCAGACAG	This manuscript
Medtr8g091720-R	GAGACACATTCTTGAACCGTTTC	This manuscript
PRS-F	TGGCAGGAAAGGGTGTTC	(64)
PRS-R	GCCACCTGAATACCAGCAG	(64)
PDF2-F	GTGTTTTGCTTCCGCCGTT	(62)
PDF2-R	CCAAATCTTGCTCCCTCATCTG	(62)
SecAgent-F	GGCAGGTCTGCCTATGGTTA	(63)
SecAgent-R	GGTCAGACGCACAGATTTGA	(63)
P122	ATGAAGACTTTACGGGTCTCACACCATGTCCTTG GATTCGGAGGT	This manuscript
P123	ATGAAGACTTGCCAAATTGCGAGAGCCAGT	This manuscript
P132	ATGAAGACTTTGGCCTCCAGTTTGCCCTTG	This manuscript
P151	TAGAAGACAATTCGGAGCTGGCATGATTAC	This manuscript
P152	TAGAAGACAACGAAGGTATTGGTCCAAGGT	This manuscript
P133	ATGAAGACTTTGTCTCTCTTAAGCAAAGGTGGA ATATTC	This manuscript

P134	ATGAAGACTTGACAATTTGGCTATTATGTTTTAAATAATC	This manuscript
P154	TAGAAGACAACTCGACTTTGTTAGCTTTACTACTTTTTC	This manuscript
P153	TAGAAGACAACGAGCATTTTATATTGAGGATGG	This manuscript
P135	ATGAAGACTTCTACCTTTCTCTTGGTGTAAATCTGGATC	This manuscript
P128	ATGAAGACTTGTAGACCATAATAGTGTTGACTTTTGC	This manuscript
P129	ATGAAGACTTTACACGAGCATCGCAAAAGG	This manuscript
P130	ATGAAGACTTTGTATTCACATTATATCTGAGGCATTG	This manuscript
P131	ATGAAGACTTCAGAGGTCTCACCTTTTCCTTTTCAGTAATCACCACAA	This manuscript
P093	ATGAAGACTTTACGGGTCTCACACCATGATACCACTACCAGTAGCAGCA	This manuscript
P094	ATGAAGACTTAGTTTTCTTCAATGGTGGCTTG	This manuscript
P095	ATGAAGACTTAACTAAGACCCTACTTCCACCACC	This manuscript
P096	ATGAAGACTTGGAGACAATAACACAAATCATAGATATAAGA	This manuscript
P097	ATGAAGACTTAGGCCCTCCTCCGTCAAA	This manuscript
P098	ATGAAGACTTGATACGACCCTCTGCCCG	This manuscript
P099	ATGAAGACTTTATCTGTCTCAATTAGTTCAGGTGG	This manuscript
P100	ATGAAGACTTCTGCAAGAGTGTCAAAATTCAG	This manuscript
P101	ATGAAGACTTGCAGACTGTATTTGGAAATAGAATT	This manuscript
P102	ATGAAGACTTGTAGACTCTCTAGGTGCCGCC	This manuscript
P103	ATGAAGACTTCTACCATTGGAGACTTTTGATTCC	This manuscript
P104	ATGAAGACTTCAGAGGTCTCACCTTATCGCCTGAAGCAATTACAA	This manuscript

P225	TTCACGGTAAACGCAAGATG	This manuscript
P226	TGGGTAATGGAATGGAGGAG	This manuscript
P252	CCATGGCGGTTCCGTGAATCTTAGG	This manuscript

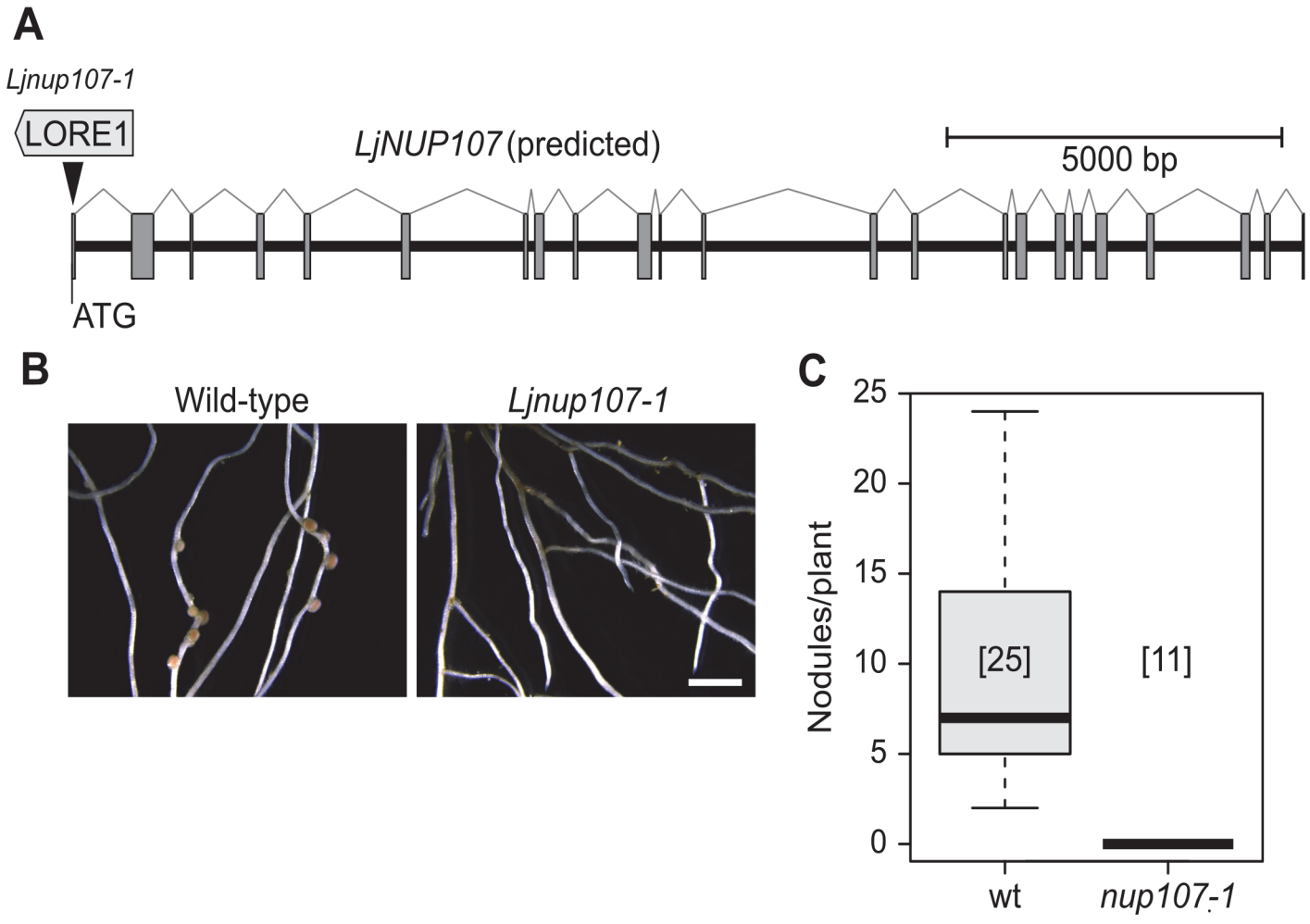

A
Ljnup107-1
LORE1
LjNUP107 (predicted)
5000 bp
ATG
B
Wild-type
Ljnup107-1
C
Nodules/plant
25
20
15
10
5
0
[25]
[11]
wt
nup107-1

Figure 1. A LORE1 retrotransposon insertion in Lotus *NUP107* leads to a defect in nodulation

(A) Gene structure prediction of *LjNUP107* depicting exons (grey bars) and introns (black line). Line *Ljnup107-1* contains a LORE1 insertion in the first predicted exon of the gene. (B) Stereomicroscopic images of wild type and homozygous *Ljnup107-1* roots. Plants were grown for 10 weeks at 18°C and inoculated after 6 weeks with *M. loti MAFF* 303099 expressing dsRED. Colonized nodules are visualized by the red fluorescence from *M. loti* (scale bar = 3 mm). (C) Box plot showing nodulation 4 weeks after inoculation with *M. loti* at 18°C. Number of analyzed plants is indicated in brackets.

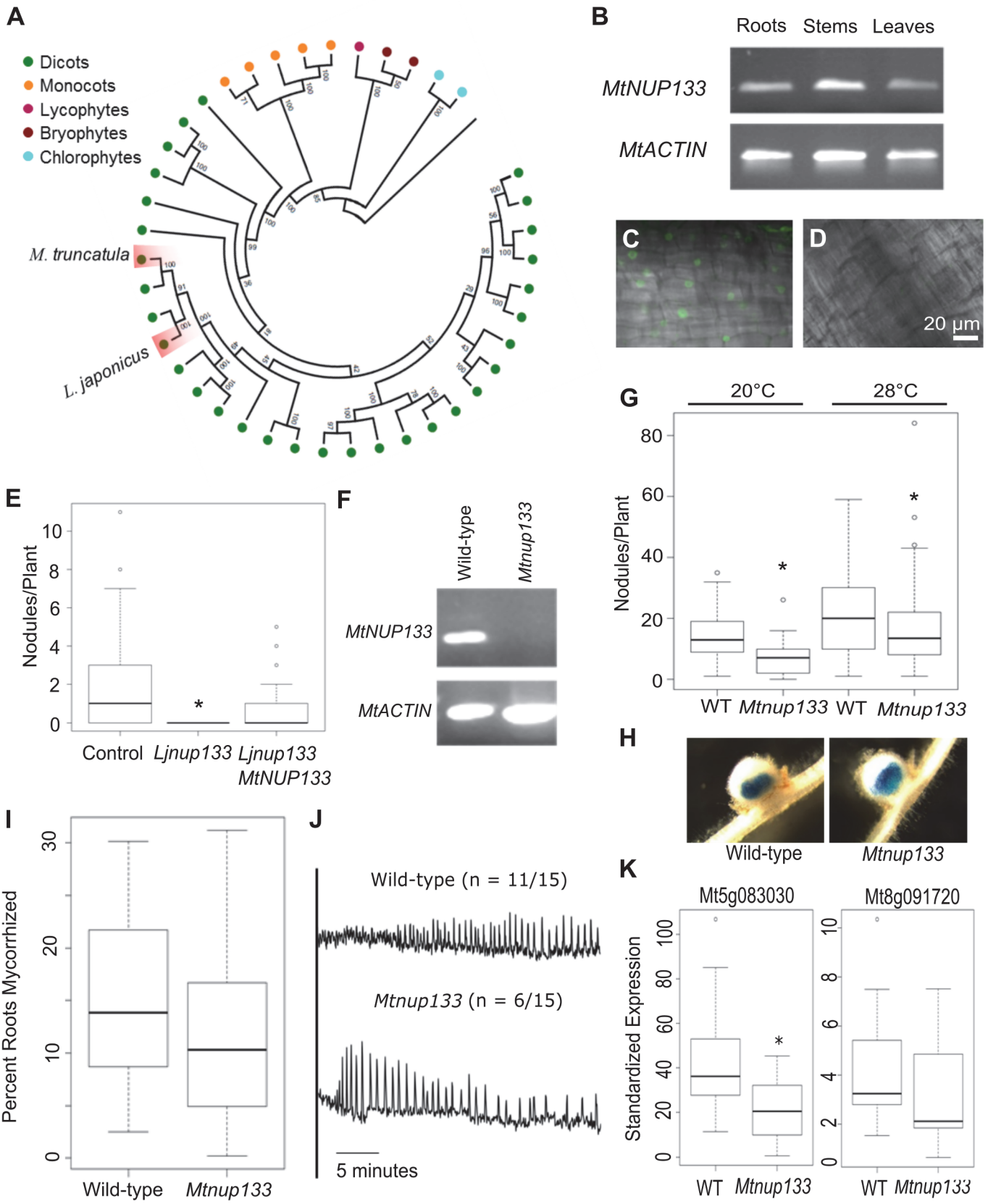

A
Dicots
Monocots
Lycophytes
Bryophytes
Chlorophytes
M. truncatula
L. japonicus
B
Roots
Stems
Leaves
MtNUP133
MtACTIN
C
D
20 µm
E
Nodules/Plant
Control
Ljnup133
Ljnup133 MtNUP133
F
Wild-type
Mtnup133
MtNUP133
MtACTIN
G
20°C
28°C
Nodules/Plant
WT
Mtnup133
WT
Mtnup133
H
Wild-type
Mtnup133
I
Percent Roots Mycorrhized
Wild-type
Mtnup133
J
Wild-type (n = 11/15)
Mtnup133 (n = 6/15)
5 minutes
K
Mt5g083030
Mt8g091720
Standardized Expression
WT
Mtnup133
WT
Mtnup133

Figure 2. Medicago LjNUP133 homolog required but not essential for symbiosis

(A) Phylogenetic analysis of NUP133 across diverse plant species. (B) Expression analysis of *MtNUP133* by RT-PCR, *ACTIN* serves as a contro. Localization of MtNUP133-GFP in transgenic *Medicago* roots (C) and untransformed control (D). (E) MtNUP133 is able to rescue *Ljnup133* nodulation defects. Asterisks (*) denote significance as compared to the wild type control as calculated by Welch two sample t-test ($p < 0.01$), $n > 25$ for all treatments. Black horizontal lines within the boxes indicate the median. (F) Expression of *Mtnup133* was not observed in the mutant line when using primers spanning the insertion site. (G) Nodulation is reduced in *Mtnup133.* Light gray (wild-type), dark gray (*Mtnup133*). Significance denoted by an asterisk (*) was determined by Welch two sample *t*-test ($p < 0.01$) $n > 40$. (H) Nodules colonized by *S. meliloti* expressing *nifH*:GUS. Blue coloration (*nifH* expression) was observed in the nodules formed on *Mtnup133* suggesting that these are functional nodules. Scale bar indicates 0.1 mm. (I) Roots were cut into approximately 1 cm pieces and the percent of root pieces which are mycorrhized was quantified in wild-type and *Mtnup133* mutants. No significant difference was observed. One representative trace shown ($n = 15$) (J) Normal Nod factor-induced calcium spiking is observed in the *Mtnup133* mutant. Horizontal black line indicates 5 minutes after Nod factor addition. Scale bar indicates 5 minutes. (K) Nod factor-induced gene expression shown as standardized to control genes in wild-type or *Mtnup133*. Significance denoted by an asterisk (*) was determined by Welch two sample *t*-test ($p < 0.05$).

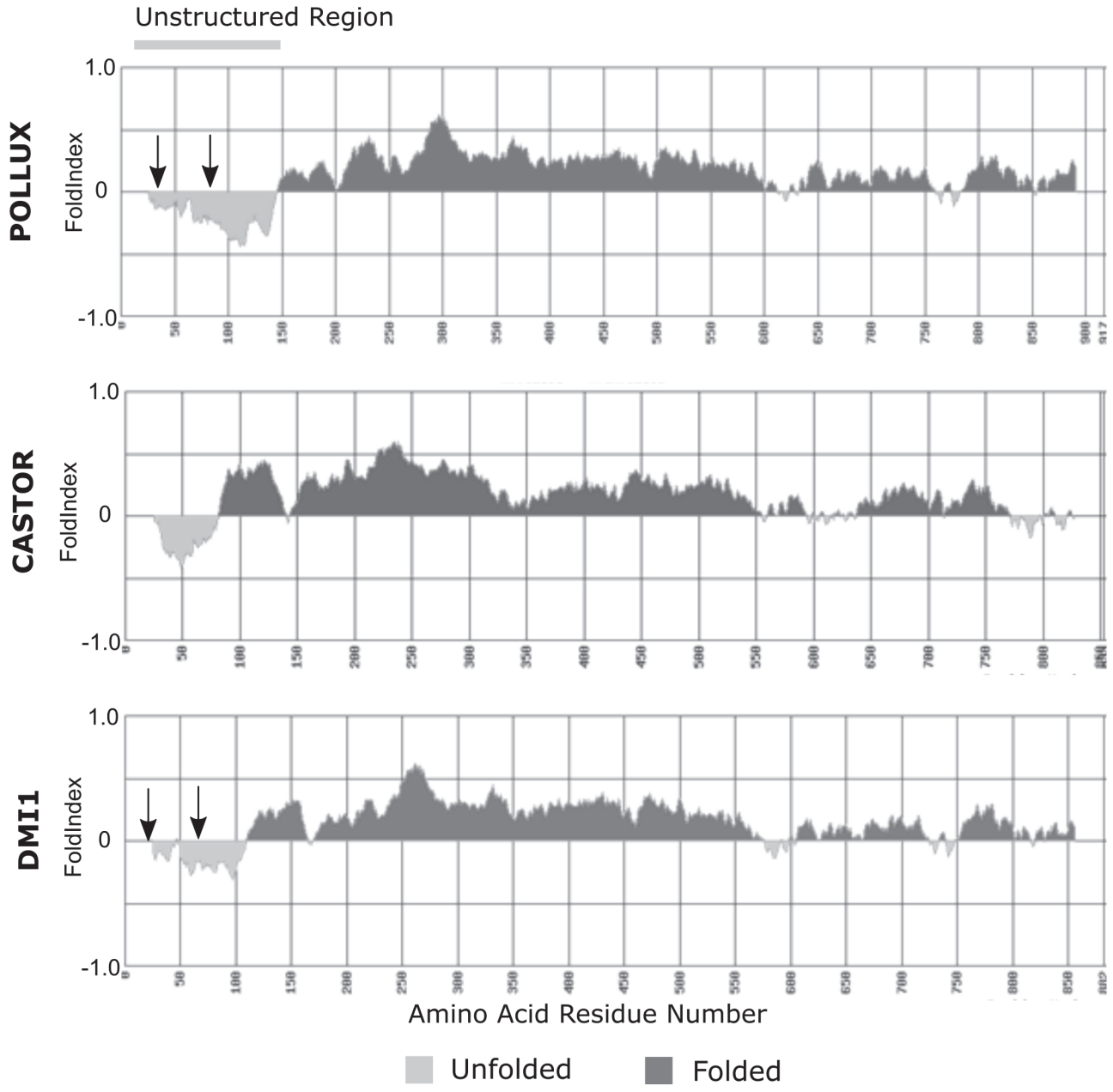

Unstructured Region
POLLUX
CASTOR
DMI1
FoldIndex
1.0
0
-1.0
Amino Acid Residue Number
Unfolded
Folded

Figure 3. Symbiotic ion channels contain putative "linker" regions at N-terminus

Prediction of the location of unstructured regions within DMI1, CASTOR, and POLLUX using FoldIndex software. The x-axis indicates amino acid number and the y-axis indicates the fold-index value. Positive values (dark gray) represent domains likely to be folded and negative values correspond to unstructured regions (light gray). Vertical arrows (black) indicate NLS sequences as determined by cNLS Mapper (score $\geq$ 6.5).

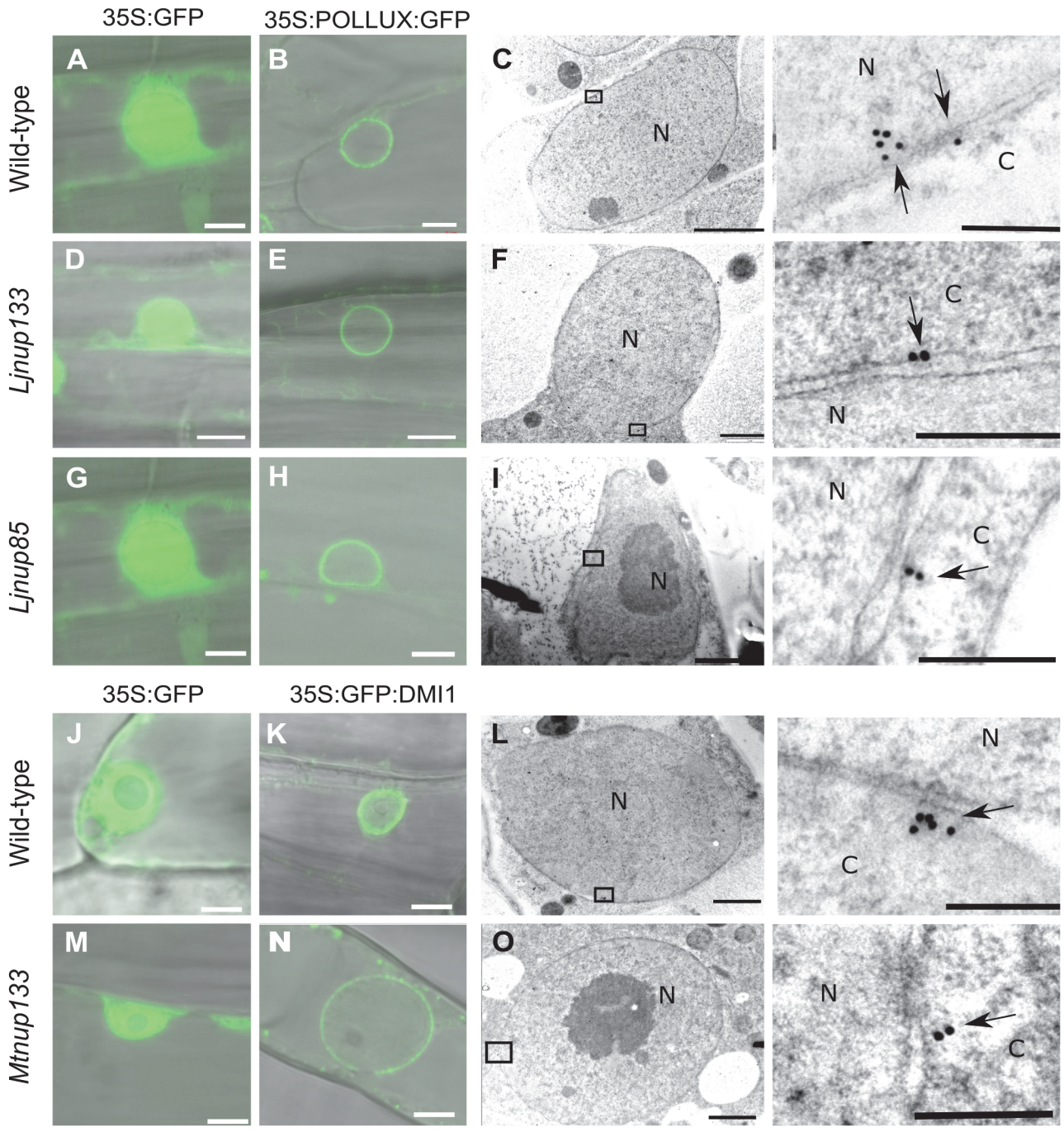

35S:GFP
35S:POLLUX:GFP
Wild-type
Ljnup133
Ljnup85
A
B
C
D
E
F
G
H
I
N
C
35S:GFP
35S:GFP:DMI1
Wild-type
Mtnup133
J
K
L
M
N
O

Figure 4. POLLUX and DMI1 localization on the nuclear envelope

Soluble GFP localizes to the cytoplasm and the nucleoplasm in Lotus wild-type (A), *Ljnup133* (D), *Ljnup85* (G), Medicago wild type (J) and *Mtnup133* (M) root cells. POLLUX-GFP localizes to the nuclear envelope in Lotus wild-type (B), *Ljnup133* (E) and *Ljnup85* (H) root cells. DMI1-GFP localizes to the nuclear envelope in Medicago wild type (K) and *Mtnup133* (N) root cells. Scale bar indicates 5 µm. Electron micrographs with anti-GFP immunolabeled sections of root cells. Gold particles (black arrows) indicating the location of POLLUX-GFP (C, F, I) or DMI1-GFP (L, O) in wild-type and mutants. Images on the left show an overview of the nucleus (scale bars = 1 µm) with the image on the right indicates a zoomed in picture of gold particles (scale bars = 250 nm).

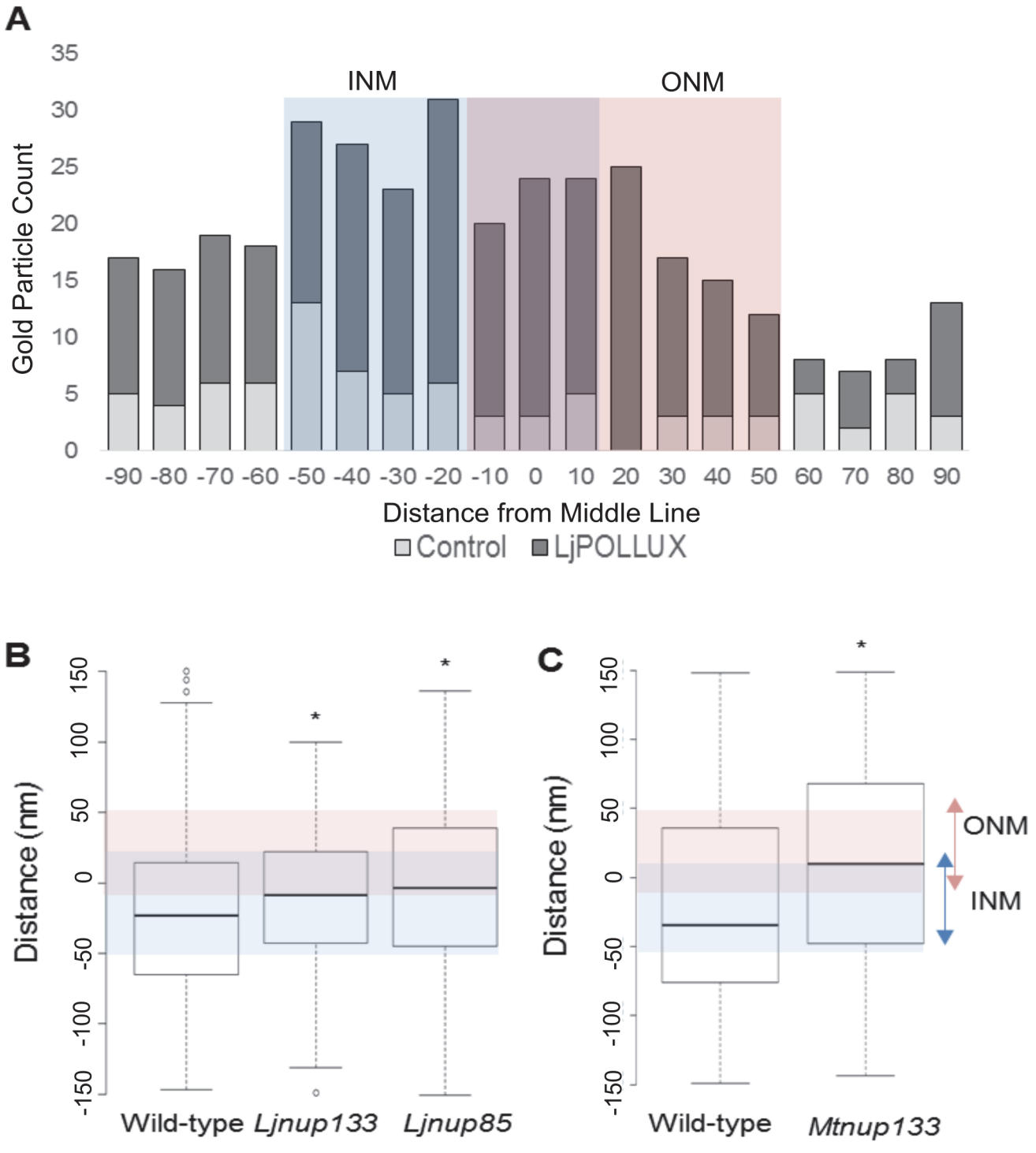
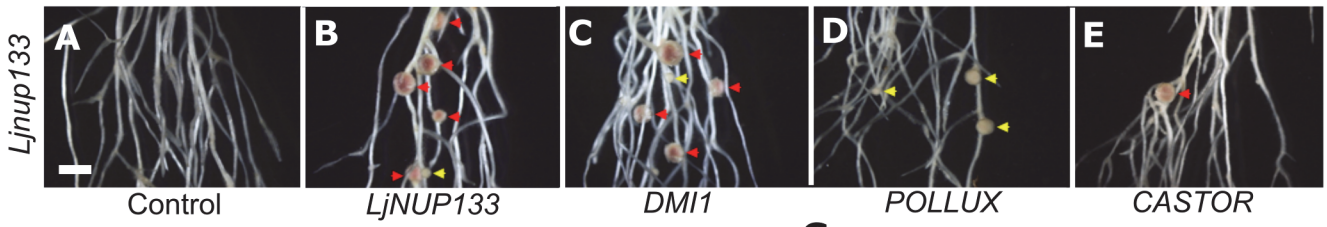

A
INM
ONM
Gold Particle Count
35
30
25
20
15
10
5
0
-90 -80 -70 -60 -50 -40 -30 -20 -10 0 10 20 30 40 50 60 70 80 90
Distance from Middle Line
Control
LjPOLLUX
B
Distance (nm)
150
100
50
0
-50
-100
-150
Wild-type
Ljnup133
Ljnup85
C
Distance (nm)
ONM
INM
Wild-type
Mtnup133

Figure 5. Mis-localization of POLLUX and DMI1 in nucleoporin mutants

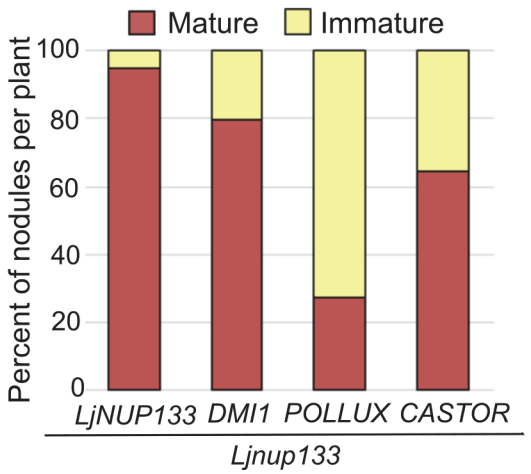
POLLUX-GFP accumulates in both the INM and ONM of transgenic Lotus roots (A). Untransformed Lotus roots immuno-labeled with anti-GFP antibodies were used as control, n = 99 nuclei. Localization of POLLUX-GFP shifts towards the ONM in the *Lotus nup85* and *nup133* (B) mutant background. The blue box highlights gold particles on the INM, while pink highlights those on the ONM. The overlap area of these boxes indicates gold particles where INM or ONM localization cannot be determined. Center of the nuclear envelope is distance = 0 nm. Mean distances of gold particles indicating DMI1 (C) localization, from the center of the nuclear envelope in wild-type and *Mtnup133*. Number of gold particles considered for the analysis (n) is > 200 for all samples. Significance as determined by Welch two sample *t*-test ($p < 0.01$) is indicated by an astericks. (*).



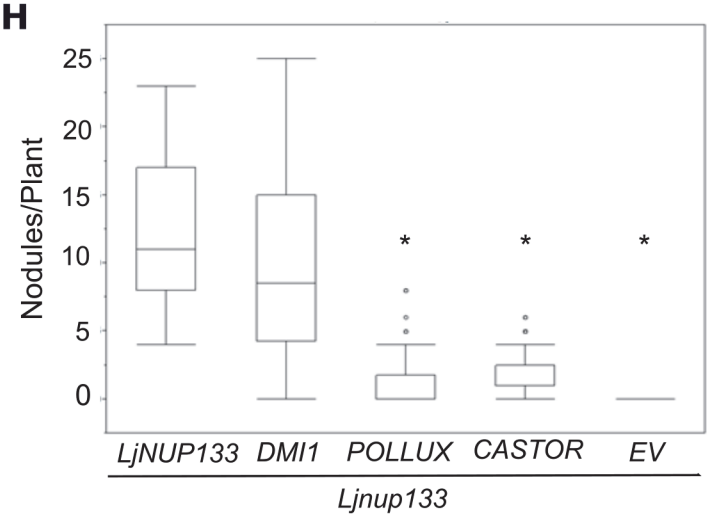
F

	Mutant line	Construct	Nodulated plants/total
A	*Ljnup133-2*	EV	0/17
B	*Ljnup133-2*	35S-NUP133	13/13
C	*Ljnup133-2*	35S-DMI1	26/28
D	*Ljnup133-2*	35S-POLLUX	23/57
E	*Ljnup133-2*	35S-CASTOR	35/57

G



H



I

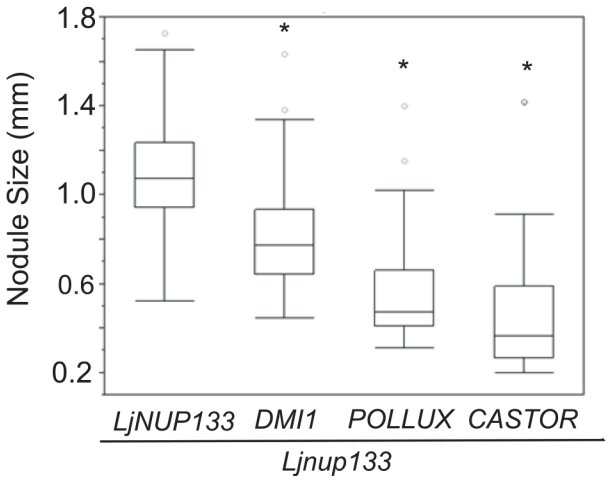


Figure 6. Complementation of *Ljnup133* with *DMI1, POLLUX* or *CASTOR*

Nodules on *Ljnup133-2* transgenic roots expressing (A) control (empty vector), (B) $35S_{pro}$*:NUP133*, (C) $35S_{pro}$*:DMI1*, (D) $35S_{pro}$*:POLLUX*, (E) $35S_{pro}$*:CASTOR*. Scale bar = 1 mm. Arrows indicate immature (yellow) and mature (red) nodules. Number of plants nodulating out of total plants transformed is indicated (F). Immature (yellow) vs mature (red) nodules were also quantified in this experiment (G). Box plot showing average number of nodules/transformed *Ljnup133-2* plants expressing *LjNUP133, DMI1, POLLUX* and *CASTOR* under the 35S promoter 4 weeks after inoculation with *M. loti.* Plants transformed with an empty vector served as controls. (H). Significance denoted was determined by Steel test. (I) Box plots showing mean nodules size in complemented *Ljnup133-2* transgenic plants expressing *LjNUP133, DMI1, POLLUX* and *CASTOR* under the 35S promoter 4 weeks after inoculation with *M. loti*. Plants transformed with *LjNUP133* served as a control. Significance denoted was determined by Steel test.

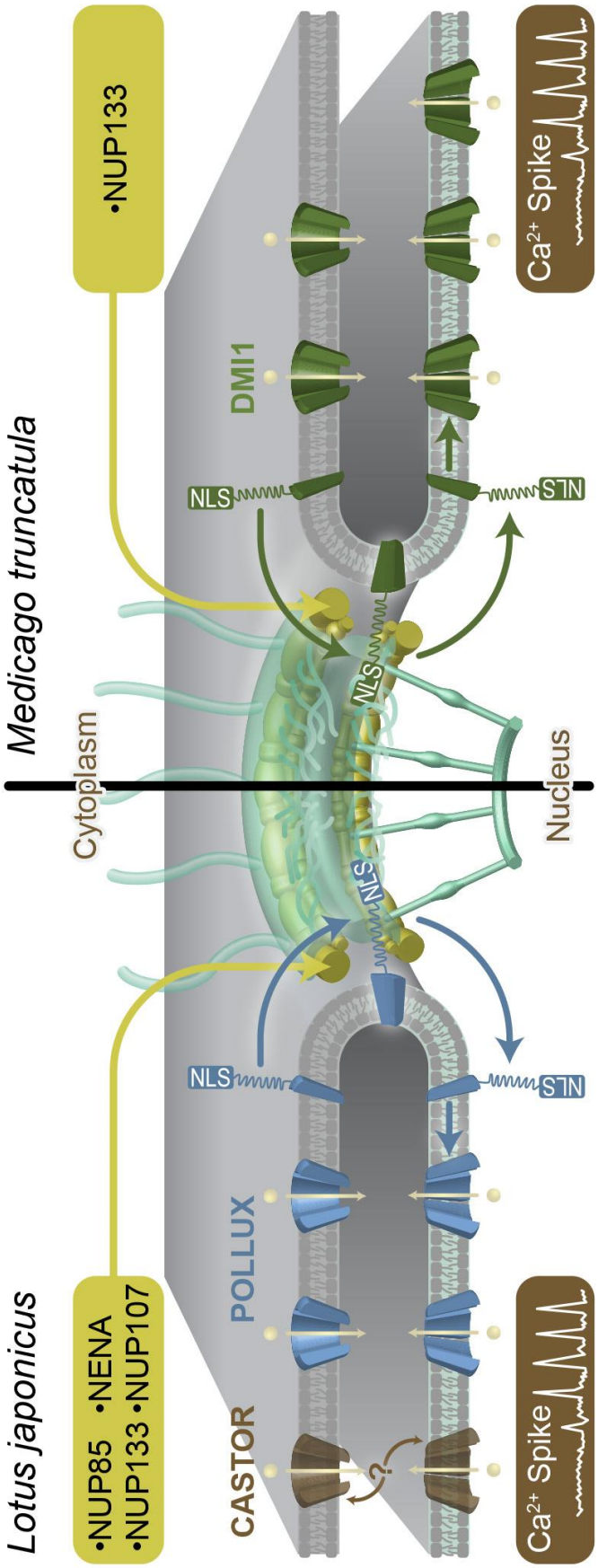

Lotus japonicus
•NUP85 •NENA
•NUP133 •NUP107
•NUP133
Medicago truncatula
Cytoplasm
Nucleus
CASTOR
POLLUX
DMI1
NLS
NLS
NLS
NLS
NLS
NLS
Ca2+ Spike
Ca2+ Spike
?

Figure 7. Model of nuclear import for *DMI1* and *POLLUX*

NUP133, NUP85, NENA and LORE1 in Lotus and NUP133 in Medicago are part of the NPC scaffold (yellow) and our data suggest that this region of the NPC is involved in transport of POLLUX (blue) and DMI1 (green) to the INM. We propose that transport to the INM is via the central channel, facilitated by the N-terminal NLS and unstructured regions of these proteins. Precise localization of CASTOR on the INM or ONM of nuclear envelope it not yet resolved. Localization to the INM is most likely critical for the function of these counter-ion channels in facilitating the accumulation of calcium within the nucleus during calcium spiking, but the role of the ONM is unclear.

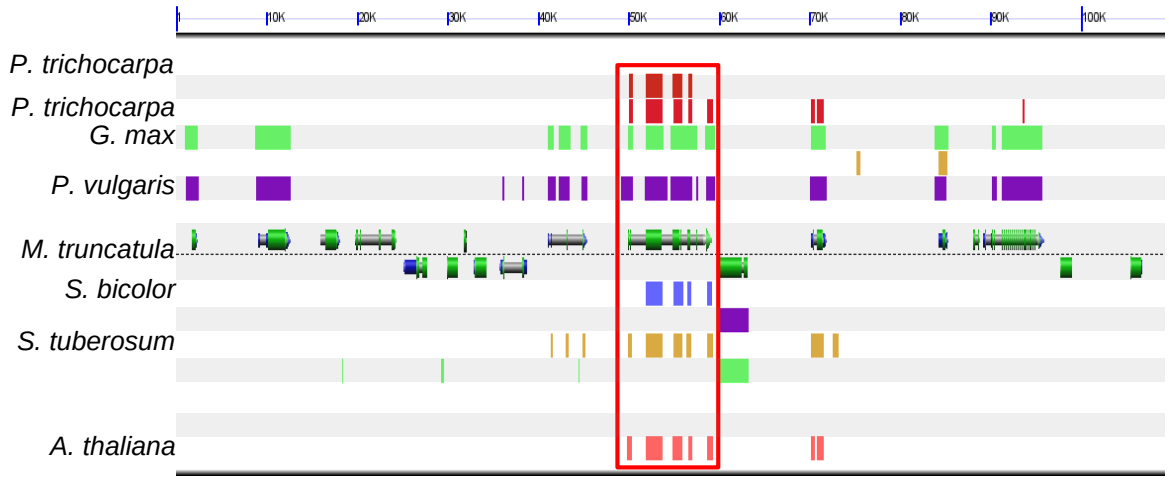
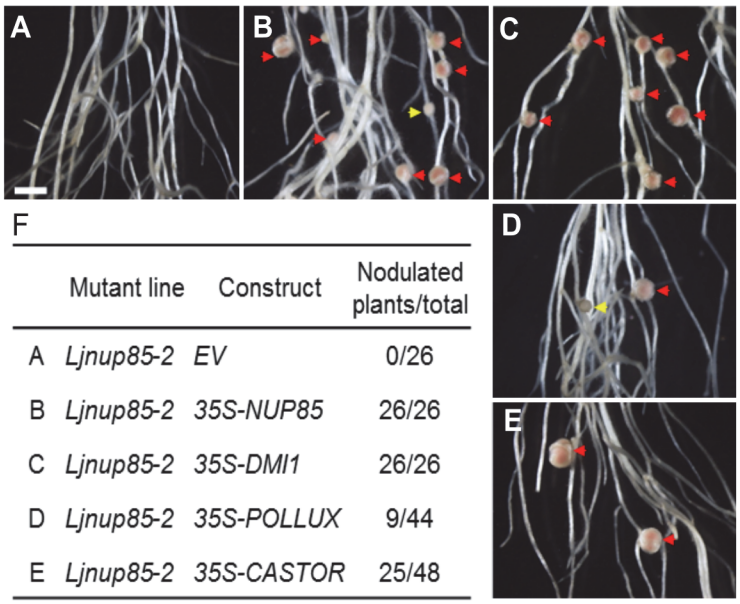

10K
20K
30K
40K
50K
60K
70K
80K
90K
100K
P. trichocarpa
P. trichocarpa
G. max
P. vulgaris
M. truncatula
S. bicolor
S. tuberosum
A. thaliana

Figure S1. Synteny analysis of the *NUP133* locus in angiosperms.

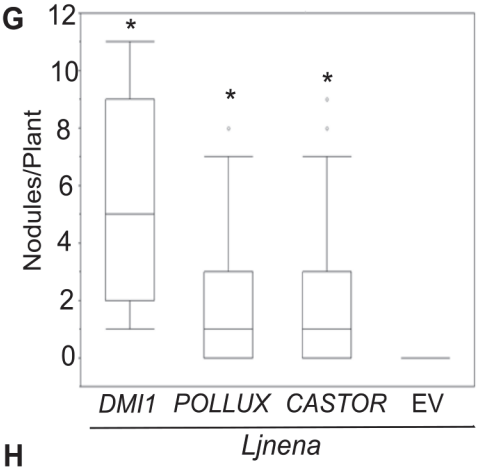
A 200Kb region surrounding the *NUP133* locus in *Medicago truncatula* was compared to the same genomic region in six other angiosperms. Synteny was confirmed for all of them but the monocot *Sorghum bicolor*.



F

	Mutant line	Construct	Nodulated plants/total
A	*Ljnup85-2*	*EV*	0/26
B	*Ljnup85-2*	*35S-NUP85*	26/26
C	*Ljnup85-2*	*35S-DMI1*	26/26
D	*Ljnup85-2*	*35S-POLLUX*	9/44
E	*Ljnup85-2*	*35S-CASTOR*	25/48

G



H

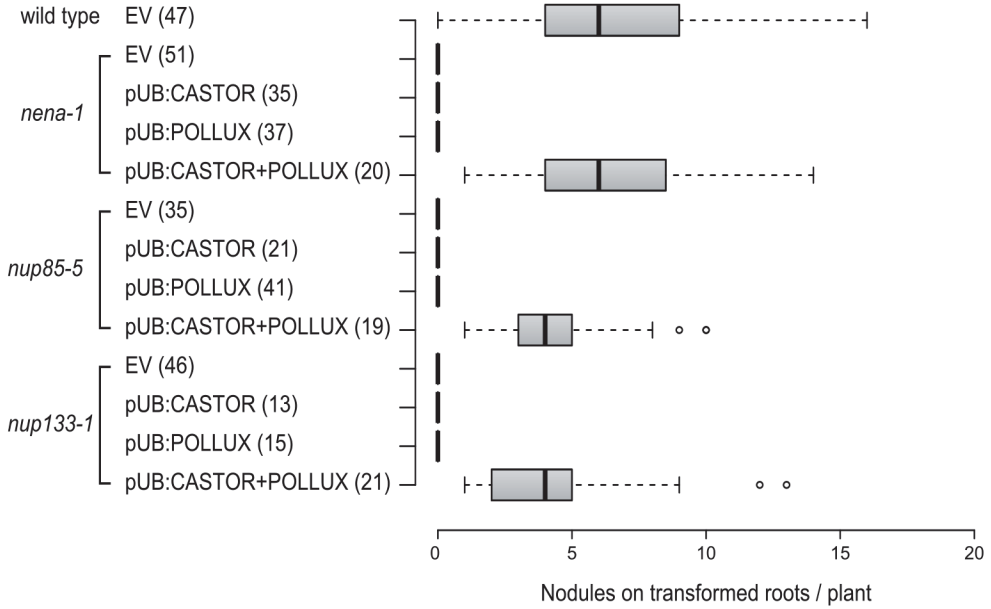
Mutant line	Construct	Nodulated plants/total
Ljnena-6	*EV*	0/17
Ljnena-6	*35S-DMI1*	39/41
Ljnena-6	*35S-POLLUX*	20/27
Ljnena-6	*35S-CASTOR*	27/43

Figure S2. Complementation of *Ljnup85 and Ljnena* with *DMI1, POLLUX* or *CASTOR*

Nodules formed on *Ljnup85-2* transformed with (A) empty vector (EV), (B) *35S$_{pro}$:NUP85*, (C) *35S$_{pro}$:DMI1*, (D) *35S$_{pro}$:POLLUX* and (E) *35S$_{pro}$:CASTOR*. Red and yellow arrows indicated mature and immature nodules, respectively. *Ljnup85-2* plants nodulated out of total transformed (F). Box plot showing average number of nodules per *Ljnena-6* plant transformed with *DMI1, CASTOR* and *POLLUX* under the 35S promoter four weeks after inoculation of *M. loti* (G). Significant difference from empty vector control (EV) denoted by asktericks (*) was determined by Steel-Dwass test. *Ljnena-2* plants nodulated out of total transformed (H).

A

mutant line	construct	Nodulated plants / total	Average nodules / transformed roots
wt	EV	47/47	6,2
nena-1	EV	1/51	0
nena-1	pUB:CASTOR	0/35	0
nena-1	pUB:POLLUX	0/37	0
nena-1	pUB:CASTOR+POLLUX	20/20	6,2
nup85-1	EV	0/35	0
nup85-1	pUB:CASTOR	0/21	0
nup85-1	pUB:POLLUX	0/41	0
nup85-1	pUB:CASTOR+POLLUX	19/19	4,9
nup133-1	EV	0/46	0
nup133-1	pUB:CASTOR	0/13	0
nup133-1	pUB:POLLUX	0/15	0
nup133-1	pUB:CASTOR+POLLUX	21/21	4,6



B

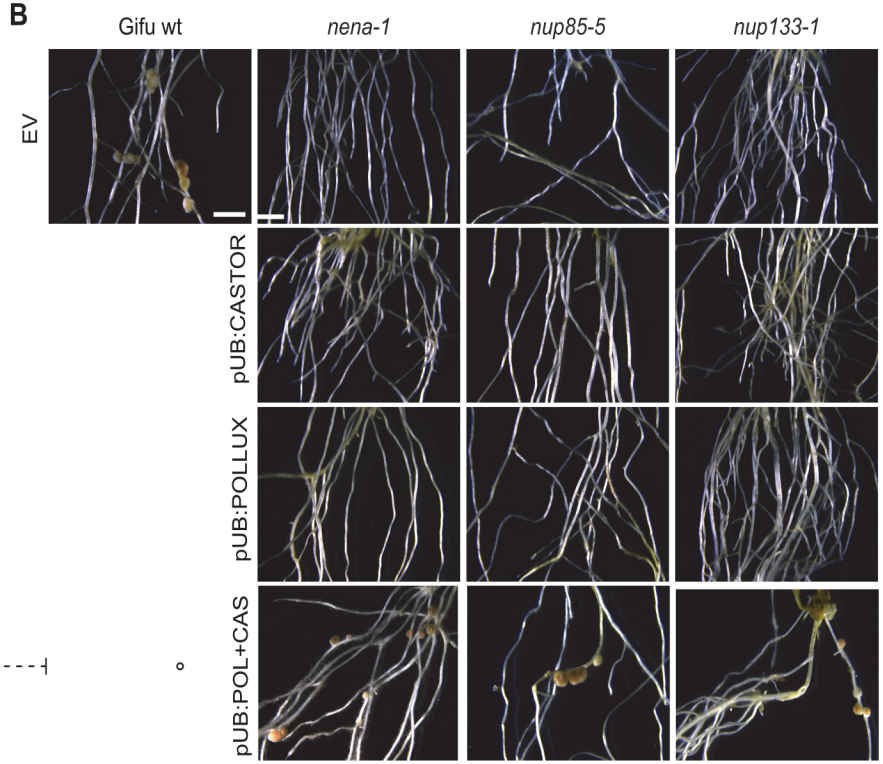


Figure S3. Co-over-expression of CASTOR and POLLUX rescues *Ljnup133, Ljnup85* and *Ljnena*

A) Over-expression of both CASTOR and POLLUX (UB_{pro}CASTOR+POLLUX) complements the nodulation phenotype in Lotus *nena-1, nup85-1* and *nup133-1* mutants, while individual overexpression of CASTOR and POLLUX (UB_{pro}CASTOR / UB_{pro}POLLUX) does not. Nodulation was analyzed 4 weeks after inoculation with *M. loti* MAFF303099. B) Stereomicroscopic images of transformed root systems. Colonized nodules are visualized by the fluorescence of dsRED expressed by *M. loti* MAFF303099. Scale bar = 3 mm. EV = empty vector control transformed with $35S_{pro}$:GFP.

CHAPTER 3: ACTIVATION OF SYMBIOTIC SIGNALING AND ROOT ARCHITECURE MODIFICATION BY ARBUSCULAR MYCORRHIZAL FUNGI IN RICE

This chapter is a modified version of the following publication:

Jongho Sun, J. Benjamin Miller, Emma Granqvist, Audrey Wiley-Kalil, Enrico Gobbato, Fabienne Maillet, Sylvain Cottaz, Eric Samain, Muthusubramanian Venkateshwaran, Sébastien Fort, Richard J. Morris, Jean-Michel Ané, Jean Dénarié, and Giles E.D. Oldroyd. Activation of Symbiosis Signaling by Arbuscular Mycorrhizal Fungi in Legumes and Rice. *Plant Cell*. 2015 Mar; 27(3):823-38.

A.W.-K. performed all the lateral root assays, analyzed data, assisted in editing the manuscript and extensively re-wrote the manuscript for inclusion in this thesis chapter. Calcium spiking assays included in the thesis chapter were conducted by J.S.

ABSTRACT

Plants establish root symbioses with arbuscular mycorrhizal fungi to facilitate nutrient acquisition. Establishment of this interaction requires plant recognition of diffusible signals from the fungus, including lipo-chito-oligosaccharides (LCOs) and chito-oligosaccharides (COs). Nitrogen-fixing rhizobia bacteria that form symbioses with leguminous plants also signal to their hosts via LCOs (Nod factors). Here we have assessed the induction of symbiotic signaling processes by the mycorrhizal (Myc)-produced LCOs and COs in rice. We show that chitin oligomers CO4 and CO8, but not Myc-LCOs, activate symbiotic calcium oscillations in rice atrichoblasts, however, CO4 and Myc-LCOs combined were required to induce calcium spiking in root hair cells. In contrast, lateral root emergence was promoted in rice by NS-LCO, S-LCO and CO4 in a DMI3 and POLLUX dependent manner. Our work suggests that COs and LCOs are perceived using the symbiotic signaling pathway and may function in a synergistic manner to promote full symbiotic responses in rice.

INTRODUCTION

Interactions with arbuscular mycorrhizal (AM) fungi are widespread within the land plant lineage and this association facilitates nutrient update, drought resistance and pathogen resistance (1). In contrast, associations with nitrogen-fixing rhizobia leading to nodules are restricted primarily to legumes (2). Both interactions require host-microbe communication for the establishment of the association which involves the release of diffusible signals from the plant, strigolactones and flavonoids, and the production of diffusible signals from the symbionts (3).

Rhizobia bacteria signal to the plant with Nod factors or lipo-chito-oligosaccharides (LCOs) that contain a chitin backbone substituted with an N-acyl chain and a number of additional groups (sulfate, sugar residues etc.) that vary based on species of rhizobia (4). Perception of Nod factors requires a signaling pathway in the host that is also involved in the establishment of mycorrhizal associations, suggesting commonalities in the mechanisms of symbiosis signaling (3). Indeed, AM fungi also produce LCOs called Myc factors, which are simpler in structure than Nod factors but nonetheless elicit symbiotic responses in *Medicago truncatula* (5). A key feature of this common symbiosis signaling pathway is the induction of nuclear calcium oscillations, termed calcium spiking, which occur during initial perception by the host plant of rhizobia and AM fungi (6-8).

In addition to the LCOs AM fungi also produce short chain chito-oligosaccharides (COs), which are capable of activating calcium oscillations in *M. truncatula* and carrot root epidermal cells (9). Only the shorter chain COs, such as tetra-acetyl chitotetraose (CO4), were found to induce repetitive calcium oscillations (9), whereas longer CO chains (CO8) did not induce calcium spiking under these conditions. It is unclear how plants discriminate between COs generated by symbiotic fungi versus those produced as part of the production or breakdown of cell walls during

fungal pathogen infection. Cell wall fractions of *Aphanomyces euteiches*, a root rot oomycete pathogen, can activate calcium responses in *M. truncatula* however germinated spore exudates from a fungal pathogen, *Collectotricum trifolii* cannot (9, 10). This suggests that there is some mechanism in place to distinguish pathogenic COs from symbiotic COs but perhaps some pathogens can elude this system.

In order to better understand the mechanisms by which AM fungi signal to the host plant we assessed the induction of symbiotic signaling in the non-legume *Oryza sativa* (rice) by the AM produced LCOs and COs. We show that rice appears to primarily respond to COs for activation of calcium oscillations, rather than LCOs like legumes, but still responds to LCOs with promotion of lateral root emergence. We conclude that mycorrhizal produced LCOs and COs activate different symbiotic signaling processes in rice that those observed in legumes.

MATERIALS AND METHODS

Seed preparation, plant growth conditions and treatment with LCOs and COs

Oryza sativa cv Nipponbare wild-type and Tos17 insertion lines in *POLLUX* (line NC6453) and *DMI3* (line 8513) were used for root architecture experiments (11,12). Seeds were prepared by sterilizing with 2% bleach for 20 minutes, followed by 3, 5 minute rinses with sterile water and then sterile seeds were imbibed overnight. Seeds were then plated on damp germination paper in petri plates under sterile conditions and germinated in the dark at 25 degrees for 7 days. Germinated rice plants were then plated on Fahraeus Medium on germination paper and grown at 22 degrees under constant light. After 5 days the plants were treated with 10^{-8}M LCOs and COs for 24 hours by submersion and re-plated onto Fåhræus Medium 1.5% agar plates (0.132 g/L $CaCl_2$, 0.12 g/L $MgSO_4.7H_2O$, 0.1 g/L KH_2PO_4, 0.075 g/L $Na_2HPO_4.2H_2O$, 5 mg/L Fe-citrate, and 0.07 mg/L each of $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $ZnCl_2$, H_3BO_3, and $Na_2MoO_4.2H_2O$, adjusted to pH 6.5

before autoclaving). As all signals were suspended in 50% ethanol, the appropriate concentration of ethanol in sterile DI water served as a control. Root system architecture was assessed after 2 weeks.

Calcium imaging

Mycorrhizal induced calcium responses were measured as described previously (7). Transgenic *Oryza sativa* nipponbare lines carrying Yellow Cameleon 3.6 (YC3.6) FRET based calcium sensor was used to detect calcium spiking. YC3.6 was imaged on a Nikon Eclipse Ti inverted microscope (Nikon, Japan) equipped with an OptoLED Illuminator (model OptoLED, Cairn Research Ltd, UK). YC3.6 was excited at a wavelength of 455nm using a royal blue LED and was captured with a charge-coupled device (CCD) camera (model RETIGA-SRV, Qimaging, Canada). Emitted fluorescence was separated by an image splitter with a dichroic mirror (model Optosplit II, Cairn Research Ltd, UK) and then passed through a cameleon filter set. Images were collected every 5 seconds with 1 second exposure and analyzed using MetaFluor (Molecular Devices, Sunnyvale, CA, USA).

Mathematical analysis of calcium oscillations

For Bayesian Spectrum Analysis we computed the most probable periods in the time series following published procedures (13). Ten traces per treatment were analyzed. The joint distributions over the period were used to characterize each group. The plots show binned data to summarize the key periods. These ten traces per treatment were also analyzed for interspike intervals. The point of maximum height for each spike was computed after detrending of the time series using a moving average algorithm. The distances between these maxima gave rise to an interspike distribution. We used the non-parametric Mann-Whitney U-test, also known as the Mann–Whitney–Wilcoxon test (14,15) to test for significant differences between the distributions.

Three traces, with altogether approximately 80 spikes, per treatment were analyzed for calcium spike characteristics. The time series have an interval of 5 seconds between data points. The traces were detrended using a moving average algorithm (16). We then characterized the spikes by the time required for each upward and downward phase. This was computed by the number of data points it took from the maximum spike height to the baseline fluctuation of the trace. The plots show the mean value of the upward and downward phases for each treatment and the associated standard deviations are indicated by the error bars.

Measurements of root architecture modifications

Total lateral roots of rice were enumerated manually two weeks after application of 10^{-8}M COs and LCOs. As COs and LCOs were suspended in 50% ethanol, the control is sterile deionized water containing the appropriate amount of ethanol. Lateral roots were defined as large and fine lateral roots emerging from crown roots, as well as fine lateral roots emerging from large lateral roots. Root system length was measured starting from the root-shoot junction to the tip of the longest root. Number of crown roots was assessed manually as those roots emerging from the root-shoot junction. Root type characterizations were based upon the descriptions in Gutjahr et al (17). Data were assessed for normality using the Shapiro-Wilk test ($\alpha = 0.01$) and statistical significance was determined using a paired *t*-test assuming unequal variances, or a Mann-Whitney-Wilcoxin Test if normality was not observed ($\alpha = 0.05$). All statistical analysis was conducted using the R software package (18).

RESULTS

Activation of calcium oscillations by Myc-LCOs and COs in rice

Mycorrhizal fungi associate with a wide range of plant species and at least in rice this association is dependent on the common symbiosis signaling pathway (11, 19-21). Hence, non-legumes should be able to recognize the mycorrhizal produced LCOs and/or COs. Two major species of LCOs have been characterized from exudates of *R. irregularis*: LCO-IV (C16:0, S or C18:1, S), which we will refer to as Sulphated (S)-LCO and LCO-IV (C16:0 or C18:1), which we will refer to as non-sulphated (NS)-LCO (5). For this study we used S-LCOs and NS-LCOs that were either purified from *R. irregularis* exudates or were synthesized in genetically-modified bacteria as previously described (5). In order to define the activation of the symbiosis signaling pathway by the AM-produced LCOs and COs, we assessed their ability to activate calcium oscillations, the pathway's earliest measurable event (3).

Calcium responses were assessed using a stably transformed line of *Oryza sativa* cv Nipponbare carrying YC3.6. AM fungi have been shown to predominantly colonize the large lateral roots (17) and therefore we focused on this root type. No calcium responses were observed following treatment with any of the LCOs assessed, but strong calcium oscillations were observed following treatment with 10^{-5}M CO4 (**Fig 1**). In order to test an array of different LCO structures we analyzed calcium spiking in response to Nod factor isolations from the broad host range rhizobial species, *Rhizobium* sp. NGR234, as well as *Rhizobium tropici*, in addition to the Myc LCOs. Considering that these LCO treatments were performed with 10^{-5}M, we are confident that rice does not respond to the Nod factors or Myc factors tested. Treatments with 10^{-8}M CO4 still showed calcium oscillations in rice epidermal cells, but the robustness of the response was reduced and the number of responsive cells was also reduced (**Fig 1**). Unlike results reported in *M.*

truncatula, rice appears to respond equally well to both CO4 and CO8 with calcium oscillations (**Fig 2**).

Mycorrhizal LCOs and COs induce rice root architecture modification

Rice has been shown to respond to AM fungi and to exudates from the spores of AM fungi, with changes to root structure, in particular the promotion of lateral root outgrowth (17, 22). These responses were independent of the common symbiosis signaling pathway. In contrast, we have observed CO4 and CO8 induction of the common symbiosis signaling pathway in rice as measured by the activation of calcium oscillations. In an attempt to understand these potentially contradictory results, we tested the promotion of lateral root outgrowth in rice by S-LCO, NS-LCO, CO4 and CO8. This study showed that NS-LCO, S-LCO, CO4 and CO8 promoted lateral root and crown root growth in rice (**Fig 3**). Interestingly, root system length was only enhanced upon application of NS-LCOs (**Fig 3**) These results imply that COs and LCOs activate two modalities of signaling in rice: calcium oscillations that are activated by COs and a separate signaling pathway activated by both LCOs and COs that is associated with changes to root architecture.

Root architecture modification by COs and LCOs are DMI3 and POLLUX dependent

To assess the role of the common symbiotic pathway in regulating root architecture modifications in response to purified LCOs and COs, we quantified root responses to mycorrhizal signals in rice knock out mutants of *pollux* (upstream of calcium spiking) and *dmi3* (downstream of calcium spiking). We found that lateral root growth promotion by LCOs and CO4 was dependent on both POLLUX and DMI3, while the response to CO8 was dependent on POLLUX (**Fig 4**). The increase in crown root growth by both LCOs and COs was dependent upon DMI3

and POLLUX, and there were significantly less crown roots in response to all treatments in the *pollux* mutant (**Fig 4**). Finally, the overall root length increase observed in response to NS-LCO was dependent upon both POLLUX and DMI3 (**Fig 4**). These results demonstrate that root architecture changes in response to purified mycorrhizal signals require proteins of the common symbiotic pathway.

Both LCOs and COs are required to induce calcium spiking in trichoblasts

Rhizobia colonize legumes by root hair cells (trichoblasts), whereas AM fungi colonize roots via non-root hair epidermal cells (atrichoblasts). Thus, these different root epidermal cell types may respond differently to COs and LCOs. To validate this we directly compared trichoblast and atrichoblast responses using high concentrations of CO4 in rice. Calcium oscillations observed in rice following treatments of CO4 were restricted to atrichoblasts, with no responses in trichoblasts even with CO4 treatments of 10^{-5}M (**Fig 5**). This preferential nature of rice atrichoblasts to respond to the AM signals is consistent with a preference for AM fungi to colonize the root via atrichoblasts (24). It is possible that either AM fungi produce signaling molecules other than S-LCO, NS-LCO and CO4 that induce calcium oscillations in rice trichoblasts or that the mix of signaling molecules is important. To test this we assessed induction of calcium oscillations by an equimolar mix of 10^{-5}M S-LCO, NS-LCO and CO4. Strikingly, we observed calcium spiking in rice root hair cells when treated with this mix of signals, yet when applied individually at 10^{-5}M these signals did not elicit calcium responses (**Fig 6**).

DISCUSSION

COs and LCOs act synergistically as symbiotic signals in rice

AM fungi signal to the host plant via diffusible signals (7, 22-24) and at least some of these signals are LCOs (5) and COs (9). In this work we show that the AM-produced COs can activate calcium oscillations in rice. S-LCO and NS-LCO were purified from exudates of AM fungi based on their capability to activate symbiotic responses in *M. truncatula* that were dependent on the common symbiosis signaling pathway (5). The fact that these LCOs do not trigger calcium spiking in rice may reflect this selectivity in their initial identification. However, it seems that rice can sense LCOs, since the mix of Myc-LCOs and CO4 activated calcium oscillations in rice root hair cells and LCOs can also promote lateral and crown root growth. Therefore, the absence of calcium responses in rice to the LCO treatments alone does not indicate a lack of response to LCOs by rice.

Our work has revealed a close correlation between cell-type and its responsiveness to LCOs and CO4. We observed that calcium responses to COs were restricted to atrichoblasts in rice. This preferential response in atrichoblasts correlates well with a preferential colonization of atrichoblasts by AM fungi (23). However, we observed calcium oscillations in rice trichoblasts in treatments where LCOs and CO4 were combined. It would appear that responses in rice trichoblasts are at least partially explained by the mix of LCOs and CO4 produced by the AM fungus. Interestingly, it was shown some years ago that a mix of Nod factors and COs was better at inducing nodulation associated gene expression in soybean than Nod factor treatments alone (25). Perhaps these earlier observations reflect responses to AM fungi, rather than what was previously thought to be a rhizobial response. Alternatively, a mix of LCOs and COs may be relevant in rhizobial interactions as well as AM associations.

Multiple pathways mediate LCO and CO responses in rice

We established that rice can distinguish between LCOs, CO4 and CO8 and responds accordingly with either calcium oscillations and/or root architecture modifications. The fact that rice responds to AM fungi with at least two separate signaling pathways has already been shown (21) and the promotion of lateral roots by AM fungi in a manner independent of the common symbiosis signaling pathway was also already shown (22). Thus there is ample evidence in rice for two pathways of symbiosis signaling. Our work shows that root architecture modification in response to LCOs and CO4 requires the common symbiotic pathway, however CO8 does not require DMI3. The ability of AM fungi to stimulate lateral root emergence independent of the symbiotic pathway may indicate that the plant responds differently to a mixture of signals and stimuli than it does to purified signals, and that during symbiosis the pathway governing root architecture modification does not require calcium spiking to be initiated.

In *Arabidopsis thaliana,* lateral root development is under the control of auxin signaling modules. Under high auxin conditions, lateral root founder cells polarize and divide (27). Further rounds of cell division result in lateral root emergence at specific sites in the root. The process leading to lateral root emergence is similar in rice and using the DR5:GUS auxin reporter system, auxin was shown to accumulate in emerging lateral roots (27). Under high auxin concentrations, AUX/IAA proteins are degraded. AUX/IAA proteins repress ARF transcriptional activators, and thus their degradation leads to the transcription of auxin responsive genes (26). Auxin positively regulates lateral root formation, as a rice plant containing a constitutively active version of IAA13 has fewer lateral roots than wild type (28). Interestingly, auxin signaling is also implicated in the production of crown roots in rice (29, 30). It seems likely, therefore, that the application of LCOs and COs activates the auxin dependent lateral root and crown root emergence programs. Given

that this phenotype was dependent on DMI3 and POLLUX in the case of Myc-LCOs and CO4, it may be that there is cross talk between the common symbiosis pathway and auxin signaling which results in increased lateral root emergence and crown root growth. Assessing expression of auxin responsive genes in *Ospollux* and *Osdmi3* mutants in response to COs and LCOs may reveal the mechanisms of this signaling pathway.

Concluding remarks

AM fungi have the distinctive capability of colonizing an extremely broad group of plants. In this work we reveal that part of this capability is reflected in the signaling molecules, CO4 and LCOs, which can activate symbiosis signaling in rice in addition to their function in legumes. This broad host range of AM fungi may be facilitated by the extent of symbiotic signaling molecules that they produce, with a mix of LCOs and COs and possibility other yet uncharacterized molecules. Clearly further work is necessary, however, we have shown that LCOs, CO4 and CO8 form at least part of the spectrum of AM symbiotic signals that can be recognized by a variety of plant species to activate a range of symbiotic signaling processes.

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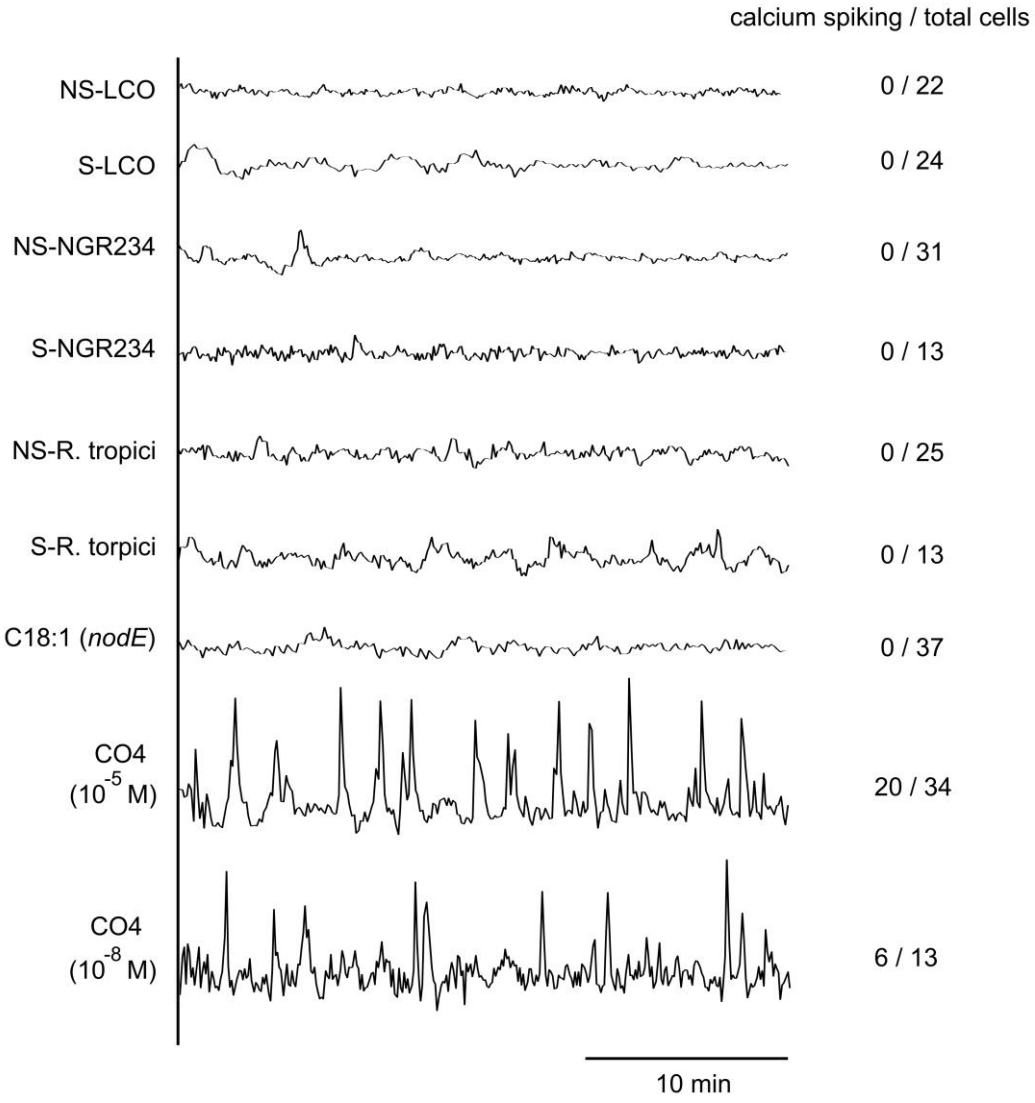
calcium spiking / total cells
NS-LCO
0 / 22
S-LCO
0 / 24
NS-NGR234
0 / 31
S-NGR234
0 / 13
NS-R. tropici
0 / 25
S-R. torpici
0 / 13
C18:1 (nodE)
0 / 37
CO4
(10-5 M)
20 / 34
CO4
(10-8 M)
6 / 13
10 min

Figure 1. Calcium responses in rice to the Myc-LCOs, Nod factors and CO4

Representative calcium traces from rice atrichoblasts treated with 10^{-5}M Myc LCOs and LCO isolations from NGR234 and *R. tropici*, as well as 10^{-5}M and 10^{-8}M treatments of CO4. The number of cells showing calcium responses, relative to the total number of cells analyzed is indicated.

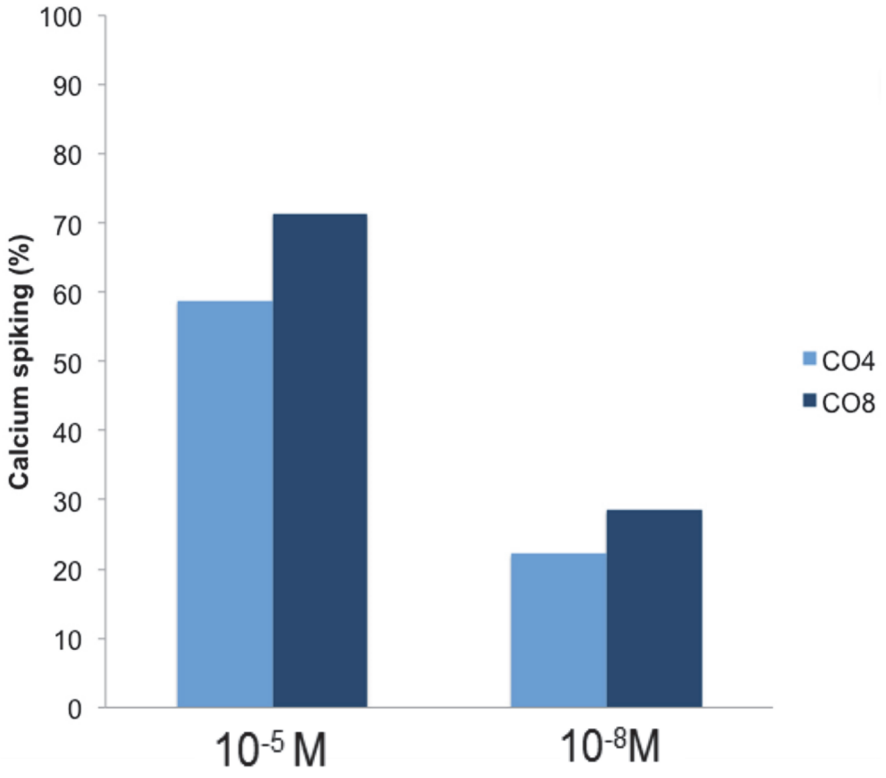
100
90
80
70
60
50
40
30
20
10
0
Calcium spiking (%)
CO4
CO8
10-5 M
10-8M

Figure 2. Calcium spiking in rice in response to CO4 and CO8

Percent of nuclei in rice atrichoblasts cells on lateral roots which undergo calcium spiking when treated with 10^{-8}M and 10^{-5} M CO4 and CO8. Plants appear to respond equally well to CO4 as to CO8.

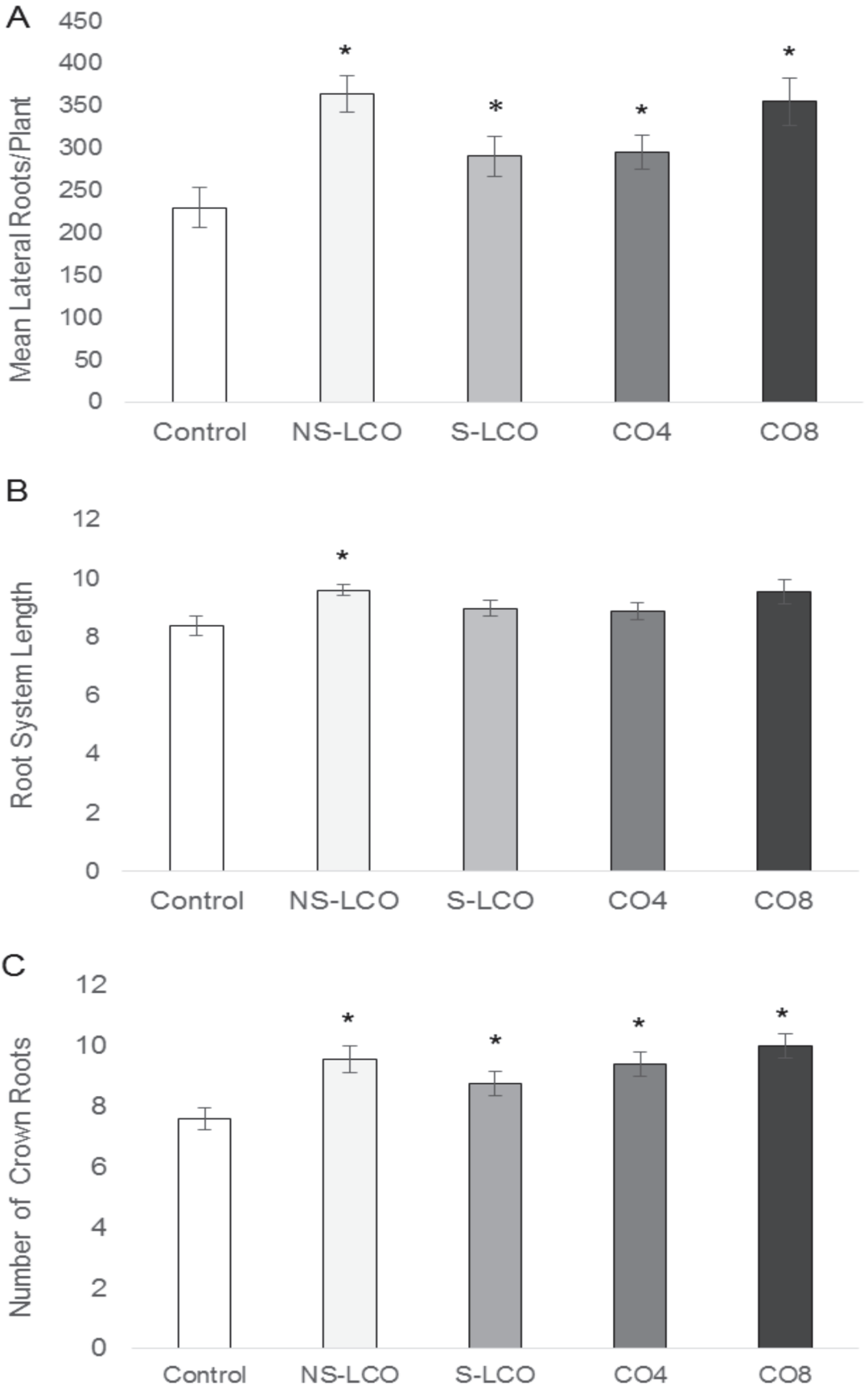
A
Mean Lateral Roots/Plant
450
400
350
300
250
200
150
100
50
0
Control
NS-LCO
S-LCO
CO4
CO8
B
Root System Length
12
10
8
6
4
2
0
Control
NS-LCO
S-LCO
CO4
CO8
C
Number of Crown Roots
12
10
8
6
4
2
0
Control
NS-LCO
S-LCO
CO4
CO8

Figure 3. Promotion of lateral root and crown root emergence in rice by LCOs and COs

The mean number of lateral roots produced per rice plant is shown in response to treatments of 10^{-8}M COs or LCOs. Plants were treated for 24 hours with each symbiotic signal and then grown for two weeks before assessing the number total number of lateral roots. Values are means ± standard error pooled from three (NS-LCO, S-LCO & CO4) or two (CO8) replicated experiments ($n \geq 28$). The *p*-value was calculated using a *t*-test, or Wilcoxon-Mann-Whitney test if data were non-normal. Significance is denoted by astericks (*) ($p < 0.05$).

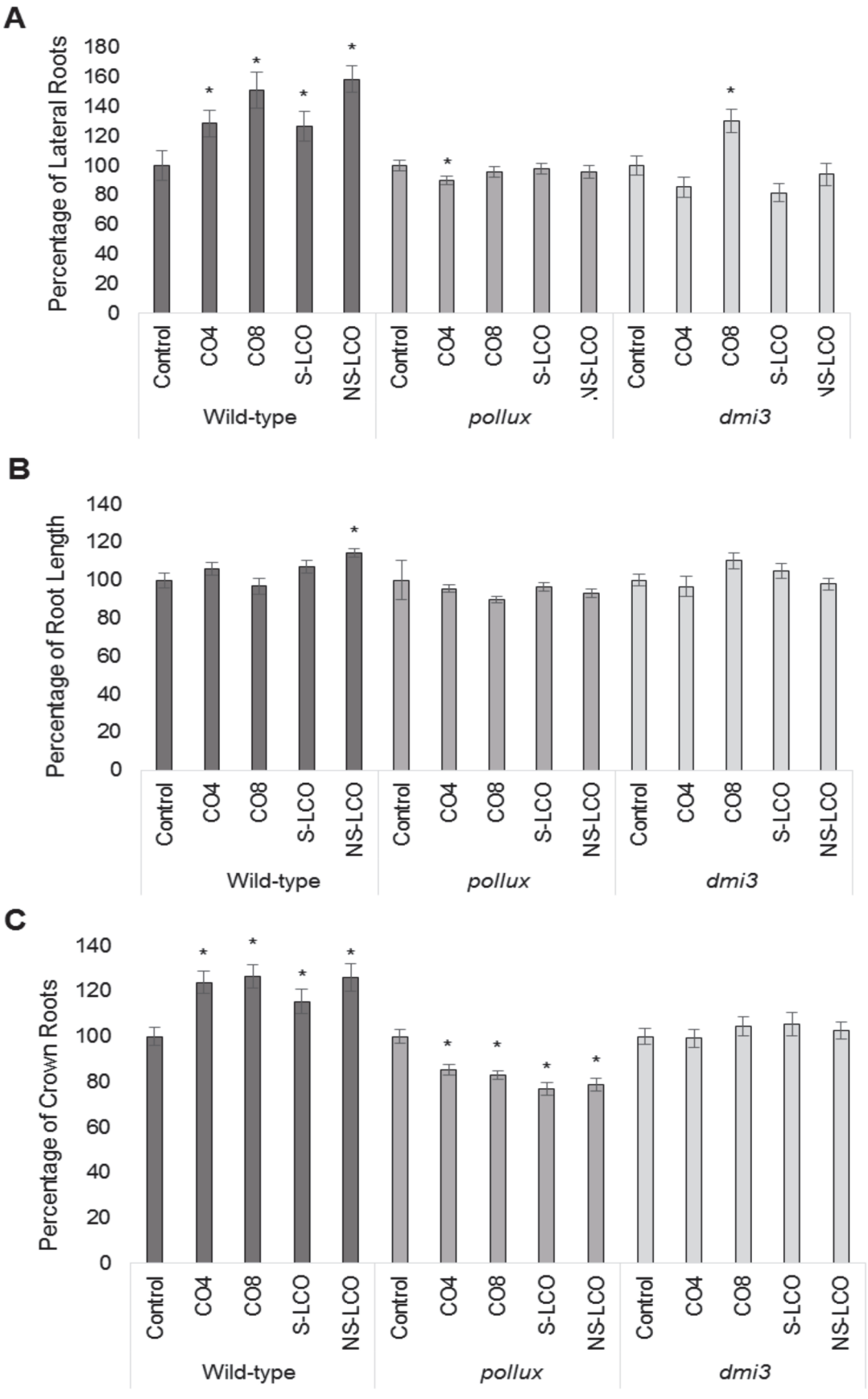
A
Percentage of Lateral Roots
180
160
140
120
100
80
60
40
20
0
Control
CO4
CO8
S-LCO
NS-LCO
Wild-type
pollux
dmi3
B
Percentage of Root Length
140
120
100
80
60
40
20
0
C
Percentage of Crown Roots
140
120
100
80
60
40
20
0

Figure 4. Root architecture responses to LCOs and COs in *Ospollux* and *Osdmi3*

The mean number of lateral roots produced per rice plant is shown in response to treatments of 10^{-8}M COs or LCOs. Plants were treated for 24 hours with each symbiotic signal and then grown for two weeks before assessing the number total number of lateral roots. Data were pooled from three replicated experiments. Values are perccents derived from dividing values by the means of the control from each respective experiment ± standard error. The *p*-value was calculated using a *t*-test. Significance is denoted by asterisks (*) ($p < 0.05$).

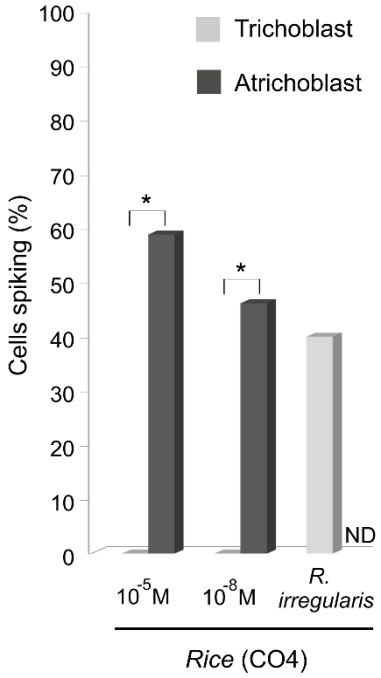
100
90
80
70
60
50
40
30
20
10
0
Cells spiking (%)
Trichoblast
Atrichoblast
*
*
ND
10-5M
10-8M
R. irregularis
Rice (CO4)

Figure 5. Calcium responses in rice trichoblasts and atrichoblasts

The percentage of calcium responsive cells among trichoblasts and atrichoblasts of rice. Treatments of 10^{-5}M CO4 and the response of trichoblasts in close proximity to *R. irregularis* hyphae were analyzed

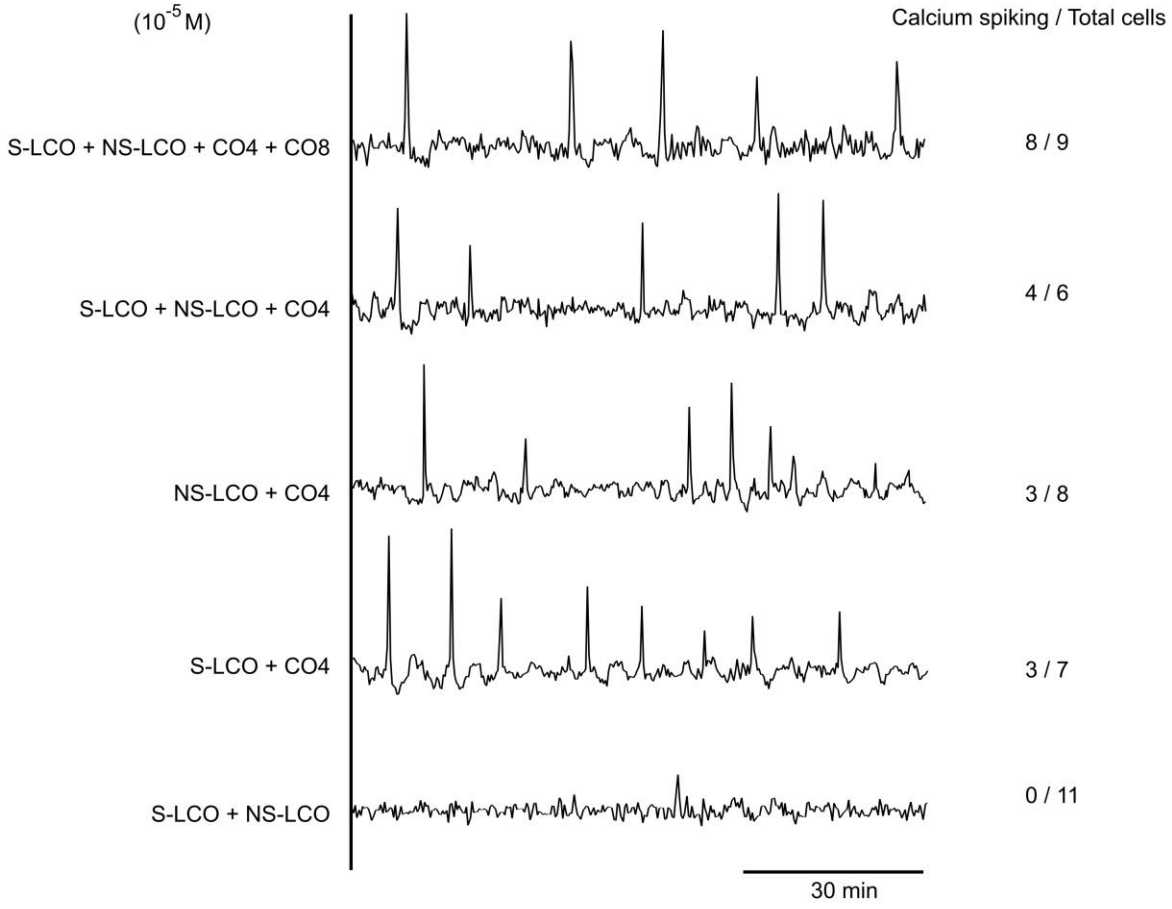
(10^{-5}M)
Calcium spiking / Total cells
S-LCO + NS-LCO + CO4 + CO8
8 / 9
S-LCO + NS-LCO + CO4
4 / 6
NS-LCO + CO4
3 / 8
S-LCO + CO4
3 / 7
S-LCO + NS-LCO
0 / 11
30 min

Figure 6. Calcium responses to LCOs and COs in rice trichoblasts

Representative calcium traces of root hair cells (trichoblasts) treated with mixes of 10^{-8}M CO4, S-LCO and NS-LCO. Note that mixes of Myc-LCOs with COs induced calcium oscillations, but the Myc-LCOs alone do not. The number of cells showing calcium responses, relative to the total number of cells analyzed is indicated.

CHAPTER 4: CHARACTERIZING THE COLONIZATION OF CEREALS BY *RHIZOBIUM* SP. IRBG74

This chapter is a preliminary version of the following manuscript:

Wiley-Kalil, A., Crook, M., Mukherjee, A., Maeda, J., Babcock, M., Erdn, S., Poinsot, V., Maillet, F., Volkening, J., Sussman, M., Prasad, G., Dénarié, J. and Ané, J.M. The Double Life of *Rhizobium* sp. IRBG74. *Plant Cell.*

Contribution: A.W.-K. participated in the experimental design, data analysis, conducted most of the colonization and all nodulation assays, as well as the RNAseq experiment. A.W.-K will write the manuscript in collaboration with M.C

ABSTRACT

Athough rhizobia are best know for their well studied associations with legumes, several species of rhizobia can colonize cereal crops. However, little is known about the host and microbial determinants which underly these interactions. Arbuscular mycorrhizal fungi also colonize both legumes and non-legumes, and signal to their hosts with lipo-chito-oligosaccharide (LCO) molecules, termed Myc factors, which are similar in structure to those produced by rhizobia during symbiosis with legumes. These fungi also produce short chitin oligomers such as CO4 and CO8, which are known to stimulate symbiotic responses in legumes. We found that while some rhizobia can colonize rice, this trait is not ubiquitous with several *Sinorhizobium* species tested being unable to colonize rice roots. This suggests that there is at least some factors which give host specificity to rice-rhizobium interactions. *Rhizobium* sp. IRBG74 can internally colonize roots of both rice and *Setaria viridis* and produces similar but not identical Nod factors to *Azorhizobium caulinodans*. Two of the Nod factor structures (fucosylated and arabinosylated) produced by *Rhizobium* sp. IRBG74 in particular are required for normal nodulation of a legume host**,** *Lotus japonicus,* however, Nod factors were not required for interactions between *Rhizobium* sp. IRBG74 and cereal hosts. Instead, internal colonization (*S. viridis)* and surface attachment (rice) were facilitated by the production of chitin oligomers as evidenced by colonization defects of the *Rhizobium* sp. IRBG74 Δ*nodC* strain. Nod factors of *Rhizobium* sp. IRBG74, however, do trigger lateral root growth responses similar to those induced by mycorrhizal fungi. Transcriptome analysis revealed that germinated spore exudates (GSEs) of mycorrhizal fungi and *Rhizobium* sp. IRBG74 Nod factors both differentially regulate a set of 43 genes, whereas GSEs had very little overlap with other LCO and CO treatments. This work suggests that *Rhizobium* sp. IRBG74 is a unique cereal

endosymbiont which may partially co-opt the mycorrhizal pathway to facilitate colonization of the non-legume host.

INTRODUCTION

Rhizobia are gram negative alpha- and-beta proteobacteria which establish a nitrogen-fixing symbiosis primarily with leguminous plants. This symbiosis is of critical importance to agriculture, where legumes are responsible for the addition of approximately 40 million metric tons of fixed nitrogen to the environment every year (1). A central feature of this symbiosis is the formation of a new root organ, called the nodule, in which the rhizobia are housed and where nitrogen fixation can take place. Cereals are also able to form associations with rhizobia, but these interactions do not result in nitrogen fixation or nodule formation. The formation of nitrogen-fixing nodules relies upon the establishment of a species-specific molecular dialogue between rhizobia and their host. Understanding how rhizobia communicate with cereals in order to facilitate root colonization is the first step to engineering nitrogen fixation in cereals and alleviating our dependence upon synthetically fixed nitrogen fertilizer.

The legume–rhizobium symbiosis is initiated upon rhizobial perception of plant root-secreted flavonoids and isoflavonoids which upregulate *nodD* gene expression in the bacteria (2, 3). *nodD* genes encode transcriptional activators that control expression of the rest of the *nod* gene family, the induction of which results in the production of Nod factors (4). Nod factors are lipo-chito-oligosaccharides (LCOs) and the *nodABC* operon, which is present in all Nod factor-producing rhizobia, is responsible for production of the basic LCO structure. The first step in Nod factor synthesis is production of the chitin oligosaccharide (CO) by NodC, followed by deacetylation of the non-reducing N-acetylglucosamine residue by NodB, which allows for the addition of the lipid chain to that residue by the acyltransferase NodA (5-7). Other Nod proteins

such as NodZ or NodU, responsible for addition of a fucose or carbamoyl residue respectively, further modify the LCO, resulting in the production of a diverse array of Nod factor structures (8, 9). While Nod factor producing rhizobia all contain the *nodABC* operon, the other *nod* genes present are species specific and in turn determine the unique cocktail of Nod factors produced (10, 11). The types of Nod factors a rhizobial species synthesizes is the primary determinant of its leguminous host range (12).

In symbiosis with legumes, production of Nod factors is synchronized with rhizobial attachment to root hairs (13). The combination of the Nod factors and rhizobial attachment triggers root hair deformations which result in a shepherd's crook shape that curls around and entraps the bacteria. From here, the rhizobia form a micro-colony and colonize the plant via a plant-derived tube-like structure, called an infection thread, which is formed at the tip of the root hair and grows down into the cortical cell layer (13).Cortical cells below the site of infection re-enter cell division cycles and initiate production of the nodule primordium. The rhizobia are then released into the cells of the nodule within a membrane bound symbiosome, where they develop into nitrogen-fixing bacteroids (14). Root hair infection is the classical mode of entry into the legume host, but some rhizobia are able to take advantage of cracks, which occur naturally due to damage or lateral root outgrowth, to gain entry to the cortical cell layer. However, Nod factors are usually (15), but not always (16, 17), still required at later stages of this symbiosis for nodule formation.

Perception of Nod factors by the legume host requires a symbiotic signaling pathway that culminates in oscillations of calcium concentration in the nucleus (calcium spiking) and subsequent expression of genes required for the formation of the symbiosis (18, 19). This signaling pathway includes a plasma membrane leucine rich repeat receptor like kinase (DMI2) and a nuclear cation channel (DMI1) required for induction of calcium spiking upon Nod factor addition, and a

calcium- and calmodulin-dependent protein kinase (DMI3), and DMI3 interactor (IPD3), which are activated by calcium spiking to induce transcription of symbiotic genes via the activity of transcription factors (NSP1, NSP2, NIN, ERN1) (19-28). This signaling pathway is also critical for the formation of arbuscular mycorrhizal symbiosis and thus is often referred to as the common symbiosis pathway (29, 30). Utilization of this pathway by arbuscular mycorrhizal fungi is likely due to the fact that they also produce LCOs (Myc factors) which induce calcium spiking (31). In addition to LCOs, mycorrhizal fungi produce chitin oligosaccharides (COs), but their role in symbiosis is unclear (32).

Arbuscular mycorrhization is widespread in the plant lineage, including cereal crops, and benefits the plant via increased access to soil nutrients, such as potassium and nitrogen (33). In addition to these benefits, mycorrhizal fungi have been shown to enhance rice lateral root production independently of the common symbiosis pathway (34). Lateral root growth promotion is likely due to the production of a diffusible signal, as the application of arbuscular mycorrhizal germinated spore exudates is sufficient to recapitulate this response (35). Interestingly, both Myc and Nod factors can induce lateral root production in *Medicago truncatula* and this response requires components of the common symbiosis pathway (31, 36). Whether or not non-legumes would respond to Nod factors with lateral root growth promotion is unknown.

Non-legumes are able to associate with several species of rhizobia. These are in many cases symbionts of legumes which are grown in close association with a non-legume, for example, maize–bean and *Sesbania* species – rice production systems. However, the extent to which diverse rhizobial species can colonize non-legumes has not been investigated in a systematic manner (37-41). Nod factors have previously been found to be dispensable for associations between

Azorhizobium caulinodans and *Arabidopsis thaliana* (42, 43), but this may not be the case for all interactions formed between non-legumes and rhizobia.

To better understand the associations formed between cereals and rhizobia, we analyzed a diverse panel of rhizobia for their ability to colonize rice roots. We also conducted a detailed analysis of the symbioses formed between cereals and *Rhizobium* sp. IRBG74 as this species can colonize rice and enhance growth, and thus is an attractive target for use as a bio-fertilizer (40). To dissect the role of specific Nod factor structures in rice–rhizobium symbiosis versus legume nodulation we performed targeted deletion of *Rhizobium* sp. IRBG74 *nod* genes and analyzed the nodulation or colonization phenotype in both legumes and non-legumes of the resultant *nod* mutant strains. This work, along with lateral root assays and gene expression analysis identified plant-, rhizobial-, and signal-specific host responses. It is clear that cereals interact with rhizobia on a species-specific basis and that in some cases, associations are enhanced by the production of signaling molecules.

MATERIALS AND METHODS

Plant Growth

To surface disinfect, *Oryza sativa* cv. Nipponbare seeds were treated with 2% sodium hypochlorite solution for 15 minutes, followed by three 5-minute rinses in sterile water. *Setaria viridis* seeds were treated with a 1% sodium hypochlorite, 0.1% sodium doecyl sulfate (SDS) solution for 15 minutes, followed by 2 rinses with sterile water, then treated with 75% ethanol for 5 minutes, followed by 5 rinses with sterile water. After sterilization, rice and *S. viridis* seeds were plated onto damp germination paper in sterile Petri dishes and germinated in the dark at 24°C for 5–7 days. *Lotus japonicus* seeds were scarified in sulfuric acid for 8 minutes and rinsed in DI water 5 times, then sterilized in 8.25% sodium hypochlorite for 2 minutes, followed by 6 rinses in sterile

water. *L. japonicus* seeds were then imbibed in sterile water overnight before plating onto 1.5% water agar containing 1 µM gibberellic acid (GA3 Agar). After 2–4 days at 4°C to synchronize germination, *L. japonicus* was germinated in the dark at room temperature for 5 days. *Sesbania cannabina* seeds were scarified in sulfuric acid for 30 minutes followed by 6 rinses in sterile water before plating onto GA3 agar and germinating in the dark at 37°C for 3 days.

Bacterial culture

Strains used in this study are listed in Table 1. *Escherichia coli* and rhizobia cultures were grown at 37°C and 30°C respectively, on Luria–Bertani (LB) medium or Tryptone-Yeast (TY) medium. TY medium contains (per liter): 6 g tryptone, 3 g yeast extract, 0.5 g $CaCl_2$, pH 7.0. Where required, the media were supplemented with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc, 60 ng/µl), ampicillin (Ap, 100 ng/µl), chloramphenicol (30 ng/µl), gentamicin (Gm, 50 ng/µl for *E. coli* and 150 ng/µl for rhizobia), kanamycin (Km, 50 ng/µl), rifampicin (Rf, 50 ng/µl), spectinomycin (Sp, 50 ng/µl), sucrose (100 µg/µl), tetracycline (Tc, 10 ng/µl).

Strain and plasmid construction

Plasmids used in this study are listed in Table 2. All custom oligonucleotides were purchased from Integrated DNA Technologies and are listed in Table 3. Mobilization of plasmids was accomplished by triparental mating with helper *E. coli* B001 (DH5α harboring plasmid pRK600). pRK600 expresses *trans*-acting proteins required for mobilization of plasmids harboring the RK2 transfer origin (*oriT*). Briefly, the *E. coli* donor (containing the plasmid of interest), the *E. coli* helper (B001), and the rhizobial recipient strains were combined and allowed to grow together in the absence of antibiotics for 18–24 hours at 30°C, following which the cells were transferred to selective plates, grown for another 18–24 hours at 30°C, and single colonies were recovered. Rifampicin-resistant colonies of rhizobia were recovered following overnight growth

in LB at 30°C at 225 rpm. These rhizobia were then tagged with the *gusA*-expressing transposon mTn*5*ss*gusA21* from pCAM121 by triparental mating (see description above) followed by selection on LB-Rf-Sp-XGluc. Blue colonies were recovered and the locations of insertions were determined by arbitrary polymerase chain reaction (PCR). For deletion of *nod* genes, 500-bp fragments up- and downstream of the target gene were cloned and fused together by overlap-extension PCR using *Pfu* polymerase, subcloned into pENTR/D-TOPO (Invitrogen), and then recombined into the *sacB* vector, pJQ200SK, by the Gateway system (Invitrogen). Deletion constructs were introduced into *Rhizobium* sp. IRBG74 by triparental mating (see description above) followed by plating on LB-Sp-Gm. The plasmid integration was resolved by selection on LB-10% sucrose, and the deletion was confirmed by loss of resistance to gentamicin and by PCR.

Root colonization assays

Rice and *S. viridis* were plated onto Fåhræus medium after germination and grown for 5 days prior to inoculation. Plants were inoculated by flooding the plates with 50 ml of an OD_{600} = 0.1 or 0.6 culture of rhizobia for ten minutes and then the excess was removed. Plants were grown at room temperature under constant light for two weeks before colonization was assessed. For the rhizobial diversity panel screen on rice, colonization was visualized using strains carrying a GUS visual reporter. Plants were vacuum infiltrated in GUS staining solution for 10 minutes followed by incubation for 6–12 hours at 37°C. The staining solution contained 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 50 mM sodium phosphate (pH 7.2), 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, and 5 mM Na-EDTA, as described by Journet *et al*. (44). Internal colonization was assessed by surface sterilization followed by grinding pre-weighed roots in 5 ml of 1× Phosphate Buffered Saline (PBS). Surface sterilization was achieved by vortexing the roots in 1× PBS for 1 minute, followed by incubation for 30 seconds in 75% ethanol, inverting to mix.

Next roots were submerged in a 1.5% sodium hypochlorite solution for 30 seconds and then roots were rinsed four times in sterile water. The water from the fourth rinse was plated to assess efficacy of surface sterilization. 10-fold serial dilutions were performed on ground plant material and plated onto LB-Sp agar. Plates were incubated for 24–48 hours at 30°C before counting colonies and calculating colony forming units (CFUs) per mg of root tissue. Surface attachment was enumerated by vortexing roots in 1× PBS for 1 minute and then removing the plant. 10-fold serial dilutions were performed on the bacteria that were detached in the PBS solution and plated onto LB-Sp agar.

Rhizobium sp. IRBG74 localization on rice and _Setaria viridis_

Plant roots inoculated with *Rhizobium* sp. IRBG74 (OD_{600} = 0.6) carrying a GUS or GUS/GFP marker were imaged directly by confocal microscopy or following GUS staining using light microscopy or sectioned prior to visualization by light or confocal microscopy (Table 1). For sectioning, plant roots were embedded in 4% low temperature melting agar, cut into blocks and sectioned using a Vibratome™ into 25 μm-thick sections. For confocal localization of *Rhizobium* sp. IRBG74 GUS/GFP, inoculated roots were stained with either FM4-64 (LifeTechnologies #T13320), a fluorescent membrane marker, or propidium iodide, a cell wall stain. Propidium iodide was also used as a live/dead cell marker as only dead cells will display nuclear staining. Prior to imaging roots were dipped into a solution of 15 μM propidium iodide in water for 5 minutes before mounting in sterile water. Roots stained with FM4-64 were dipped into a 15 μM dye solution in water on ice before immediately imaging. The 488 nm laser (GFP) and the 536 nm laser (propidium iodide/FM4-64) were used to excite the sample, and emission was captured in the 510–550 nm (GFP), the 550–600 nm (FM4-64), or the 600–650 nm (propidium iodide) range using a Zeiss LSM510 microscope.

Nodulation Assays

Lotus japonicus grown on Fåhræus medium plates containing minimal ammonium nitrate (0.5 mM) for 5 days was inoculated with 50 ml of an OD_{600} = 0.1 culture of rhizobia per plate. Excess was poured off, plates were wrapped in parafilm, and roots were covered using aluminum foil. Plants were grown at room temperature on light shelves under constant light. Three weeks after inoculation, plates were harvested and assessed for nodule number and surface colonization using the GUS staining protocol described above. *Sesbania cannabina* seedlings were potted into a 1:1 perlite:vermiculite mixture and grown for one week before inoculation with 10 ml of an OD_{600} = 0.1 rhizobium culture per pot, applied directly at the base of the seedling. Plants were grown at the greenhouse and watered alternately with tap water and Fåhræus medium twice weekly to keep plants under submerged conditions. Eight weeks after inoculation plants were harvested and nodules counted.

Nod factor and Germinated Spore Exudate (GSE) production

Rhizophagus irregularis spores (PremierTech Mycorrhizae PT L-ASP-A, 4000 spores/ml) were rinsed three times in sterile water before germinating at 30°C for 5–7 days. Dehydration allowed for the GSEs to be concentrated to 8,000 spores/ml and this concentrated solution was collected by filter sterilization of the germinated spores–water solution. Nod factors were prepared from *Rhizobium* sp. IRBG74 harboring pA28 (**Table 1**) grown in Vincent Mannitol Medium (45) containing 10 µM Naringenin, 2 µM Apigenin, and 2ug/ml Tetracycline for 48 hours at 30°C until the OD_{600} was between 1.5 and 2.0. The culture was centrifuged and the pellet discarded. To extract the LCOs, the supernatant was mixed with butanol twice and the water phase discarded. The butanol phase was then evaporated at 45°C with a Rotavapor® and the remaining precipitate was re-suspended in water. This solution was mixed with ethyl acetate (final concentration 55%)

twice and the water phase was kept. The water was then evaporated and the remaining precipitate re-suspended in 50% ethanol and filtered using a 0.45 mm filter. The Nod factors were then purified from this solution using an acetonitrile gradient by HPLC. UV detection was between 206 and 220 nm, and the fractions collected corresponded to the major peaks eluting between 30 and 50 minutes. The acetonitrile was evaporated and the precipitate containing purified Nod factors re-suspended in 50% ethanol and stored at -20°C.

Measurements of rice root architecture modification

Total lateral roots of rice were enumerated manually two weeks after application of 10^{-8} M *Rhizobium* sp. IRBG74, *Sinorhizobium meliloti* and *Azorhizobium caulinodans* Nod factors dissolved in 50% ethanol or a control treatment with sterile deionized water containing the appropriate amount of ethanol. Lateral roots were defined as large and fine lateral roots emerging from crown roots, as well as fine lateral roots emerging from large lateral roots. Lateral root counts were assessed for normality using the Shapiro–Wilk test ($p = 0.01$) and statistical significance was determined using a paired *t*-test assuming unequal variances ($p = 0.05$). All statistical analysis was conducted using the R software package (http://www.r-project.org/).

RNAseq

Rice roots grown on Fåhræus medium plates for 5 days were treated with 10^{-8} M non-sulfated (NS) and sulfated mycorrhizal (S) LCOs, 10^{-8} M *Rhizobium* sp. IRBG74 Nod factors, 10^{-6} M chitotetraose (CO4), 10^{-6} M chitooctaose (CO8), as well as *Rhizophagus irregularis* GSEs (diluted 1:10 with 1 µl/20 ml ethanol added) for 24 hours before root RNA was harvested. As signals were suspended in 50% ethanol two controls were used, one for 10^{-6} M COs (100 µl/20 ml ethanol) and one for the 10^{-8} M LCOs, 10^{-8} M Nod factors and GSE (1 µl/20 ml ethanol). The roots from four plants were pooled for each treatment and RNA was extracted using GenCatch

Total RNA Extraction Kit (Epoch). DNA contaminants were removed using the Ambion DNA free DNase treatment kit (Invitrogen). Four biological replicates for all treatments and controls were submitted for sequencing on the Illumina HiSeq 2000, generating 100-bp single reads. Adapter and quality trimming was conducted on the sequence data using the fastx_toolkit and reads were mapped to the rice genome using TopHat (46). Reads were counted using the HTSeq (47) program and analysis was done in the R program using DESeq (48). Using DESeq, reads were normalized and filtered to remove those genes with an upper quartile read count of less than 10. Principal component plot analysis was conducted to determine consistency of treatment groupings. Based on this analysis, one control treatment was removed for the S-LCO, NS-LCO, IRBG74 Nod factors and GSE treatments, and two controls were removed for the CO4 and CO8 treatments. Two S-LCO treatments were also removed, along with one NS-LCO, and one GSE treatment. In all experiments at least two, if not three, biological replicates remained. Hierarchical clustering analysis was conducted using Gene Cluster 3.0 (49) using the genes differentially expressed in the GSE treatment as the reference gene set.

RESULTS

Rhizobium sp. IRBG74 colonizes rice root hairs and lateral root junctions

To better understand the formation of the symbiosis between *Rhizobium* sp. IRBG74 and rice we visually assessed root colonization at 24, 36, and 96 h post-inoculation by confocal microscopy using GFP-labeled strains. *Rhizobium* sp. IRBG74 cells were observed around some root hairs at 24 h (**Fig 1A**), inside of some root hairs at 36 hours (**Fig 1B**), and inside root hairs that appeared to be dead as determined by propidium iodide staining at 96 hours (**Fig 1C**). Bacterial colonization was also observed at the base of root hairs and lateral roots at a later time point (5

days) (**Fig 1D, E, and F**). At two weeks post inoculation, roots colonized with either GFP or GUS-expressing *Rhizobium* sp. IRBG74 were sectioned to observe internal colonization by light and confocal microscopy. *Rhizobium* sp. IRBG74 was observed in the epidermal, exodermal, sclerenchymal, and cortical cells (**Fig 1G, H**).

Colonization of *Setaria viridis* by *Rhizobium* sp. IRBG74

To determine whether *Rhizobium* sp. IRBG74 can colonize cereals other than rice, we inoculated the model C4 plant *Setaria viridis* with a strain carrying a GUS marker. *Setaria viridis* is closely related to switchgrass, an important biofuel crop, as well as agronomically important millets and thus represents another important group of cereal crops (50). *Rhizobium* sp. IRBG74 colonizes the entire root system, including adventitious roots, the primary root, and lateral roots (**Fig 2A**). We observed that *Rhizobium* sp. IRBG74 colonizes the surface of lateral roots extensively in a loosely attached manner (**Fig 2B)**. Cross sections of inoculated roots revealed that *Rhizobium* sp. IRBG74 is primarily limited to epidermal cells, although some blue coloration was observed in cortical cells (**Fig 2C**). Intense blue coloration was observed at the base of root hairs, suggesting that *Rhizobium* sp. IRBG74 preferentially concentrates at this site on the roots (**Fig 2C, E, and F**).

***Rhizobium* sp. IRBG74 produces a diverse mixture of Nod factor structures**

Nod factors produced by *Rhizobium* sp. IRBG74 were analyzed by mass spectrometry and simple structures without substitutions (typically LCOs with n = 2) as well as more complex molecules containing multiple glycosylation sites (typically LCOs with n = 3) were identified (**Fig 3A**). The majority of the Nod factors were N-methylated structures (n = 3) and 75% of the structures contained a carbamoyl residue. There were multiple lengths and saturations of the acyl chain present in the Nod factor mixture, most notable is the long di-unsaturated acyl chain (C20:2)

which was previously identified in *Neorhizobium galegae*, another rhizobium closely related to *Agrobacterium* species (51, 52).

Rhizobium sp. IRBG74 Nod factors induce lateral root formation in rice

Mycorrhizal LCOs induce lateral root responses in rice, and thus we hypothesized that rice may respond similarly to Nod factors. We treated rice with 10^{-8} M *Rhizobium* sp. IRBG74 Nod factors as well as Nod factors from *A. caulinodans* and *Sinorhizobium meliloti* as controls. *Rhizobium* sp. IRBG74 Nod factors induced lateral root formation, however, those produced by *A. caulinodans* and *Sinorhizobium meliloti* did not (**Fig 3B**). Thus, it would appear that there is structure specificity in this response, with not all LCOs being equivalent. Lateral root growth enhancement was POLLUX dependent (**Fig 3C**) indicating the common symbiosis pathway in mediating this response.

Rhizobium sp. IRBG74 Nod factors, LCOs and COs induce distinct transcriptional responses in rice

To determine what genes are induced upon perception of symbiotic signals in rice, we conducted an RNAseq experiment on rice root samples that had been treated for 24 hours with LCOs, COs, GSEs and Nod factors from *Rhizobium* sp. IRBG74. We compared differential gene expression between the treatments and found that there were generally more genes that were distinct to each treatment than those that were common (**Fig 4**). One exception to this would be the CO8 treatment, where most of the genes up or down-regulated in response to this signal were also differentially expressed in response to CO4 (**Fig 4B**). Of all the signals, NS-LCOs seemed to induce the most unique responses, as this treatment shared the fewest genes with the other signals (**Fig 4A & B**). According to these data, NS-LCOs and S-LCOs are not perceived similarly by rice, despite being very similar in structure (**Fig 4A**). *Rhizobium* sp. IRBG74 Nod factors behave most

similar to GSEs in terms of gene expression, suggesting that they are most similar to the active signaling molecules found in the mycorrhizal spore exudates (**Fig 4A, B, D**).

Aberrant nodulation observed in *Lotus japonicus* inoculated with Δ*nodZ* and Δ*noeP*

The diverse array of Nod factors produced by *Rhizobium* sp. IRBG74 may indicate that this rhizobium is capable of nodulating a diverse array of legumes. To better understand the relative importance of some of the Nod factor structures produced by this bacterium in nodulation of legume host, we conuducted nodulation assays with wild-type and *nod* mutant strains in the model legume *Lotus japonicus*. While *Rhizobium* sp. IRBG74 was isolated originally from *Sesbania cannabina*, nodulation experiments with these plants is labor-intensive, challenging to maintain under sterile conditions, and takes over two months. Fortunately, collaborators of ours found that *Rhizobium* sp. IRBG74 can also form nodules on *L. japonicus* (Dr. Jens Stougaard, personal communication). As these plants are much smaller and thrive in an *in vitro* environment, this provides a convenient, high-throughput method to assess nodulation in parallel with experiments using the native host. We inoculated *L. japonicus* with *Rhizobium* sp. IRBG74 strains that had deletions in *nodA, nodB, nodC, nodZ, noeP,* and *nodU* (**Table 1**). A rhizobium native to *L. japonicus, Mesorhizobium haukuii* MAFF303099, was used as a control (**Table 1**). Wild-type *Rhizobium* sp. IRBG74 nodulated at a rate similar to *M. loti*, demonstrating the validity of our experimental system. As expected, the mutants that do not produce LCOs (Δ*nodA,* Δ*nodB,* and Δ*nodC*) did not form nodules on *L. japonicus* (**Fig 5A**). Nodulation was almost abolished in the Δ*nodZ* strain, which does not produce fucosylated Nod factors (**Fig 5A**). In contrast, Δ*noeP*, which does not produce arabinosylated Nod factors, exhibited a super-nodulation phenotype (**Fig 5A**).

Wild-type nodulation was observed in response to the Δ*nodU* strain, which does not produce carbamoylated Nod factors (**Fig 5A**).

Fucosylated and carbamoylated Nod factors are not required for nodulation in *Sesbania cannabina*

Different legume hosts require different Nod factor structures from their rhizobium symbionts, thus, we did not expect that *Sesbania cannabina* would exhibit the same nodulation phenotypes in response to the various *nod* mutants of *Rhizobium* sp. IRBG74 that we observed in *L. japonicus*. To test this hypothesis, we inoculated *S. cannabina* grown in pots under greenhouse conditions with wild-type (positive control), Δ*nodA* (negative control), Δ*nodZ*, and Δ*nodU*. Nodulation rates in response to Δ*nodZ* and Δ*nodU* were similar to those triggered by the wild-type strain and, as expected, there was no nodulation in response to inoculation with Δ*nodA* (**Fig 5B**). The nodules formed upon inoculation were of similar size and pink in color, indicating production of leghemoglobin and therefore, active nitrogen fixation (**Fig 5C**). These results demonstrate the variation in Nod factor structures required by *L. japonicus* and *S. cannabina* from the same rhizobium, *Rhizobium* sp. IRBG74 and reveal the relative importance of specific Nod factor structures during nodulation.

LCOs are dispensable for internal colonization of rice and *S. viridis*

As Nod factors are required for legume nodulation and induce lateral root formation in rice, we hypothesized that they might play a role in the colonization of a cereal host by activating the mycorrhizal pathway. To test this hypothesis, we inoculated rice plants with the Δ*nodA* strain under sterile conditions and internal colonization was determined two weeks post-inoculation by surface sterilizing roots, grinding the roots and serially plating this mixture. As the Δ*nodZ* strain did not

form nodules on *L. japonicus*, we also inoculated plants with this strain to determine if fucosylated Nod factors would play a role in colonization of rice. There was no difference in the levels of internal colonization among wild-type, Δ*nodZ*, and Δ*nodA* (**Fig 6A**). Due to this result, we only tested Δ*nodA* on *S. viridis*, and found the same result on that host (**Fig 6B**). We concluded that, in contrast to nodulation in legumes, Nod factors, or LCOs, are not critical for internal colonization of the cereal host.

COs are required for internal colonization of *S. viridis* but dispensable in rice

While LCOs are required for nodulation of legumes, COs induce calcium spiking and lateral root growth responses in rice (53). Thus, we hypothesized that COs may play a role in colonization of cereals by rhizobia. To test this hypothesis we inoculated rice and *S. viridis* with the Δ*nodC* strain of *Rhizobium* sp. IRBG74 and conducted internal colonization assays. In *Setaria* internal colonization by the Δ*nodC* strain was lower than wild-type, however this was not the case in rice (**Fig 7A)**. Based on this data we propose that there is host specificity in the rhizobium–cereal interaction in terms of chemical structures that facilitate host–symbiont communication.

COs are required for proper surface attachment of *Rhizobium* sp. IRBG74 in rice

Previously, a Δ*nodD*$_1$*ABC* strain of *Sinorhizobium meliloti* was shown to have a reduction in biofilm formation (54). As biofilm formation is critical for bacterial colonization of plants, we hypothesized that *Rhizobium* sp. IRBG74 Δ*nodC* would be more easily detached from the roots of its host (55). During our experiments to assess internal colonization we vortexed the roots in phosphate buffer for one minute prior to surface sterilization. The bacteria found in the buffer afterwards are those that are only loosely attached to the roots and thus are easily detached by the vortex treatment. Assessment of inoculated rice revealed a much higher accumulation of bacteria

in the buffer fraction in plants colonized by the Δ*nodC* strain; however, this was not the case for *S.viridis* (**Fig 7B**). As one would expect biofilm formation to be critical in the colonization of both host plants, this result was unexpected, but highlights that the role of Nod factors in biofilm formation is still not fully understood.

Colonization of rice is not ubiquitous among species of rhizobia

While several strains of rhizobia have been reported to colonize rice, it is not at all clear how widespread this phenomenon is among nodule-forming bacteria. We suspected that the ability to colonize rice is specific to certain species, and so we assembled a panel of rhizobia that included one reported to colonize rice (*Azorhizobium caulinodans*), another *Sesbania*-nodulating rhizobium (*Sinorhizobium saheli*), and rhizobia that have a broad legume host range and thus may have developed superior colonization abilities (*Sinorhizobium fredii*, *Sinorhizobium* sp. BR816, *Rhizobium tropici*, *Mesorhizobium haukuii* MAFF303099, and *Mesorhizobium loti* R7A). We labeled these strains with a GUS marker and visually assessed colonization of rice. Two weeks post inoculation *Mesorhizobium loti, M. haukuii* and *R. tropici* all colonize rice roots at levels similar to *Rhizobium* sp. IRBG74 or *A. caulinodans*, as determined by visual assessment of blue coloration (**Fig 8**). *Sinorhizobium* sp. BR816 and *S. fredii* were poor colonizers of rice **(Fig 8).** Interestingly, despite being a *Sesbania* symbiont like *Rhizobium*. sp. IRBG74 and *A. caulinodans*, *S. saheli* also colonized rice very poorly, with little to no blue coloration visible (**Fig 8**). These results suggest that in addition to production of COs, other bacterial determinants may play an important role in the ability of rhizobia to colonize the roots of cereals, and that this ability is by no means ubiquitous among this group. Comparing the genomes of this panel might reveal novel proteins that are important for the colonization of cereal roots by beneficial rhizobacteria.

DISCUSSION

Rhizobium sp. IRBG74 is an endophyte of rice and *Setaria viridis*

We have found that *Rhizobium* sp. IRBG74 colonizes rice externally near root hairs and at lateral root emergence sites and can penetrate the sclerenchymatous layer to reach cortical cells. The presence of *Rhizobium* sp. IRBG74 near the xylem in rice raises the question of whether these rhizobia can traverse the vasculature and ascend to the above-ground tissue. This has been reported in several other instances of rhizobium–rice interactions, and thus seems plausible (56). Given the intimacy of this association, it would likely indicate the necessity of host immune response suppression. Whether or not perception of LCOs or COs would play a role in the ability of rhizobia to colonize the cereal host vasculature is yet unknown, but it has been documented that immune responses are suppressed during legume–rhizobium interactions and Nod factors can reduce MAMP-associated responses in *Arabidopsis* (57-59). If COs are important for suppression of host immune responses in cereals, this may explain the reduction of internal colonization we observe in *Setaria viridis* inoculated with the Δ*nodC* strain.

Colonization of lateral root emergence sites by *Rhizobium* sp. IRBG74 in rice is consistent with external colonization observed in *Sesbania cannabina,* the native legume host, and reports of other rhizobia on cereals (**Fig S1**) (56, 60, 61). Interestingly, this colonization pattern resembles lateral root junction crack entry nodulation found in *Sesbania* and *Parasponia* species, and thus *Rhizobium* sp. IRBG74 may be an excellent candidate for engineering a less complex nitrogen fixing symbiosis in cereals (15).The root hair colonization we observed is similar to reports of the colonization of rice by *S. meliloti*, where the rhizobia was found in lysed root hairs (56). It is tantalizing to propose that *Rhizobium* sp. IRBG74 could be colonizing the plant via root hair entry, however, it is not clear whether this is endophytic or saprophytic behavior.

Our work also revealed a novel host for *Rhizobium* sp. IRBG74: *Setaria viridis*. *Rhizobium* sp. IRBG74 grew in a thick layer on the root surface and was also able to colonize epidermal cells. Most intriguing was the dense proliferation of rhizobia observed at root hair junctions, suggesting that these sites may be an entry point into the root. A previous report claimed infection of rice by *Rhizobium leguminosarum* via pseudo-infection threads, however, it was unclear whether bacteria were inside of the so-called infection thread structures and the root hairs were alive (61). During rhizobia-legume symbiosis, infection thread formation initiates at the top of the root hair, rather than at the base. Given our data show bacterial accumulation primarily at the root hair base, infection thread formation in this case seems unlikely.

Colonization of cereals by rhizobia demonstrates symbiont and host specificity

Inoculation of rice with a panel of diverse rhizobia revealed that the ability to colonize rice roots is not a ubiquitous trait. For example, a soybean symbiont *Sinorhizobium fredii* and a symbiont of *Phaseolus* species, *Sinorhizobium* sp. BR816, were unable to colonize rice. Based on phylogenies built on the 16S ribosomal DNA sequence, both *Sinorhizobium fredii* and *Sinorhizobium* sp. BR816 are close relatives of *S. meliloti*, which is a rice endosymbiont. However, all these bacteria produce different types of Nod factor structures (52, 62, 63). *Sinorhizobium* sp. BR816 produces LCO pentamers, which are carbamoylated and sulfated, whereas *Sinorhizobium fredii* produces fucosylated LCOs which are three to five GlcNAc residues in length (64-66). These not only differ from the Nod factors produced by *S. meliloti,* but also those produced by *Rhizobium* sp. IRBG74 (67). It would be therefore interesting to test whether transferring the Sym plasmid of *Rhizobium* sp. IRBG74 to *Sinorhizobium fredii* and *Sinorhizobium* sp. BR816, would allow these strains to colonize rice.

Lateral root growth promotion was only observed in response to Nod factors produced by *Rhizobium* sp. IRBG74 and *S. meliloti*, suggesting that rice preferentially perceives certain Nod factor structures. Using a transcriptomics approach in rice, we identified 323 genes differentially expressed in response to *Rhizobium* sp. IRBG74 Nod factors. Some of the genes are also differentially expressed in response to GSEs [41], S-LCOs [49], CO4 [48] and CO8 [23], but there was very little overlap with genes regulated by NS-LCO [7] (**Fig 4**). The treatment most similar to GSEs of mycorrhizal fungi was the *Rhizobium* sp. IRBG74 Nod factors, suggesting that these exudates contain LCO structures similar to those produced by *Rhizobium* sp. IRBG74. While colonization of rice by *Rhizobium* sp. IRBG74 was LCO-independent, it is clear that rice can perceive and respond to the *Rhizobium* sp. IRBG74 Nod factors. The transcriptomics data suggests that Nod factor perception in rice may share some components with the mycorrhizal pathway.

A differential requirement for *Rhizobium* sp. IRBG74 COs was found during internal colonization and surface attachment of rice and *Setaria*, suggesting that the relative importance of COs may differ based on the host plant was well as the rhizobial symbiont. This seems particularly likely given that *A. caulinodans nodC* and *nodD* mutants seem unaffected in their ability to colonize rice (43) and thus have most likely evolved a CO-independent mode of colonization. In addition to being responsible for the formation of the chitin backbone, *nodC* is also involved in determining the length of the chitin chain (68). The length of the chitin chain can confer host specificity in legume nodulation, and thus would be interesting to test if this is also the case in the colonization of cereals. Comparative genomics between *Azorhizobium caulinodans* and *Rhizobium* sp. IRBG74 may also reveal factors important for Nod factor-independent rice colonization.

Fucosylated and arabinosylated Nod factor structures required for wild-type nodulation of *Lotus japonicus*

The Sym plasmid of *Rhizobium* sp. IRBG74 contains many *nod* genes that confer the ability to produce a diverse array of Nod factor structures (51). In theory, this allows *Rhizobium* sp. IRBG74 to nodulate not only its native host *Sesbania cannabina*, but also many other species in the *Sesbania* genus, and *L. japonicus* **(Fig S2).** *Rhizobium* sp. IRBG74 *nodZ* was required for nodulation of *L. japonicus*. Our data are consistent with delayed nodulation of *Lotus* species by a *M. loti* Δ*nodZ* strain and suggest that if the experiment duration were longer we may eventually find nodulation (69). A delayed nodulation phenotype has been previously observed in *Parasponia* species inoculated with strains isolated from other legumes, with the conclusion that the Nod factors produced by these strains are not ideal for induction of nodules on *Parasponia* (70). Thus it would seem that fucosylated Nod factors are an important determinant in host specificity for *L. japonicus*. Given that *Azorhizobium caulinodans* also produces fucosylated Nod factor structures, we expected that they would be important in nodulating *Sesbania*, however, we did not observe a reduction in nodulation upon inoculation with the *Rhizobium* sp. IRBG74 Δ*nodZ* strain (9). *Azorhizobium caulinodans* and *Rhizobium* sp. IRBG74 have vastly different host ranges within the *Sesbania* genus, however, so it may be that fucosylated Nod factors are important for nodulation of species other than *S. cannabina* (**Fig S2**).

Arabinosylated Nod factor structures are unique to the *Sesbania* species symbionts *Azorhizobium caulinodans, Sinorhizobium saheli, Sinorhizobium terangae, Rhizobium* sp. MUS10, and *Rhizobium* sp. IRBG74 and likely, this modification has been highly selected by this plant genus (51, 71-73). In *Rhizobium* sp. IRBG74, the arabinosyltransferase responsible for this modification is encoded by *noeP* and, upon deletion of this gene, we observed an enhanced ability to nodulate *Lotus japonicus*. Regulation of nodule number in plants is controlled by auto-regulation of nodulation and the ability of the plant to regulate nodule number is dependent upon

detection of Nod factors (74). This gene may, depending on nitrogen fixing rates, be a good target for enhancing nitrogen fixation in plants nodulated by these strains and raises the idea of tweaking Nod factor composition to enhance efficacy of rhizobial inoculants.

Concluding remarks

This work has revealed that host specificity in both legumes and cereals is complex and determined by multiple factors, including LCO and CO structures. Despite producing very similar Nod factor structures, *Azorhizobium caulinodans* and *Rhizobium* sp. IRBG74 display very different host specificity patterns in *Sesbania* and the requirement for COs during colonization of cereals seems to be specific to *Rhizobium* sp. IRBG74. *Sinorhizobium saheli* also produces very similar Nod factor structures to *Rhizobium* sp. IRBG74 (71) but shows limited ability to colonize rice roots. The plant host has evolved to differentiate between friend and foe in the root rhizosphere, and while for some symbionts LCOs and COs are an important asset to colonizing both internally and externally, there are clearly also other factors required for the host specific colonization of cereals by rhizobia.

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Table 1. Strains used in this study

Strain	Relevant characteristics	Source or Reference
B001	*Escherichia coli* DH5α harboring helper plasmid pRK600	(75)
DB3.1	*Escherichia coli* cloning strain	Invitrogen
DH5α	*Escherichia coli* cloning strain	(76)
OneShot TOP10	*Escherichia coli* cloning strain	Invitrogen
GPR76	*Rhizobium* sp. IRBG74GU*S* harboring pHC60; expresses gusA and gfp; SpR, TcR	Gyaneshwar Prasad
IRBG74GUS	*Rhizobium* sp. IRBG74GUS*,* cured of pHC60, expresses gusA; SpR	This study
MC152	*Rhizobium* sp. IRBG74GUS harboring pA28; SpR, TcR	Fabienne Maillet
MC153	*Rhizobium* sp. IRBG74GUS; RfR, SpR	This study
MC304	*Rhizobium* sp. IRBG74GUS harboring pRF771; SpR, TcR	This study
MC172	*Rhizobium* sp. IRBG74GUS nodA::pMBC229; SpR, GmR	This study
MC302	*Rhizobium* sp. IRBG74GUS *ΔnodA; SpR*	This study
MC318	*Rhizobium* sp. IRBG74GUS ΔnodA harboring pMBC250; SpR, TcR	This study
MC322	*Rhizobium* sp. IRBG74GUS ΔnodA harboring pRF771; SpR, TcR	This study
MC331	*Rhizobium* sp. IRBG74GUS nodB::pMBC248; SpR, GmR	This study
MC333	*Rhizobium* sp. IRBG74GUS ΔnodB; SpR	This study
MC358	*Rhizobium* sp. IRBG74GUS ΔnodB harboring pMBC277; SpR, TcR	This study
MC359	*Rhizobium* sp. IRBG74GUS ΔnodB harboring pRF771; SpR, TcR	This study
MC511	*Rhizobium* sp. IRBG74GUS 1.26 nodC::pMBC279; SpR, GmR	This study
MC512	*Rhizobium* sp. IRBG74GUS ΔnodC; SpR	This study
MC533	*Rhizobium* sp. IRBG74GUS ΔnodC harboring pMBC408; SpR, TcR	This study
MC534	*Rhizobium* sp. IRBG74GUS ΔnodC harboring pRF771; SpR, TcR	This study
MC332	*Rhizobium* sp. IRBG74GUS nodU::pMBC249; SpR, GmR	This study
MC334	*Rhizobium* sp. IRBG74GUS ΔnodU; SpR	This study
MC360	*Rhizobium* sp. IRBG74GUS ΔnodU harboring pMBC278; SpR, TcR	This study
MC361	*Rhizobium* sp. IRBG74GUS ΔnodU harboring pRF771; SpR, TcR	This study
MC313	*Rhizobium* sp. IRBG74GUS nodZ::pMBC231; SpR, GmR	This study
MC314	*Rhizobium* sp. IRBG74GUS ΔnodZ; SpR	This study
MC319	*Rhizobium* sp. IRBG74GUS ΔnodZ harboring pMBC251; SpR, TcR	This study

MC323	*Rhizobium* sp. IRBG74GUS ΔnodZ harboring pRF771; SpR, TcR	This study
MC173	*Rhizobium* sp. IRBG74GUS noeP::pMBC230; SpR, GmR	This study
MC301	*Rhizobium* sp. IRBG74GUS ΔnoeP; SpR	This study
MC320	*Rhizobium* sp. IRBG74GUS ΔnoeP harboring pMBC252; SpR, TcR	This study
MC321	*Rhizobium* sp. IRBG74GUS ΔnoeP harboring pMBC253; SpR, TcR	This study
MC324	*Rhizobium* sp. IRBG74GUS ΔnoeP harboring pRF771; SpR, TcR	This study
ORS571	*Azorhizobium caulinodans* ORS571	(77)
MC130	*Azorhizobium caulinodans* ORS571; Rf^R	This study
MC325	*Azorhizobium caulinodans* ORS571 tagged with mTn*5*ss*gusA21*; Rf^R, Sp^R	This study
7653R	*Mesorhizobium huakuii* 7653R	(52)
MC345	*Mesorhizobium huakuii* 7653R; Rf^R	This study
MC364	*Mesorhizobium huakuii* 7653R tagged with mTn*5*ss*gusA21*; Rf^R, Sp^R	This study
MAFF303099	*Mesorhizobium huakuii* MAFF303099	(78)
MC343	*Mesorhizobium huakuii* MAFF303099; Rf^R	This study
MC356	*Mesorhizobium huakuii* MAFF303099 tagged with mTn*5*ss*gusA21*; Rf^R, Sp^R	This study
R7A	*Mesorhizobium loti* R7A	(79)
MC341	*Mesorhizobium loti* R7A; Rf^R	This study
MC355	*Mesorhizobium loti* R7A tagged with mTn*5*ss*gusA21*; Rf^R, Sp^R	This study
PRF81	*Rhizobium freirei* PRF81	(80)
MC337	*Rhizobium freirei* PRF81; Rf^R	This study
MC351	*Rhizobium freirei* PRF81 tagged with mTn*5*ss*gusA21*; Rf^R, Sp^R	This study
CIAT 899	*Rhizobium tropici* CIAT 899	(81)
MC335	*Rhizobium tropici* CIAT 899; Rf^R	This study
MC349	*Rhizobium tropici* CIAT 899 tagged with mTn*5*ss*gusA21*; Rf^R, Sp^R	This study
USDA257	*Sinorhizobium fredii* USDA257	(82)
MC178	*Sinorhizobium fredii* USDA257; Rf^R	This study
MC329	*Sinorhizobium fredii* USDA257 tagged with mTn*5*ss*gusA21*; Rf^R, Sp^R	This study
ORS609	*Sinorhizobium saheli* bv. *sesbaniae* ORS 609	(83)
MC141	*Sinorhizobium saheli* bv. *sesbaniae* ORS 609 Rf^R	This study
MC157	*Sinorhizobium saheli* bv. *sesbaniae* ORS 609 tagged with mTn*5*ss*gusA21*; Rf^R, Sp^R	This study
BR816	*Sinorhizobium* sp. BR816	(84)

MC175	*Sinorhizobium* sp. BR816; Rf^R	This study
MC327	*Sinorhizobium* sp. BR816 tagged with mTn*5*ss*gusA21*; Rf^R, Sp^R	This study

Table 2. Plasmids used in this study.

Plasmid	Relevant characteristics	Source
pRK600	Self-transmissible helper plasmid; Cm^R	(85)
pJQ200SK	Vector for construction of *sacB*-mediated deletions; Gm^R	(86)
pRF771	Vector for P_{trp} transcriptional fusions; Tc^R	(87)
pDEST24	Gateway vector expressing the bacteriocin gene *ccdB*; Cm^R	Invitrogen
pCAM121	mTn*5*ss*gusA21* (P_{aph}:*gusA*:Ter_{trpA}); Ap^R, Sp^R, DAP^S	(88)
pA28	pRK7813 expressing $nodD_1$ of *Sinorhizobium* sp. NGR234	(89)
pMBC219	pJQ200SK with the Gateway cassette from pDEST24 cloned into XbaI; Gm^R	This study
pMBC223	pRF771 with the Gateway cassette from pDEST24 cloned into XbaI; Tc^R	This study
pMBC229	For deletion of nodA of *Rhizobium* sp. IRBG74; GmR	This study
pMBC248	For deletion of nodB of *Rhizobium* sp. IRBG74; GmR	This study
pMBC279	For deletion of nodC of *Rhizobium* sp. IRBG74; GmR	This study
pMBC249	For deletion of nodU of *Rhizobium* sp. IRBG74; GmR	This study
pMBC231	For deletion of nodZ of *Rhizobium* sp. IRBG74; Gm	This study
pMBC230	For deletion of noeP of *Rhizobium* sp. IRBG74; GmR	This study
pMBC250	pRF771; P_{trp}:*nodA*; Tc^R	This study
pMBC277	pRF771; P_{trp}:*nodB*; Tc^R	This study
pMBC409	pRF771; P_{trp}:*nodC*; Tc^R	This study
pMBC278	pRF771; P_{trp}:*nodU*; Tc^R	This study
pMBC251	pRF771; P_{trp}:*nodZ*; Tc^R	This study
pMBC252	pRF771; P_{trp}:*noeP* (short); Tc^R	This study
pMBC253	pRF771; P_{trp}:*noeP* (long); Tc^R	This study

Table 3. Primers used in this study.

Name	Sequence	Purpose	Source or Reference
oMBC001	CGACTCACTATAGGGCGAATTG	verification of pJQ200SK inserts	This study
oMBC002	ACAGGAAACAGCTATGACCATG	verification of pJQ200SK inserts	This study
oMBC026	GACATTTGCATTACACGGTTGG	verification of pRF771 inserts	This study
oMBC027	ACCTGCGTTCAGCAGTTCTG	verification of pRF771 inserts	This study
oMBC073	GTCACGACGTTGTAAAACGACG	verification of pENTR/D-TOPO inserts	This study
oMBC074	CAGAGCTGCCAGGAAACAGC	verification of pENTR/D-TOPO inserts	This study
oMBC032	CGCTCTAGAACAAGTTTGTACAAAAAAGCTG	clone the Gateway cassette from pDEST24 into various rhizobial vectors	This study
oMBC049	CGCTCTAGACCACTTTGTACAAGAAAGC	clone the Gateway cassette from pDEST24 into various rhizobial vectors	This study
TSP1	GTTTACTTTGCAGGGCTTCCCAAC	Tn*5* primer for arbitrary PCR	(90)
TSP2	AGCTGGCAATTCCGGTTCGCTTG	nested Tn*5* primer for arbitrary PCR	(90)
ARB1A	GCCACGCGTCGACTAGTACNNNNNNNNNNACGCC	degenerate primer for arbitrary PCR	(90)
ARB2	GCCACGCGTCGACTAGTAC	nested primer for arbitrary PCR	(90)
oMBC056	cacCGATCCCAATTGCGATCTCG	in-frame deletion of nodA of *Rhizobium* sp. IRBG74	This study
oMBC004	tcatagctctggCACGTCAGAACACATTTCATCT	in-frame deletion of nodA of *Rhizobium* sp. IRBG74	This study
oMBC005	tgttctgacgtgCCAGAGCTATGAAACAGCTG	in-frame deletion of nodA of *Rhizobium* sp. IRBG74	This study
oMBC057	TGGCTGAACTGAATTCAGC	in-frame deletion of nodA of *Rhizobium* sp. IRBG74	This study
oMBC007	CTCAATGCGCATGGCACTGG	check primer for *nodA* deletion	This study
oMBC008	GAACCTGCATGCCTTTATAGAC	check primer for *nodA* deletion	This study
oMBC077	cacCGAAGTATAGGTAGATGAAATGTG	clone *Rhizobium* sp. IRBG74 nodA + RBS	This study
oMBC078	GTCAGATAAACACTGCGGTCG	clone *Rhizobium* sp. IRBG74 nodA + RBS	This study
oMBC107	cacCGTTCTTTCGGAATACCTATGG	in-frame deletion of nodB of *Rhizobium* sp. IRBG74	This study
oMBC108	cctttaattgtgCAGCTGTTTCATAGCTCTGG	in-frame deletion of nodB of *Rhizobium* sp. IRBG74	This study
oMBC109	atgaaacagctgCACAATTAAAGGACGGTATTGC	in-frame deletion of nodB of *Rhizobium* sp. IRBG74	This study
oMBC110	CTATCGATGGATCGCGCATC	in-frame deletion of nodB of *Rhizobium* sp. IRBG74	This study
oMBC111	CAAGGGATGAATGCGTTTCATC	check primer for *nodB* deletion	This study
oMBC112	GTCTCGTATTGGTCGAGCAG	check primer for *nodB* deletion	This study

oMBC136	cacCACGCTCGATGGACGAATGG	clone Rhizobium sp. IRBG74 nodB + RBS	This study
oMBC079	GCTGTACCAAATAGATCCATGC	clone Rhizobium sp. IRBG74 nodB + RBS	This study
oMBC173	caCCAGCACTTATCCAACGTATGG	in-frame deletion of nodC of *Rhizobium* sp. IRBG74	This study
oMBC174	ttaatccatcggACCAAATAGATCCATG CAATACC	in-frame deletion of nodC of *Rhizobium* sp. IRBG74	This study
oMBC175	gatctatttggtCCGATGGATTAACCTTT TCGCG	in-frame deletion of nodC of *Rhizobium* sp. IRBG74	This study
oMBC176	CATTTCAGCAACGCTCTCAAGG	in-frame deletion of nodC of *Rhizobium* sp. IRBG74	This study
oMBC177	CTGATTGATCGTAATGGCCCAG	check primer for *nodC* deletion	This study
oMBC178	GTAAGCAAACAGTCTTCGTTGG	check primer for *nodC* deletion	This study
oMBC137	cacCATCCGATTGCTCTCCCAGC	clone *Rhizobium* sp. IRBG74 nodC + RBS	This study
oMBC080	GACCAAAATCTCGGATGCTCG	clone *Rhizobium* sp. IRBG74 nodC + RBS	This study
oMBC113	caccGCAGCAATCCATCACACACG	in-frame deletion of nodU of *Rhizobium* sp. IRBG74	This study
oMBC016	gcgtgtcgtatgCGTGAGTTTAATTCCG CAAATG	in-frame deletion of nodU of *Rhizobium* sp. IRBG74	This study
oMBC017	attaaactcacgCATACGACACGCCCGG AATG	in-frame deletion of nodU of *Rhizobium* sp. IRBG74	This study
oMBC114	GAAGCACTCTCCTTTTTTGACG	in-frame deletion of nodU of *Rhizobium* sp. IRBG74	This study
oMBC115	GCTTTGGATGTTGCAGCTGAC	check primer for *nodU* deletion	This study
oMBC020	GTTCTCGCGCACTGTGAATTC	check primer for *nodU* deletion	This study
oMBC142	cacCGTTTCTCAATGATCCGGCTAC	clone *Rhizobium* sp. IRBG74 nodU + RBS	This study
oMBC143	CACAGTCCGGTCGATATAGG	clone *Rhizobium* sp. IRBG74 nodU + RBS	This study
oMBC197	CCTAAACGTGTTTCGGGAGC	*Rhizobium* sp. IRBG74 nodU sequencing primer	This study
oMBC075	cacCGAATGCTGGCTGAAGCTGC	in-frame deletion of nodZ of *Rhizobium* sp. IRBG74	This study
oMBC010	ctttgacactccGTCAGCAAGCATTTGC GGTG	in-frame deletion of nodZ of *Rhizobium* sp. IRBG74	This study
oMBC011	atgcttgctgacGGAGTGTCAAAGGATC TCTG	in-frame deletion of nodZ of *Rhizobium* sp. IRBG74	This study
oMBC076	CTCACCTGGACATGGCTTTG	in-frame deletion of nodZ of *Rhizobium* sp. IRBG74	This study
oMBC013	TCGTCCGCACCGAAAACTGC	check primer for *nodZ* deletion	This study
oMBC014	CATTTTGCGGGACTTCTTGAGC	check primer for *nodZ* deletion	This study
oMBC081	caccGTCGATCAATAACCGATCGAG C	clone *Rhizobium* sp. IRBG74 nodZ + RBS	This study
oMBC082	GATCTGTACCACATGGGAAGC	clone *Rhizobium* sp. IRBG74 nodZ + RBS	This study
oMBC060	cacCATGCGTAGCAACTACGAAGG	in-frame deletion of noeP of *Rhizobium* sp. IRBG74	This study

oMBC061	ctccaaataaccCCATATCAGGCCGAACATTCC	in-frame deletion of noeP of *Rhizobium* sp. IRBG74	This study
oMBC062	ggcctgatatggGGTTATTTGGAGTGAGTTGTGG	in-frame deletion of noeP of *Rhizobium* sp. IRBG74	This study
oMBC063	GAGGATGTCGTTCAGACATTGG	in-frame deletion of noeP of *Rhizobium* sp. IRBG74	This study
oMBC064	CAATCAGAAACACTCGATGCTG	check primer for *noeP* deletion	This study
oMBC065	CAGCTCCGCAACAAGGTTCC	check primer for *noeP* deletion	This study
oMBC083	caccGCTGTATGTTTCATCATTGCTG	clone *Rhizobium* sp. IRBG74 noeP + RBS (short)	This study
oMBC084	caccGCTTTTCAAGAGGGTAAGGATG	clone *Rhizobium* sp. IRBG74 noeP + alternative RBS (long)	This study
oMBC085	GTCAAAGCCACAACTCACTCC	clone *Rhizobium* sp. IRBG74 noeP + RBS	This study

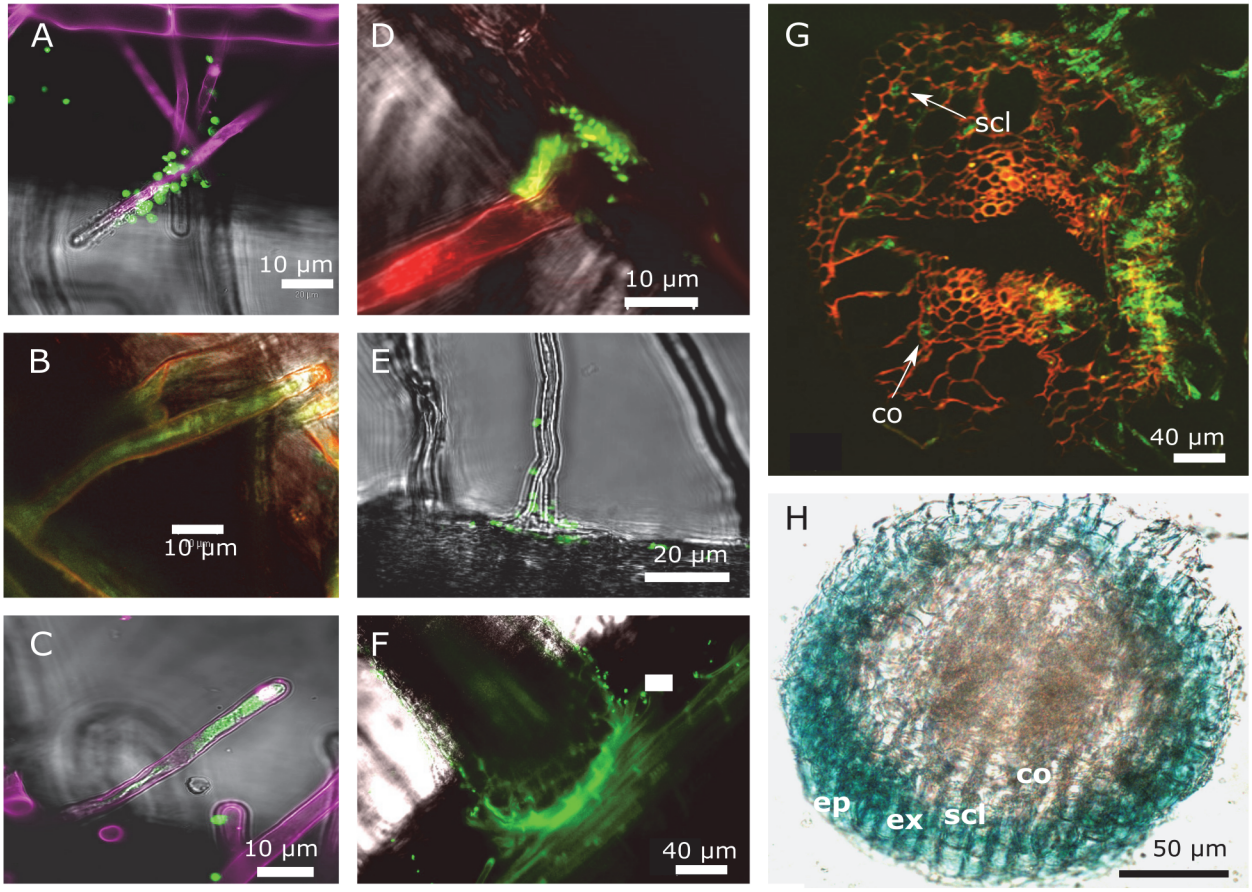

A
10 µm
D
10 µm
G
scl
co
40 µm
B
10 µm
E
20 µm
H
co
ep
ex
scl
50 µm
C
10 µm
F
40 µm

Figure 1. External and internal colonization of rice by *Rhizobium* sp. IRBG74

Rhizobium sp. IRBG74 expressing GFP accumulated at root hair tips 24 hours post inoculation (hpi) (A), and inside root hairs 36 hpi. (B) and 96 hpi (C). Colonization at root-root hair junctions (D, E) is similar to what is observed at the lateral root junction (5 days pi) (F). Cross sections of rice roots using GFP (G) or GUS (H) labeled strains 5 days pi. Green is GFP fluorescence (510–500 nm), red is FM4-64 (550–600 nm) membrane stain and purple is propidium iodide (600–650 nm) cell wall stain. ep = epidermis, ex = exodermis, scl = sclerenchyma, and co = cortex.

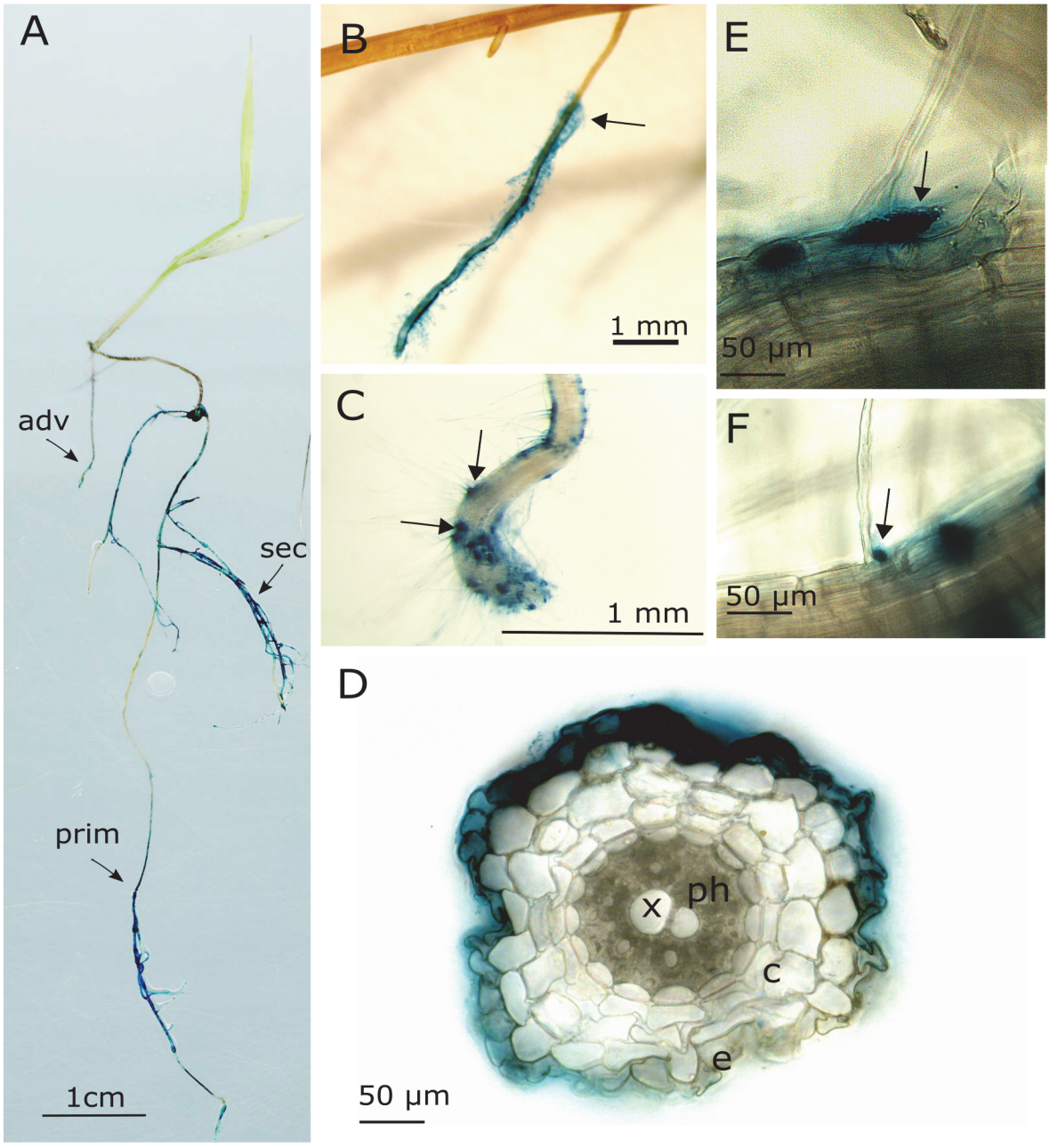
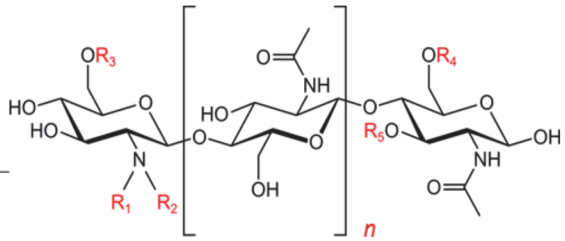

A
adv
sec
prim
1cm
B
1 mm
C
1 mm
D
x
ph
c
e
50 µm
E
50 µm
F
50 µm

Figure 2. ***Rhizobium* sp. IRBG74 colonization of *Setaria viridis* roots**

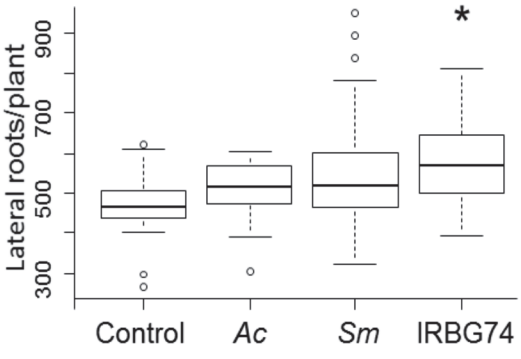
Roots two weeks post inoculation with *Rhizobium* sp. IRBG74 expressing the GUS reporter (A). adv = adventitious roots, prim = primary roots, sec = secondary roots. Surface colonization on lateral roots (B) and accumulation at root hairs (black arrows) (C). Cross section of colonized root, e = epidermis, c = cortex, ph = phloem, x = xylem. Magnification of root hair, arrow indicating IRBG74 colonization (E,F).

A

Rhizobium	R1	R2	R3	R4	R5	n
Rhizobium sp. IRBG74	N-Me	C16:1	Cb	Fuc	D-ara	2
		C18:0	H	MeFuc		3
		C18:1				
		C18:3				
		C20:2				
Azorhizobium caulinodans ORS571	N-Me	C16:0	Cb	D-ara	D-ara	2
		C18:0	H	Fuc	H	3
		C18:1		H		



B



C

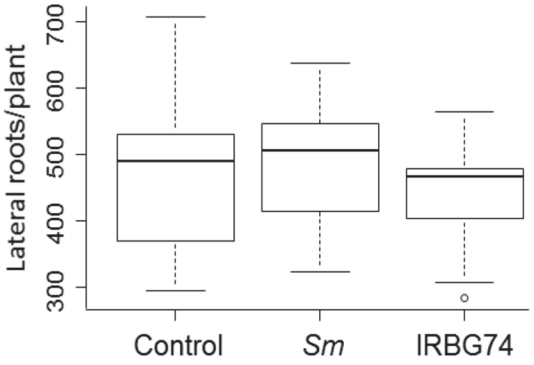


Figure 3. Nod factor structures of *Rhizobium* sp. IRBG74 promote lateral root outgrowth

(A) Chitin backbone length (*n*), acyl chain length and number of saturations (R2), and chitin backbone substitutions (R1, R3-R5) found in *Rhizobium* sp. IRBG74 Nod factors as determined by mass spectrometry compared to those produced by *Azorhizobium caulinodans*. Letters are indicated in generalized Nod factor diagram. Rice wild-type (B) and *pollux* mutants (C) treated with *Rhizobium* sp. IRBG74 (IRBG74), *Azorhizobium caulinodans* (*Ac*), and *Sinorhizobium meliloti* (*Sm*) Nod factors with a water control (Control). Lateral roots were enumerated after 2 weeks. Data presented from a representative experiment out of two total. $n \geq 16$. Significance, denoted by astericks (*), was determined by *t* test for normal data and Wilcoxon-Mann-Whitney test for non-normal data ($p < 0.05$).

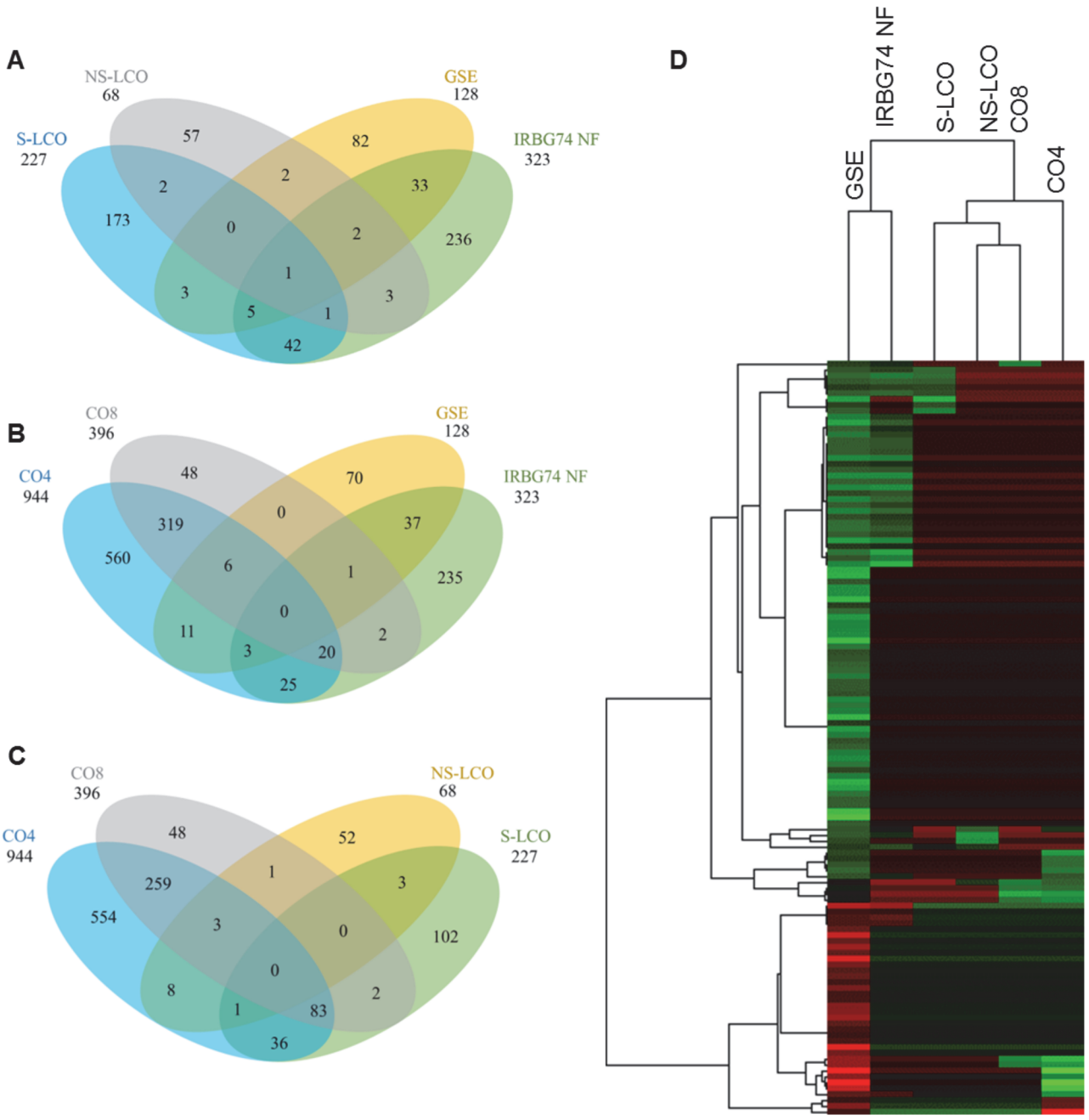

A
NS-LCO
68
GSE
128
S-LCO
227
IRBG74 NF
323
57
82
2
2
33
173
0
2
236
1
3
3
5
1
42
B
CO8
396
GSE
128
CO4
944
IRBG74 NF
323
48
70
0
319
37
560
6
1
235
0
11
2
3
20
25
C
CO8
396
NS-LCO
68
CO4
944
S-LCO
227
48
52
1
259
3
554
3
0
102
0
8
2
1
83
36
D
IRBG74 NF
S-LCO
NS-LCO
CO8
GSE
CO4

Figure 4. LCOs, COs, GSEs and *Rhizobium* sp. IRBG74 Nod factors trigger distinct transcriptomic responses in rice

Differential expression measured by RNAseq on samples treated with 10^{-8} M NS-LCO, S-LCO, GSEs, *Rhizobium* sp. IRBG74 Nod factors (IRBG74 NF), CO4 and CO8 for 24 hours. Significantly differentially expressed genes ($p < 0.05$) were compared among treatments. Numbers underneath treatment names indicate total number of differentially expressed genes. Hierarchical clustering of genes in all treatments relative to differentially expressed genes in the GSE treatment (D). Red indicates up-regulated genes, green indicates down regulated genes and black indicates no change between treatment and control.

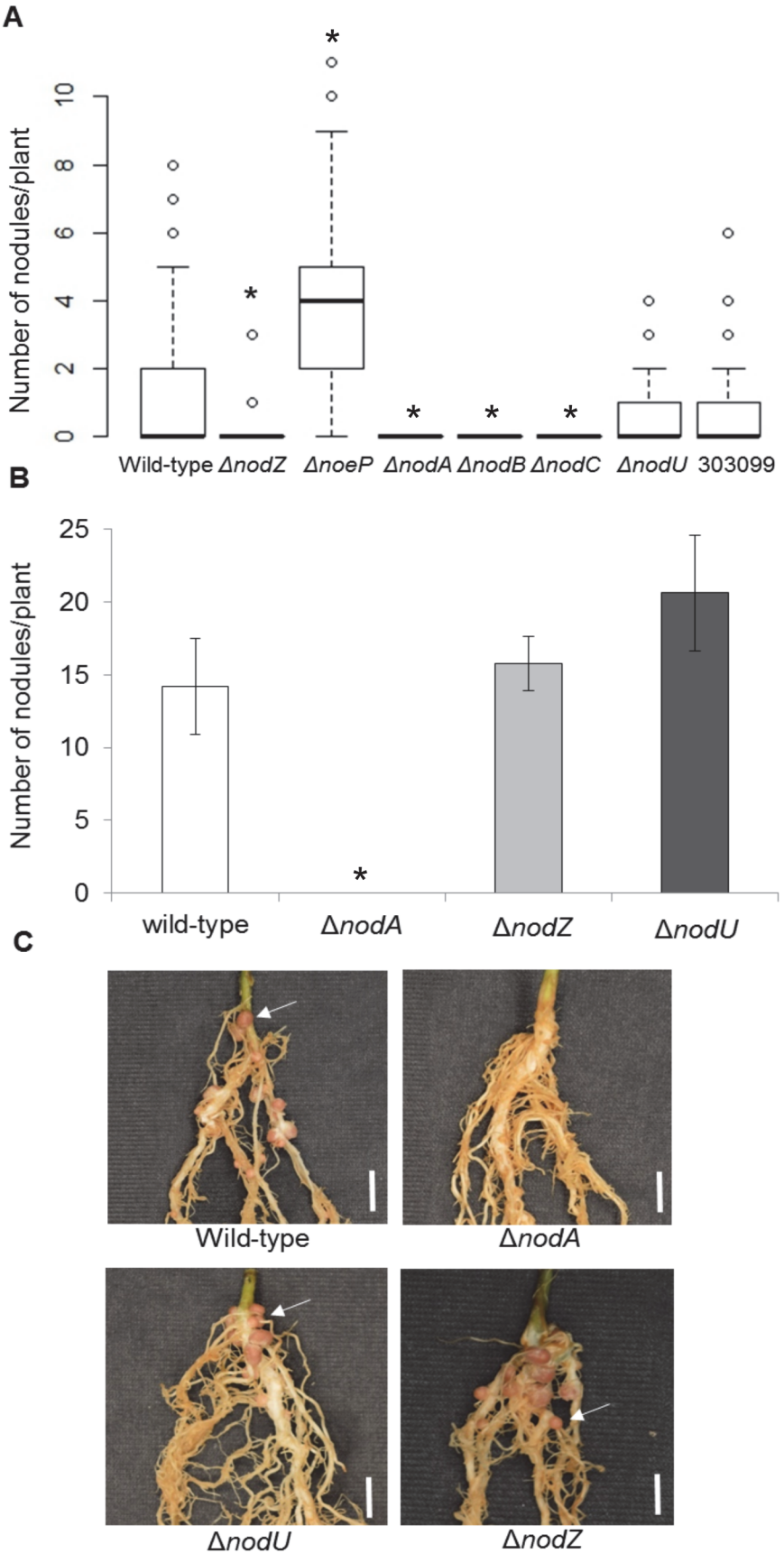

A
Number of nodules/plant
0
2
4
6
8
10
Wild-type
ΔnodZ
ΔnoeP
ΔnodA
ΔnodB
ΔnodC
ΔnodU
303099
B
Number of nodules/plant
0
5
10
15
20
25
wild-type
ΔnodA
ΔnodZ
ΔnodU
C
Wild-type
ΔnodA
ΔnodU
ΔnodZ

Figure 5. Nodulation of *Lotus japonicus* and *Sesbania cannabina* by *Rhizobium* sp. IRBG74 wild-type and *nod* mutants

(A) *Lotus japonicus* inoculated with *Rhizobium* sp. IRBG74 wild-type (WT) and *nod* mutants, as well as *Mesorhizobium huakuii* 303099 (303099) were assessed for nodulation after 3 weeks. Data represented is number of nodules pooled from at least two experiments. $n \geq 47$. Error bars are standard error, *p*-value was determined by Wilcoxon–Mann–Whitney test of significance ($p < 0.01$). (B) Average number of nodules formed per plant of *S. cannabina* inoculated with *Rhizobium* sp. IRBG74 wild-type and Δ*nodA*, Δ*nodZ* and Δ*nodU*. Significance determined by *t* test ($p < 0.01$). Plants were grown in the greenhouse for 8 weeks post-inoculation before harvesting. $n \geq 5$. (C) Images of representative *S. cannabina* nodulated roots showing large, pink nodules (white arrows). Scale bars are 1 cm.

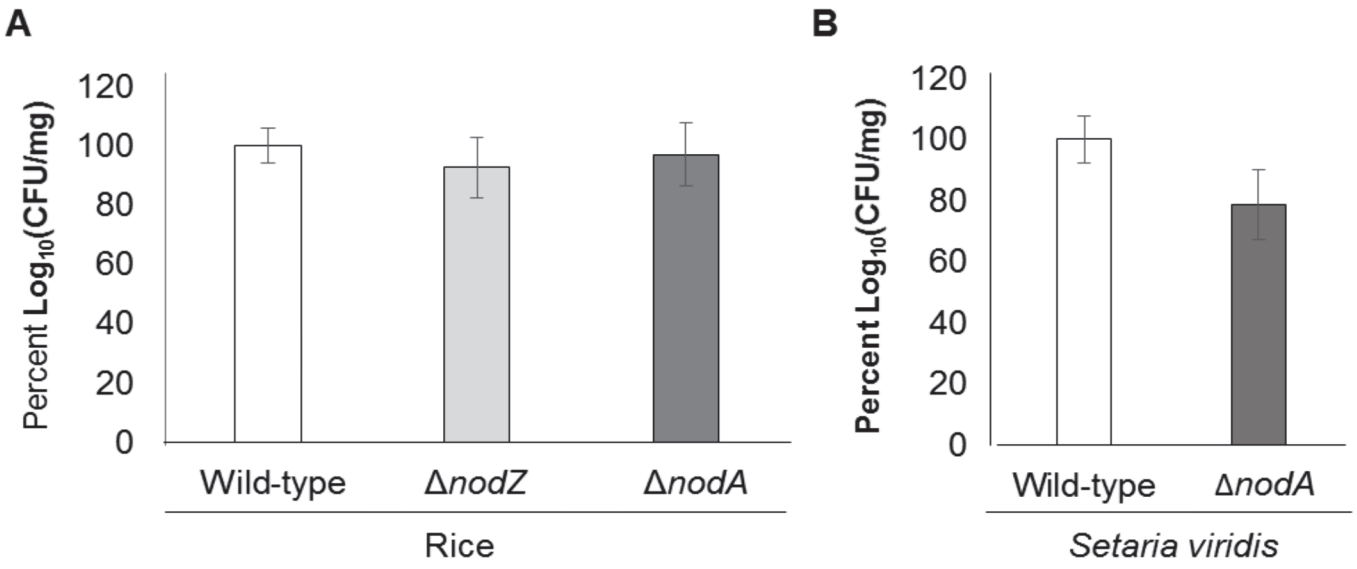

A
Percent Log10(CFU/mg)
120
100
80
60
40
20
0
Wild-type
ΔnodZ
ΔnodA
Rice
B
Percent Log10(CFU/mg)
120
100
80
60
40
20
0
Wild-type
ΔnodA
Setaria viridis

Figure 6. Internal colonization of rice and *Setaria viridis* by *Rhizobium* sp. IRBG74 is not LCO-dependent

A) Rice plants inoculated with *Rhizobium* sp. IRBG74 wild-type, Δ*nodZ* (no fucosylated LCOs) and Δ*nodA* (no LCOs) were assessed for internal colonization after 2 weeks. Data represented were pooled from at least four replicated experiments standardized to the mean of each experiment and represented as percent Log_{10}(CFU/mg) relative to wild-type. $n > 20$. B) *Setaria viridis* inoculated with *Rhizobium* sp. IRBG74 wild-type and Δ*nodA* were assessed for internal colonization after 2 weeks. Data represented were pooled from five experiments standardized to the mean of each experiment and represented as percent Log_{10}(CFU/mg) relative to wild-type. $n \geq 14$. Error bars are standard error, *p*-value was determined by *t* test ($p < 0.05$).

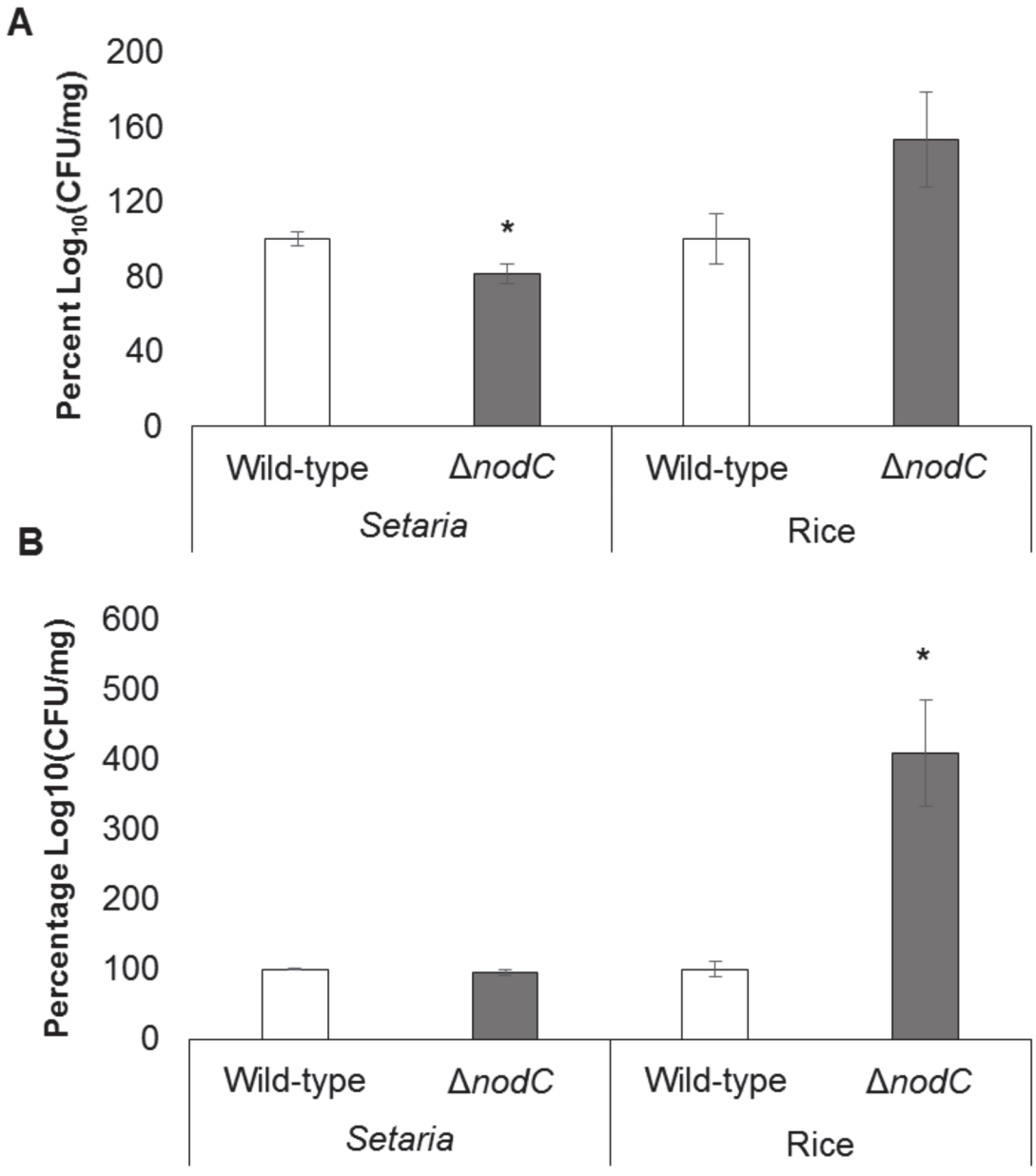

A
Percent Log10(CFU/mg)
200
160
120
80
40
0
*
Wild-type
ΔnodC
Setaria
Wild-type
ΔnodC
Rice
B
Percentage Log10(CFU/mg)
600
500
400
300
200
100
0
*
Wild-type
ΔnodC
Setaria
Wild-type
ΔnodC
Rice

Figure 7. COs required for internal colonization in *Setaria* and surface attachment in rice

Plants inoculated with *Rhizobium* sp. IRBG74 wild-type and Δ*nodC* were assessed for internal colonization of roots (A) and quantity of rhizobia detached by vortexing roots (B) after 2 weeks. Internal colonization was reduced in *Setaria* inoculated with the Δ*nodC* strain, while more rhizobia were detached by vortexing in rice inoculated with Δ*nodC*. Data represented pooled from at least four (A) or three (B) experiments standardized to the mean of each experiment and represented as percent Log_{10}(CFU) relative to wild-type. $n \geq 21$. Error bars are standard error, *p*-value was determined by *t* test on transformed data ($p < 0.01$).

Rhizobium sp. IRBG74
Sinorhizobium sahelii
Sinorhizobium fredii
Sinorhizobium sp. BR816
Azorhizobium caulinodans
Mesorhizobium loti
Mesorhizobium huakuii
Rhizobium tropici

Figure 8. **The ability to colonize rice is not ubiquitous among rhizobia**

Various rhizobia expressing the beta-glucuronidase marker were tested for their ability to colonize rice roots. *Rhizobium* sp. IRBG74 (WT) and *Azorhizobium caulinodans* served as positive controls. GUS stained roots are shown 2 weeks post inoculation. Scale bar is 1 cm.

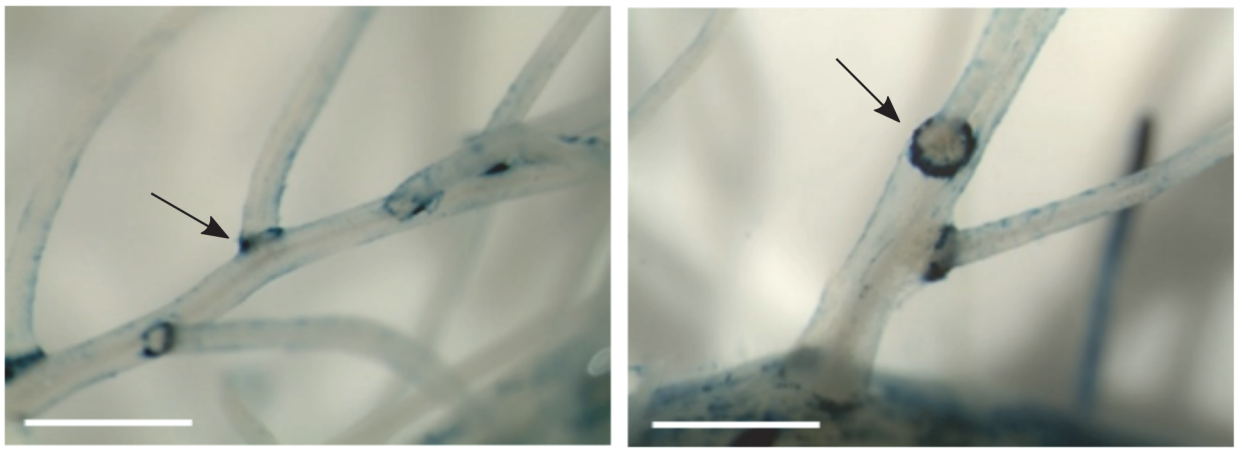


Figure S1. Colonization of *Sesbania bispinosa* by *Rhizobium* sp. IRBG74

GUS-stained *Sesbania bispinosa* root colonized by *Rhizobium* sp. IRBG74 expressing beta-glucuronidase. *Rhizobium* sp. IRBG74 accumulates at lateral root junctions (black arrows) . Images were taken two weeks post inoculation. Scale bars are 1 mm.

	S. bispinosa	*S. cannabina*	*S. exasperata*	*S. formosa*	*S. grandiflora*	*S. punicea*	*S. rostrata*	*S. virgata*
Azorhizobium caulinodans	N^{+} F^{-}	$N^{+/-}$ F^{-}	$N^{-[s]}$	N^{+} F^{-}	N^{+} F^{-}	$N^{-[s]}$	N^{+} F^{+}	$N^{+/-}$ F^{+}
Rhizobium sp. IRBG74	N^{+} F^{+}	N^{+} F^{+}	N^{+} F^{+}	N^{+} F^{+}	N^{+} F^{+}	N^{-}	$N^{+/-}$ F^{+}	$N^{+/-}$ F^{+}

Figure S2. *Sesbania* host range of *Azorhizobium caulinodans* and *Rhizobium* sp. IRBG74

Analysis of the nodulation and nitrogen fixation pattern among species of *Sesbania* based on published literature. The associations are described as exhibiting nodulation and fixation (dark green, N^{+} F^{+}), forming smaller nodules but some nitrogen is fixed (light green, $N^{+/-}$ F^{+}), forming nodules but not fixing nitrogen (yellow, N^{+} F^{-}), studies are conflicting about nodulation but nitrogen is not fixed (orange, $N^{+/-}$ F^{-}), no stem nodules form and formation of root nodules and fixation not reported (dark blue, $N^{-[s]}$), no nodules form (light blue, N^{-}). (83, 91-95)

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

This study set out to address gaps in our knowledge of the formation of plant-microbe symbiosis and to further understanding of the perception and function of chitin based signaling molecules in plants. Rhizobia and mycorrhizal fungi signal to plants using LCOs and COs, and perception of these signals results in nuclear calcium spiking, symbiotic gene expression and initiation of host colonization. In the well-studied model plant *Lotus japonicus*, nucleoporins are required for LCO induced calcium spiking, however their function was unknown. Based on the Lotus *nup* mutant phenotype and the role of nucleoporins in the trafficking of proteins to the nucleus and inner nuclear membrane, it had been speculated that they could be required for transport of proteins required for LCO induced calcium spiking to the inner nuclear membrane. Up until now, however, evidence to support this hypothesis had been lacking.

The discovery that mycorrhizal fungi not only produce LCOs but also COs which can both activate calcium spiking in the model legume *Medicago truncatula* opened the door for questions regarding the relative role of these compounds during mycorrhizal symbiosis. The activity of these molecules during mycorrhizal symbiosis in non-legumes for example was unclear. Non-legumes such as rice are able to form associations with rhizobia which do not result in nodulation, however internal root colonization does take place. It seems likely that rhizobia may co-opt the mycorrhizal signaling pathway to facilitate this symbiosis using Nod factors, but previously this had not been observed. The goal of this thesis research was to answer the following questions:

1) What is the role of nucleoporin proteins during LCO induced calcium spiking?
2) What is the primary signaling molecule inducing symbiotic responses in rice?
3) What is the role of Nod factors during rhizobial symbiosis in rice?

Plant nucleoporins involved in ion channel localization to the inner nuclear membrane

To understand the role of nucleoporins during legume-nodulation and arbuscular mycorrhization we used a reverse genetics approach to identify and assess the symbiotic phenotype of a Medicago *nup133* mutant. Transmission electron microscopy was employed to determine the precise localization of nuclear membrane localized ion channels (DMI1 and POLLUX) in a nucleoporin mutant background. While the approach required the localization of GFP fusion constructs, rather than the native protein, this allowed us to detect the protein in sufficient quantities to allow for quantification and statistics. While the Medicago *nup133* mutant had a less severe symbiotic phenotype than the Lotus *nup133* mutant, mis-localization of DMI1 and POLLUX was observed in both respective nucleoporin mutant backgrounds (**Fig 1**). Combined with the complementation of the nodulation phenotype by over-expression of DMI1 but not POLLUX, this result is consistent with differences in ion channel behavior between these two proteins and suggests that the superior ion channel, DMI1, has a reduced need for nucleoporins during symbiosis (**Fig 1**). Future work must include the localization of the other nuclear localized ion channel required for symbiosis in Lotus, CASTOR, to determine the precise localization of this protein on the nuclear envelope as well as the role of nucleoporins in its localization. Similar work should also be conducted on the Medicago calcium ATPase MCA8, to determine whether mis-localization of this protein may contribute to the phenotype observed in Medicago *nup133*.

This research was the first demonstration in plants of the requirement for nuclear pore complex components in the localization of inner nuclear membrane proteins, and presents an excellent opportunity to dissect this process further. Selective mutagenesis of the NLS sequences in the soluble N termini of POLLUX and DMI1 and subsequent localization of these soluble protein constructs via confocal microscopy could identify the critical amino acids required for their

nuclear import. Split-YFP or yeast two-hybrid interaction assays could be employed to identify the nuclear trafficking components which interact with the NLS domains. Graduated truncation of the linker region could demonstrate whether this region is necessary for inner nuclear membrane localization and if it is, the approximate length that is required. Due to the labor intensive nature of the preparation of samples for electron microscopy, initial screens of these constructs in nodulation rescue assays of *pollux* and *dmi1* lines could serve as proxy for disruption of localization, prior to TEM analysis. Finally, a reverse genetics approach could be utilized to assess the role of other nuclear pore complex members and nuclear transport machinery proteins in the translocation DMI1 and POLLUX to the inner nuclear membrane. Transcriptomic analysis in the Lotus and Medicago nucleoporin mutants could be employed to identify potential candidates for this approach.

Mycorrhizal chitin oligomers induce symbiotic responses in rice

Application of mycorrhizal LCOs and COs to rice and subsequent calcium spiking analysis demonstrated that COs, and not LCOs, act as the primary signaling molecules during mycorrhization in this crop (**Table 1: Signaling**). However, gene expression and root architecture modification was induced in response to both Myc LCOs and COs demonstrating that rice can still detect and respond to LCOs. While the CO receptors that mediate calcium spiking in legumes remains a mystery, in rice, two chitin binding proteins (OsCERK1 and OsCeBIP) are likely candidates (1). Assessing gene expression and lateral root formation in *Oscerk1* and *Oscebip* lines might help identify if these or another receptor protein are involved in mediating these responses in rice.

LCO based products are currently commercialized for use in plant growth enhancement, however COs are not. CO mediated enhancement of lateral root production may result in overall improvement of growth, and thus other assessing other phenotypes such as yield and biomass may provide evidence for their usefulness in agriculture. While we found root growth enhancement in response to chitin oligomers in rice, this response may or may not occur in other crops. Thus, application of COs to roots of maize, barley and millet for example, may provide insight into the conservation this response in valuable commodity cereal crops.

Mycorrhizal pathway may facilitate colonization of cereals by *Rhizobium* sp. IRBG74

Through assessment of a diverse panel of rhizobia in their ability to colonize rice roots, we found that this was not a ubiquitous trait (**Table 1: Establishment**). This raises questions about what bacterial components allow for colonization of rice roots and secondly, whether the ability to colonize rice indicates a similar ability to interact with other plants or not. Including more rhizobia and other potential host plants in this screen might reveal novel mechanisms via which rhizobia are able to colonize plant roots, or establish that known mechanisms such as EPS production, Type III secretion, production of Nod factors, or perception of flavonoids are involved. The nature of these symbioses has not yet been established, therefore assessing plant biomass after inoculation would reveal whether these interactions are beneficial, neutral, or parasitic in nature. It is also not clear how, and to what degree, these associations increase the fitness of the microbe (**Table 1: Maintenance**).

This study described the colonization of rice and *Setaria viridis* by *Rhizobium* sp. IRBG74. Accumulation around lateral root junctions was observed in rice, whereas root hair colonization was commonplace in *Setaria viridis* (**Table 1: Establishment**). This is the first report of COs

being involved in the colonization of cereal roots by rhizobia. Interestingly, we observed host specificity in the requirement for COs in external attachment (rice), or internal colonization (*S. viridis*). Differential means of entry may, along with differences in root architecture and compounds produced in root exudates (e.g. Flavonoids) between rice and *S.viridis*, may explain the disparity in these plants in their requirement for COs during external and internal colonization (**Table 1: Signaling**). Characterizing the colonization behavior of the *Rhizobium* sp. IRBG74 Δ*nodC* strain by GFP labeling and confocal microscopy may help determine if these bacteria develop new means of colonizing the plant upon defects in CO based signaling. Analysis of the root exudates produced by rice and *S. viridis* upon colonization by the *Rhizobium* sp, IRBG74 wild-type and Δ*nodC* strains for example could perhaps reveal differential production of plant defense compounds that may otherwise be suppressed by proper signaling.

Transcriptomic analysis of rice samples which had been treated with COs, LCOs, GSEs and *Rhizobium* sp. IRBG74 for 24 hours generated a large data set of differentially expressed genes and revealed that rice can distinguish all of these signaling molecules by responding with distinct sets of up and down regulated genes. Through this analysis we found that differential gene expression induced by GSEs is most similar to that induced by *Rhizobium* sp. IRBG74 Nod factors. This experiment provides an excellent resource for generating marker genes for future experiments and identifying missing components required for mycorrhizal symbiosis. Confirming the expression of some of these genes by qRT-PCR in wild-type rice followed by analyzing their expression pattern in *Oscastor*, *Ospollux, Osdmi3* and *Osipd3* lines may reveal whether or not they are involved in the common symbiosis pathway. Once reliable markers are generated, they can also be used to identify the receptors required for perception of chitin based signaling molecules in rice.

Understanding of the plant-microbe signaling required for establishment of beneficial symbiosis facilitates the use of these microbes and the compounds they produce for enhancing plant growth in agriculture. The findings presented in this thesis address long standing questions regarding the role of common symbiosis pathway members in mycorrhization and legume nodulation and paves the way for future research regarding the formation of symbiotic interactions in cereals. While much work needs to be done before nitrogen fixing cereals become a reality, clear evidence of perception and response to Nod factors by rice is encouraging and suggests that indeed some rhizobia may be co-opting the mycorrhizal pathway in cereals.

REFERENCES

1. Shimizu T, *et al.* (2010) Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. Plant Journal 64(2):204-214.

Table 1. Comparison of rhizobial associations with legumes and cereals

	Legume-Rhizobium Symbiosis	Cereal-Rhizobium Interactions
Signaling	Nod factors (LCOs)	Chitin oligomers (COs)
	Flavonoids	?
	Common symbiosis pathway	?
Establishment	Host Specificity	
	Suppression of host defenses	?
	Crack entry and root hair entry	Crack entry and root hair colonization
Maintenance	Host access to fixed nitrogen	No fixed nitrogen transfer
	Rhizobial access to fixed carbon	?
	Host regulation of symbiosis	?

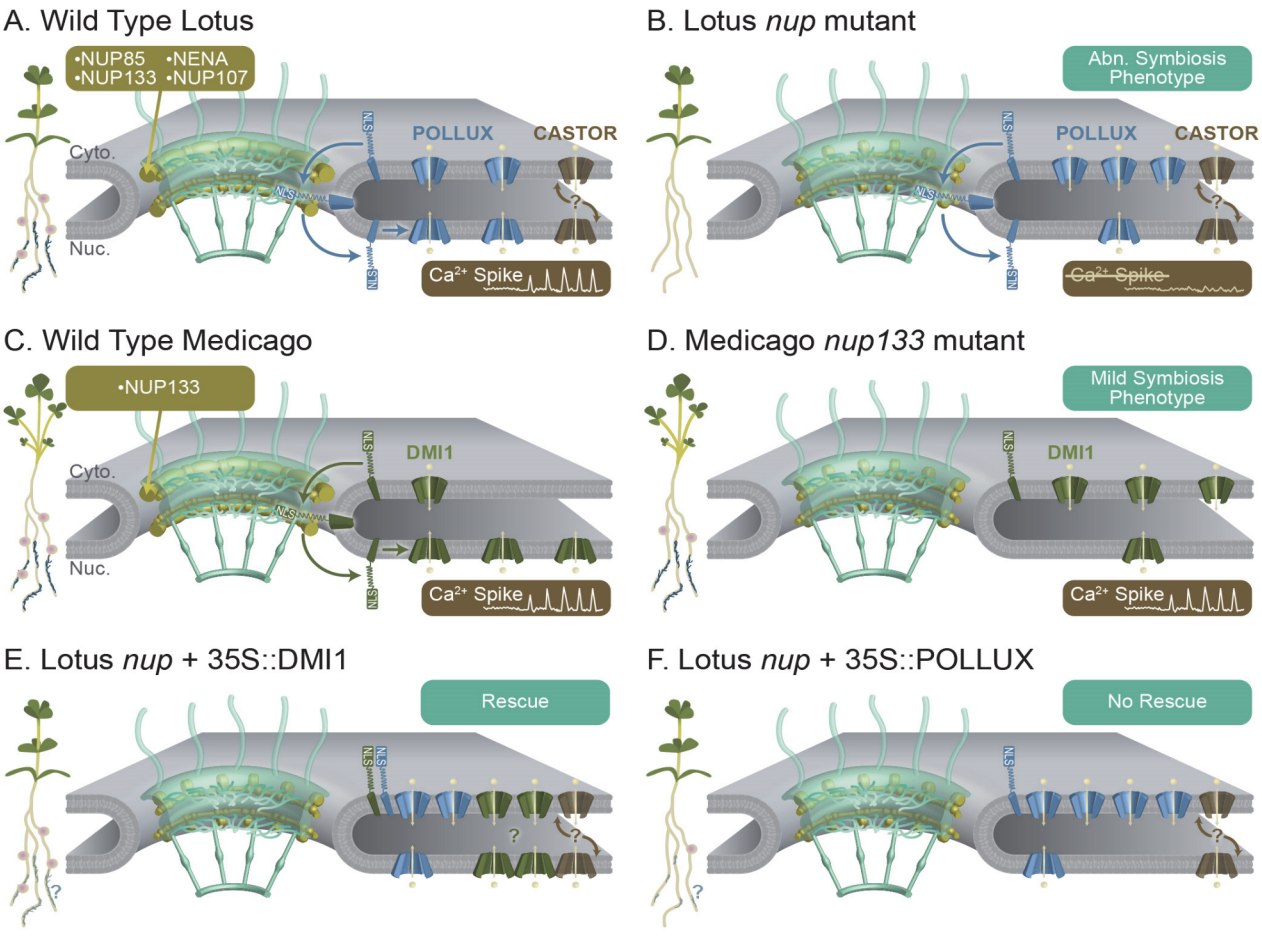

A. Wild Type Lotus
•NUP85 •NENA
•NUP133 •NUP107
Cyto.
Nuc.
NLS
POLLUX
CASTOR
Ca2+ Spike
B. Lotus nup mutant
Abn. Symbiosis Phenotype
POLLUX
CASTOR
Ca2+ Spike
C. Wild Type Medicago
•NUP133
Cyto.
Nuc.
DMI1
Ca2+ Spike
D. Medicago nup133 mutant
Mild Symbiosis Phenotype
DMI1
Ca2+ Spike
E. Lotus nup + 35S::DMI1
Rescue
F. Lotus nup + 35S::POLLUX
No Rescue

Figure 1. Nuclear envelope regulation of nodulation and mycorrhization

A) Nuclear envelope in wild-type Lotus. LjNUP85, LjNUP133, LjNENA and LjNUP107 are outer ring scaffold nucleoporins (yellow). POLLUX (blue) contains an unstructured linker region (wavy line) and two N-terminal NLS sequences, and is localized to the outer and inner nuclear membranes. CASTOR (brown) precise membrane localization is unknown. B) Lotus *nup133*, *nup85*, *nup107* and *nena* mutants are non-nodulating, do not form mycorrhizal associations at restrictive temperatures and calcium spiking is abolished (Abn. = abnormal). POLLUX (blue) showed slight mis-localization towards the outer nuclear membrane. The effect of these mutations on CASTOR localization is unknown. C) MtNUP133 is an outer ring scaffold nucleoporin. DMI1 also contains an unstructured linker region and two N-terminal NLS sequences. DMI1 has a slight preference for inner membrane localization. D) The *Mtnup133* mutants had reduced nodulation, but mycorrhization and calcium spiking occurred as in the wild-type. DMI1, however, showed a shift in localization towards the outer nuclear membrane. E) Over-expression of DMI1 in the Lotus *nup133, nup85* and *nena* mutants rescued the nodulation phenotype. Localization of DMI1 has not been conducted in this background, but we hypothesize that the distribution would be similar to that found in *Mtnup133*. The effect of DMI1 over-expression on mycorrhization is unknown. F) Over-expression of POLLUX in the Lotus nup mutant background does not fully rescue the nodulation phenotype although nodulation was slightly increased compared to empty vector. Whether or not POLLUX over-expression rescues the mycorrhization phenotype is unknown.