***Medicago truncatula* IPD3 Is a Member of the Common Symbiotic Signaling Pathway Required for Rhizobial and Mycorrhizal Symbioses**

Beatrix Horváth,¹ Li Huey Yeun,² Ágota Domonkos,¹ Gábor Halász,¹ Enrico Gobbato,³ Ferhan Ayaydin,⁴ Krisztina Miró,¹ Sibylle Hirsch,³ Jongho Sun,³ Million Tadege,⁵ Pascal Ratet,⁶ Kirankumar S. Mysore,⁵ Jean-Michel Ané,² Giles E. D. Oldroyd,³ and Péter Kaló¹

¹Agricultural Biotechnology Center, Gödöllő 2100, Hungary; ²Department of Agronomy, University of Wisconsin, 1575 Linden Drive, Madison 53706, U.S.A.; ³John Innes Centre, Norwich NR4 7UH, United Kingdom; ⁴Cellular Imaging Laboratory, Biological Research Center, Szeged 6726, Hungary; ⁵Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, U.S.A.; ⁶Institut des Sciences du Végétal, CNRS, 91198 Gif sur Yvette, France

Submitted 25 January 2011. Accepted 7 June 2011.

Legumes form endosymbiotic associations with nitrogen-fixing bacteria and arbuscular mycorrhizal (AM) fungi which facilitate nutrient uptake. Both symbiotic interactions require a molecular signal exchange between the plant and the symbiont, and this involves a conserved symbiosis (Sym) signaling pathway. In order to identify plant genes required for intracellular accommodation of nitrogen-fixing bacteria and AM fungi, we characterized *Medicago truncatula* symbiotic mutants defective for rhizobial infection of nodule cells and colonization of root cells by AM hyphae. Here, we describe mutants impaired in the interacting protein of DMI3 (*IPD3*) gene, which has been identified earlier as an interacting partner of the calcium/calmodulin-dependent protein, a member of the Sym pathway. The *ipd3* mutants are impaired in both rhizobial and mycorrhizal colonization and we show that *IPD3* is necessary for appropriate Nod-factor-induced gene expression. This indicates that *IPD3* is a member of the common Sym pathway. We observed differences in the severity of *ipd3* mutants that appear to be the result of the genetic background. This supports the hypothesis that *IPD3* function is partially redundant and, thus, additional genetic components must exist that have analogous functions to *IPD3*. This explains why mutations in an essential component of the Sym pathway have defects at late stages of the symbiotic interactions.

Legumes form nitrogen-fixing symbioses with soil bacteria collectively called rhizobia and, like most plant species, they also establish interactions with arbuscular mycorrhizal (AM) fungi. Both symbioses facilitate the uptake of mineral nutrients. Host plants and their symbionts form specialized structures

during these interactions: rhizobia induce the development of root nodules on the host plant wherein the reduction of atmospheric nitrogen takes place, while AM fungal hyphae form a densely ramified structure in the inner cortical cells of the root where the transfer of nutrients occurs.

The exchange of diffusible signaling molecules between symbionts and host plant takes place prior to physical contact. The flavonoid and isoflavonoid contents of root exudates activate rhizobia to produce nodulation (Nod) factors (NF), which induce the host plant to initiate nodulation (Oldroyd and Downie 2008; Riley et al. 2006). The perception of NF activates ion fluxes (Felle et al. 1998) and calcium oscillations in the cytoplasm (Ehrhardt et al. 1996), modulates the cytoskeleton (Timmers et al. 1999), and induces early nodulin genes (Catoira et al. 2000; Journet et al. 1994) in root epidermal cells. NF also induce root hair deformations, cortical cell divisions, and the formation of preinfection structures (Oldroyd and Downie 2008). As a signal for mycorrhizal fungi, roots release strigolactones that promote hyphal branching of AM fungi (Akiyama et al. 2005). Physiological and molecular data presumed the existence of fungal factors which elicit responses on the host plant (Kosuta et al. 2003; Kuhn et al. 2010; Navazio et al. 2007; Olah et al. 2005), including calcium oscillations and gene expression in root epidermal cells (Kosuta et al. 2008). The recent study by Maillet and associates (2011) identified lipochitooligosaccharide molecules acting as the fungal signaling factor. The structural similarity of these mycorrhizal factors with NF further supports the idea that the root nodule symbiosis recruited the components of the ancient developmental program of AM symbiosis (Parniske 2008).

Both rhizobia and mycorrhizal fungi exist as intracellular symbionts, which requires the coordinated development of both partners to allow invasion and appropriate differentiation (Jones et al. 2007; Oldroyd and Downie 2008; Parniske 2008). The best-characterized rhizobial infection strategy occurs via root hairs. Rhizobia become trapped in root hair curls and induce invaginations from these curled root hairs, forming tubular structures called infection threads (IT). The IT grows toward the newly divided cells of the developing nodule primordium, wherein the bacteria are released and colonize the host cells through endocytosis. The bacteria become enveloped with a plant-derived peribacteroid membrane, forming a cytoplasmic structure referred to as the symbiosome. In the symbiosomes, the bacteria undergo morphological changes and metabolic dif-

Current address for S. Hirsch: VIB, Department of Plant Systems and Biology, B-9052 Ghent, Belgium

Current address for M. Tadege: Department of Plant and Soil Sciences, Oklahoma State University, Stillwater 74078, U.S.A.

Corresponding author: P. Kaló; Telephone: + 36 28-526104; Fax: +36 28-526101; E-mail: kalo@abc.hu

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures and one supplementary table are published online and that Figure 7 appears in color online.

ferentiation to transform into their symbiotic state, termed bacteroids. The symbiosome is the site of nitrogen fixation and functions in nutrient and signal exchange between the plant and the bacteroid (Lodwig and Poole 2003). Signal exchanges and the contact of AM hyphae with the root also induce morphogenetic changes in the partners. Upon contact with the root, the hyphae swell to develop the hyphopodium, a penetration apparatus that facilitates the invasion of epidermal cells (Lima et al. 2009; Parniske 2004; Smith et al. 2006). AM fungal hyphae cross the epidermis through intracellular invasion and traverse the outer cortex by intercellular invasion, before penetrating inner cortical cells wherein highly branched hyphal structures, the arbuscules, are formed. The arbuscules function as an interface to facilitate nutrient exchange between the fungus and the host plant.

Genetic dissection of nodulation in the model legumes *Medicago truncatula* and *Lotus japonicus* has identified many of the components of the NF-signaling pathway. This analysis revealed that several plant genes are required for both the bacterial and fungal symbioses and, thus, both symbiotic interactions share common signaling components (the common Sym pathway). In the legume-rhizobia symbiotic model systems, ion channel proteins DMI1 (Ané et al. 2004) and CASTOR and POLLUX (Imaizumi-Anraku et al. 2005); nucleoporins *NUP85*, *NUP133*, and *NENA* (Groth et al. 2010; Kanamori et al. 2006; Saito et al. 2007); and a membrane-anchored leucine-rich repeat receptor-like kinase, DMI2/NORK/SYMRK (Endre et al. 2002; Stracke et al. 2002) are essential for the induction of calcium spiking (Wais et al. 2000). A calcium/calmodulin-dependent protein kinase (CCaMK) (DMI3) (Lévy et al. 2004; Mitra et al. 2004) functions downstream of calcium spiking and CCaMK is postulated to decode and transmit the calcium signal (Oldroyd and Downie 2006). The Sym pathway is required throughout the process of rhizobial invasion and symbiosome formation (Capoen et al. 2005; Godfroy et al. 2006; Limpens et al. 2005).

The formation of fully effective symbiotic nodules requires coordinated regulation between the processes of rhizobial infection initiated in epidermal cells and nodule organogenesis induced in the root cortex (Oldroyd and Downie 2008). Gain-of-function alleles of *CCaMK* induce spontaneous nodulation in the absence of either rhizobia or NF (Gleason et al. 2006; Tirichine et al. 2006), indicating that activation of CCaMK is sufficient for nodulation signaling. In addition to *CCaMK*, a gain-of-function allele of the cytokine receptor gene (*LHK1*) (Tirichine et al. 2007) is also able to active nodule organogenesis in the absence of rhizobia. The functional hierarchy of symbiotic plant genes in coordinating nodule formation and infection was defined on the basis of spontaneous nodulation and infection phenotypes using these gain-of-function mutants (Hayashi et al. 2010; Madsen et al. 2010). The functional analysis revealed the central regulatory position of CCaMK in connecting the infection and organogenetic pathways in *L. japonicus*. In addition, it was also demonstrated that the components of the common Sym pathway upstream of calcium spiking are only required for activation of CCaMK.

CCaMK interacts with the interacting protein of DMI3 (IPD3) (Messinese et al. 2007) and, like CCaMK (Kaló et al. 2005), IPD3 was localized to the nucleus. The ortholog of *M. truncatula* IPD3 has been identified recently in *L. japonicus* (Yano et al. 2008) and the analysis of *cyclops* mutants demonstrated the function of CYCLOPS in invasion and accommodation of both rhizobial and fungal symbiotic partners. The failure of IT growth and the absence of rhizobial infection on spontaneous nodules developed on *L. japonicus cyclops* roots suggested the importance of CYCLOPS in the CCaMK-mediated cross talk between the IT and the nodule developmental pathways (Hayashi et al. 2010; Madsen et al. 2010).

The functional analysis of *IPD3* was not feasible until now because of the lack of available *ipd3* mutants. In this article, we report the identification of mutant alleles of *IPD3* and demonstrate that *IPD3* is required for IT growth and the invasion of nodules by rhizobia. *IPD3* is also indispensable for arbuscule formation in AM symbiosis. Based on the mutant phenotypes, the detected physical interaction between IPD3 and CCaMK, and the requirement of *IPD3* for proper induction of early nodulin gene expression, we conclude that *IPD3* is a member of the common symbiotic signaling pathway and is necessary for the invasion of the host cell by both rhizobial and fungal symbiotic partners.

RESULTS

A novel *M. truncatula* allele required for rhizobial infection.

Fast-neutron mutagenized *M. truncatula* Jemalong plants were screened for mutant plants ineffective in nodulation (Marsh et al. 2007). From this screen, we identified a mutant that showed symptoms of nitrogen starvation (yellowish leaves and a stunted growth habit) and developed small white nodules on roots, indicating the absence of leghemoglobin (Fig. 1A and K to M). Acetylene reduction assays in mutant nodules showed the complete absence of nitrogenase activity (data not shown). A significant increase in nodule number was detected in the mutant compared with wild-type plants following 6 weeks of inoculation with *Sinorhizobium meliloti* (Supplementary Fig. S1). For reasons described below, we called the responsible locus *IPD3*, with this mutant allele referred to as *ipd3-1*.

The kinetics of nodule development and bacterial infection were analyzed in wild-type and *ipd3-1* plants using *S. meliloti* 1021 (pXLGD4), which constitutively expresses the *lacZ* gene. The *ipd3-1* plants showed delayed nodule development and delayed and defective bacterial infection (Fig. 1B, D, and F). No evident difference was observed in root length, number of rhizobial microcolonies, and nodule primordia per root length (data not shown) between wild-type plants and *ipd3-1* at 5 or 10 days following inoculation. The number of growing IT in root hairs per measure of root length was higher in *ipd3-1* mutants (1.9 ± 0.8 ; mean $\pm$ standard deviation [SD]) than in wild-type plants (0.5 ± 0.45) 3 days postinoculation (dpi). The *ipd3-1* mutant showed an extended infected region of the root: 5.6 ± 1.8 cm for wild-type roots and 8.7 ± 1.3 for *ipd3-1* (mean $\pm$ SD) 10 dpi. After four or more weeks, two types of white nodules developed on mutant plants; most nodules were small and round in shape (Fig. 1L) and these nodules often showed abnormal morphology at the nodule apex, with IT limited to this region (Fig. 1F). A small number of nodules developed into elongated cylindrical structures (Fig. 1M) but without the characteristic zonation of indeterminate nodules. Behind the meristematic region, these nodules contained an extended invasion zone with hypertrophied IT and a narrow zone of cells at the base of the nodules which were not penetrated by IT (Fig. 1H). The frequency of these elongated nodules varied between experiments and individuals but generally was between 5 and 10% of all nodules.

To further investigate the progression of the infection process in the elongated nodules of *ipd3-1*, we used the nucleic acid-binding dye SYTO13 that reveals plant cell nuclei and bacteria (Haynes et al. 2004). Nodules on wild-type plants showed cells largely occupied by bacteria (Fig. 2A). In contrast, only plant nuclei fluoresced in the cells in the basal part of the *ipd3-1* elongated-type nodules (Fig. 1H), indicating that these cells were not invaded by rhizobia (Fig. 2B). The images taken at the boundary of the enlarged invasion zone and the basal part of *ipd3-1* nodules showed cells with fluorescent

plant nuclei and branched IT (Fig. 2C), with bacteria retained inside of the IT (Fig. 2D). Most IT displayed an abnormal morphology; they were thick and highly crooked with enlarged, blister-like formations (Fig. 2D) when compared with wild-type ones. Ultrathin sections in the nitrogen fixation zone of wild-type nodules and especially the central region of elongated-type mutant nodules were analyzed using transmission electron microscopy. Wild-type nodule cells were fully packed with bacteroids (Fig. 2E) whereas cells in *ipd3* nodules were devoid of bacteroids (Fig. 2F). IT in *ipd3-1* showed unusually thick layers between the lumen and the plant cell walls and tortuous plasma membrane (Fig. 2H) which were never observed in wild-type ones. From these analyses, we conclude that the major function of *IPD3* is to promote appropriate IT growth.

Positional cloning identifies *IPD3*.

A map-based cloning approach was used to identify the gene affected in the *ipd3* mutant. The segregation ratio of the symbiotic phenotype in the backcrossed (40 Fix+/13 Fix-) and mapping populations (323 Fix+/135 Fix-) indicated that

the mutant phenotype was inherited as a single recessive locus ($\chi^2 = 0.062$ and probabilities [*P*] between 0.975 and 0.9 in the backcrossed population and $\chi^2 = 4.89$ and *P* = 0.025 to 0.05 in the F2 population). Initially, the mutant locus was positioned on the upper arm of chromosome 5 (LG 5) between markers MtB108 and MtB93 (Mun et al. 2006). Fine mapping positioned the locus in the genomic region covered by BAC clones mth4-3n15 (accession number CU914135) and mth4-28c15 (CU469555) (Supplementary Fig. S2A). Intron-targeted markers were designed based on predicted coding sequences on these two BAC clones, and genetic markers generated for the Golgi transport-related protein (*GTP*) and the *BREVIS RADIX*-like (*BRL*) genes identified a genomic region of 152 kb that contained the locus. Oligonucleotide primers were synthesized for the seven predicted genes in the region and polymerase chain reactions (PCR) were performed to amplify genomic DNA and cDNA samples prepared from the mutant plant.

The amplification of a part of the *IPD3* gene detected a deletion in the *IPD3* transcript. Sequence analysis of the *IPD3*

Fig. 1. Nodulation phenotypes of *ipd3-1* and wild-type plants. **A**, The *ipd3-1* mutant showed stunted growth compared with the wild type following 6 weeks of inoculation with *Sinorhizobium meliloti* strain. Kinetics of nodule development in **B**, **D**, **F**, and **H**, *ipd3-1* and **C**, **E**, and **G**, wild-type plants inoculated with *S. meliloti* expressing the *lacZ* gene. Nodule sections (60 μ m thick) were prepared with a vibration microtome and stained with X-gal at 5 (**B** and **C**), 10 (**D** and **E**), and 21 (**F** and **G**) days postinoculation (dpi). **I**, *ipd3-1* and **J**, wild-type root hairs with infection threads. The infection thread in *ipd3-1* is delayed in development compared with the wild type. **H**, Light micrograph of an X-gal-stained elongated-type mutant nodule 4 weeks postinoculation. **K**, Pink nodules developed on wild-type root following 4 weeks of inoculation. **L** and **M**, Two types of white nodules, small spherical (**L**) and elongated (**M**), were formed on *ipd3-1* roots. Scale bars: 2 cm in **A**, 200 μ m in **B** to **H**, and 2 mm in **K** to **M**. Bars in the images of **I** and **J** = 20 μ m.

cDNA revealed that the entire fourth exon of *IPD3* was missing in the *ipd3-1* mutant. A single base-pair change and a 6-bp deletion at the junction of the fourth exon and fourth intron in *IPD3* caused imperfect splicing and a transcript lacking exon 4 (Fig. 3A). The deletion of the fourth exon of the *IPD3* transcript generates a frameshift and a premature translational termination in exon 5 and, thus, the mutant transcript encodes a truncated protein with eight additional non-*IPD3*-specific residues following the third exon. The predicted incomplete gene product lacks the terminal nuclear localization signal and a region determined to be sufficient for the interaction with CCaMK (Messinese et al. 2007).

Novel mutant allele of *IPD3*.

To validate the genetic identity of *IPD3*, we identified another *ipd3* allele (NF5939) from the *Tnt1*-insertional population of *M. truncatula* (Tadege et al. 2008). PCR-amplified fragments were sequenced to identify the exact insertion site of the 5.3-kb *Tnt1* retrotransposon in the NF5939 line (Fig. 3A) and this was used to generate homozygous mutant lines. F1 plants from a cross of *ipd3-1* and NF5939 were all Fix⁻ ($n = 4$), indicating that they belong to the same complementation group; therefore, NF5939 is hereafter termed *ipd3-2*. Mutant and wild-type roots were inoculated with *S. meliloti* 1021 (pXLGD4) to analyze the function of *IPD3* in root-nodule symbiosis. On wild-type roots, IT and fully developed nodules were observed (Fig. 3B and D). Nodule-like structures were developed on mutant roots (Fig. 3C), suggesting the induction of cortical cell divisions. Although *ipd3-2* mutants displayed root hair deformations, no IT growth was observed in the presence of rhizobia (Figs. 3C and 2E), indicating defects in IT development in the *ipd3-2* mutant.

Genetic complementation of *ipd3* mutants.

In order to further prove that the mutation in *IPD3* caused the mutant phenotypes, we carried out genetic complementation experiments. The open reading frame of *IPD3* was fused either to the native *IPD3* or 35S promoters and introduced into *ipd3-1* and *ipd3-2* roots using *Agrobacterium rhizogenes*-mediated hairy root transformation. The roots were inoculated with *S. meliloti* 1021 (pXLGD4). Transformed roots were detected by either the green fluorescent protein (GFP) or DsRed fluorescent markers. Because rhizobia induce noninvaded nodules in the *ipd3-1* mutant, we assessed the infection of the nodules on the transformed roots following X-Gal staining. White undeveloped nodules formed on *ipd3-1* and *ipd3-2* roots transformed either with the empty vector or the 35S::*IPD3*, and these nodules lacked the proper zonation of indeterminate nodules with bacteria restricted to IT (Fig. 4A to F), indicative of the mutants. The inactivity of the 35S promoter in the nodule tissue was reported by Auriac and Timmers (2007). Therefore, the unsuccessful complementation of *ipd3* mutants with the 35S promoter construct suggests the requirement of the proper spatiotemporal expression of *IPD3* during nodule development.

The nodules on the roots of *ipd3-1* and *ipd3-2* mutants transformed with *IPD3* driven by its native promoter were pink, suggesting that these were functional nodules (Fig. 4G and P). The nodules showed the zones of typical indeterminate nodules and cells in the infection and the nitrogen fixation zones stained blue, indicating that these cells were invaded by rhizobia (Fig. 4I). *S. meliloti* CSB357 containing a *nifH::uidA* fusion (Bright et al. 2005) revealed normal *nifH* expression in these complemented nodules (Fig. 4R), indicating a full rescue of the mutant phenotype.

IPD3 is closely homologous to *L. japonicus* *CYCLOPS* (Yano et al. 2008). However, the *ipd3-1* and *cyclops* mutants are not identical in phenotype. Although only development of

nodule primordia and block of mycorrhizal intracellular infection was observed on *cyclops* mutant, nodule and arbuscule development is more advanced on *ipd3-1* roots. To assess whether *CYCLOPS* is orthologous to *IPD3*, we transformed *ipd3-1* with *L. japonicus* *CYCLOPS* driven by the *CYCLOPS* native promoter. The nodules on transformed roots showed patterns of *S. meliloti* expressing *lacZ* activity indicative of wild-type nodules (Fig. 4L) and we could detect bacterial release into the nodule cells, suggesting that *L. japonicus* *CYCLOPS* can complement the *ipd3-1* mutant.

IPD3 functions in NF signaling.

It has been proposed that *CYCLOPS* functions in the Sym pathway downstream of calcium spiking in a complex with CCaMK/DMI3 (Yano et al. 2008). In a similar fashion, we found that the *ipd3-1* mutant showed a pattern of calcium spiking similar in periodicity and upward phase of the spikes to the wild-type plants following NF treatment (Fig. 5A). The proposed position of *IPD3/CYCLOPS* would place it upstream of NF-induced gene expression. To test this, we crossed the *pENOD11::β-glucuronidase* (*GUS*) reporter (Journet et al. 2001) into the *ipd3-1* mutant. In contrast to wild-type plants, *ipd3-1* showed a very limited NF induction of *pENOD11::GUS*; activity was restricted to a smaller region than the wild-type and the intensity of *GUS* staining was always reduced (Fig. 5B). This was further validated using quantitative reverse-transcription (qRT)-PCR that also allowed us to test additional genes (Fig. 5C). A significant reduction of NF-inducible *ENOD11* expression was detected following 16 h of NF treatment in the *ipd3-1*, *ipd3-2* and *ipd3-3* (*Mtsym1*) (Ovchinnikova et al. 2011) alleles. In addition, a lower relative transcript level of *NIN* and *HAP2.1* (Comber et al. 2006) was also detected in the three *ipd3* mutants (Fig. 5C). As was demonstrated earlier, *DMI2* and *NSP2* are required for NF-induced gene expression (Catoira et al. 2000; Oldroyd and Long 2003) and, accordingly, no or reduced induction of *ENOD11*, *HAP2*, and *NIN* was detected in *dmi2* and *nsp2* mutants (Fig. 5C). *LIN* is suggested to function specifically in the IT pathway (Kiss et al. 2009) and, accordingly, the induction of genes in the organogenic pathway (*ENOD11*, *HAP2* and *NIN*) was strongly induced in *lin* mutant. To examine gene expression during nodule formation and bacterial infection, we monitored the relative transcript levels of these marker genes in wild-type and *ipd3* mutant plants after 1, 8, and 14 days of inoculation with *S. meliloti*. We observed reductions in the expression of *ENOD11*, *NIN*, and *HAP2.1* in both *ipd3-1* and *ipd3-3* but the nature of this reduction was not consistent across all genes (Fig. 5C).

ipd3 mutant alleles show distinct defects in the interaction with mycorrhizal fungi.

CCaMK/DMI3 and *L. japonicus* *CYCLOPS* are important for mycorrhizal symbiosis; therefore, we assessed wild-type, *ipd3*, and *ccamk* colonization by *Glomus intraradices*. The roots of wild-type plants were highly colonized with intraradical hyphae, arbuscules, and vesicles in inner cortical cells (Fig. 6A, B, and G) and these were completely lacking in *ccamk-1*. Light microscopy did not show abnormal branching or other clear visible differences in vesicle and arbuscule formation in *ipd3-1* mutant roots (Fig. 6C and D). However, quantification of colonizing fungal structures revealed that the frequency of colonization was significantly reduced in the *ipd3-1* mutant line compared with wild-type roots (Fig. 6E). The *ipd3-2* mutant showed more severe defects in the mycorrhizal interaction than *ipd3-1*; arbuscules were not observed in mutant roots co-cultivated with *G. intraradices* despite the presence of extraradical and intraradical fungal

hyphae (Fig. 6I). Brown-pigmented cells were frequently observed on mutant roots, suggesting the production of phenolic compounds (Fig. 6I). Very little brown pigmentation was observed in roots with functional arbuscules or in roots that were not inoculated with fungal spores (Fig. 6F and H).

Interaction between IPD3 and CCaMK.

IPD3 of *M. truncatula* and its ortholog in *L. japonicus* CYCLOPS both interacted with CCaMK in yeast two-hybrid systems and in transiently transformed *Nicotiana benthamiana* leaves (Messinese et al. 2007; Yano et al. 2008). To gain more

Fig. 2. Bacterial invasion of nodules is impaired in *ipd3-1*. **A**, Nodule cells in the nitrogen fixation zone of a wild-type nodule. Most cells are fully occupied with differentiated bacteria. **B**, Nodule cells in the central part of an *ipd3-1* elongated nodule contained no bacteria and only plant nuclei fluoresced in these cells. **C**, Cells in the boundary of the extended invasion zone and the empty central region of a mutant nodule. Plant nuclei and infection threads with nonreleased, undifferentiated bacteria are visible. **D**, Higher magnification of infection threads with nonreleased bacteria. Note the abnormal blister-like formations of the infection threads (arrowhead). **E**, Electron micrograph of cells in the nitrogen fixation zone of a wild-type nodule. Nodule cells were invaded and occupied by differentiated bacteria. **F**, Cells in the central region of a mutant nodule were not invaded by rhizobia. **G**, Higher magnification of a cross-section of the infection thread shown in **E**. The asterisk indicates rhizobia being released. **H**, Higher magnification of the infection thread with a thickened layer between the lumen of the infection thread and plant cell wall from **F** (arrow). Double arrowheads indicate the folds of the plasma membrane around the cell wall which was not observed on wild-type infection threads. Scale bars: 10 μ m in **A** to **D**, 2 μ m in **E** and **F**, and 500 nm in **G** and **H**; n = plant nucleus, v = vacuole, it = infection thread with nonreleased bacteria, and b = bacteroids.

insight into the physical interaction between IPD3 and CCaMK, further interaction studies were carried out by yeast two-hybrid systems. The interaction of wild-type or mutated versions of IPD3 and CCaMK were investigated and, as reported earlier, we detected that *M. truncatula* CCaMK fused to either the DNA binding domain (BD) or the activation domain (AD) of GAL4 interacts with IPD3 (Fig. 7). No homodimerization of CCaMK was detected in this system, contrary to previous bimolecular fluorescence complementation (BiFC) studies (Messinese et al. 2007). IPD3 causes autoactivation of the GAL4 system when fused to the GAL4 DNA BD and we were able to delimit the region responsible for this autoactivation between residues 129 and 384 of IPD3 (Supplementary Fig. S3). The interaction between autoactive BD-IPD3 and AD-CCaMK was demonstrated by quantifying the induction of the reporter gene *lacZ*. IPD3 fused to the DNA-BD of GAL4 showed increased *lacZ* reporter gene induction when it was co-expressed with AD-CCaMK (14.85 ± 0.58 β -galactosidase units; mean $\pm$ standard error [SE]) compared with the combination of BD-IPD3 with empty AD of GAL4 (3.31 ± 0.23 β -galactosidase units), indicating interaction

of autoactive BD-IPD3 with AD-CCaMK. Yeast growth on histidine-free (LWH) but not on histidine and adenine-free (LWHA) medium revealed a low-affinity protein interaction between IPD3 385 and 513 fused to the BD and full-length IPD3 fused to AD, indicating that the 1-to-384 region of IPD3 promotes homodimerization of IPD3 in yeast (Fig. 7). Autoactive forms of CCaMK were analyzed for their interaction with IPD3. CCaMK T271D, mutated at the putative autophosphorylation site (Gleason et al. 2006), interacted with IPD3. However, the truncated autoactive form of CCaMK (CCaMK 1-311; containing only the kinase domain and lacking the auto-inhibition domain and the three elongation factor hand motifs) was not able to interact with IPD3 (Fig. 7), which indicates that the activated kinase domain of CCaMK is not sufficient for the interaction with IPD3.

DISCUSSION

IPD3 was initially identified in an interaction screen with CCaMK/DIM3, a component of the NF signal transduction

Fig. 3. Nodulation phenotype of the *Tnt1* retrotransposon insertion allele of *ipd3*. **A**, Gene structure of *IPD3* and the position of the *Tnt1* retrotransposon insertion are shown. Black and open boxes represent exons, lines represent introns, dotted lines represent untranslated regions, and a triangle represents insertion of *Tnt1* transposon in *ipd3-2*. Positions of the oligonucleotide primers used to detect the *Tnt1* insertion (IPD3-fw3 and IPD3-rv2) or the deletion (IPD3_F2 and IPD3_R2) are indicated by horizontal arrows. Open box shows the fourth exon of *IPD3*, of which the boundary with the fourth intron contains the mutation in *ipd3-1*. The wild-type and mutant sequence of the splice site between the fourth exon and intron is shown below; nucleotides in capital letters represent exon region and noncapital letters represent the intron sequence. The G-C nucleotide change in the splice site in the *ipd3-1* sequence is highlighted in black and the deleted nucleotides are represented by dashed lines. **B** to **E**, Nodulation development is impaired in the *Tnt1* transposon insertion mutant *ipd3-2*. **B**, Wild-type nodule and **C**, *ipd3-2* nodule. **D**, Wild-type root hair with an infection thread. **E**, *ipd3-2* with an infection focus within a curled root hair. Scale bars: B and C, 500 μ m; D and E, 20 μ m.

pathway (Messinese et al. 2007). We report here the identification of *ipd3* mutants and the characterization of their symbiotic defects. The nodulation phenotypes of *ipd3* mutants described here and in the accompanying manuscript (Ovchinnikova et al. 2011) indicate that *IPD3* functions in IT growth and release of

bacteria from IT. Nodule development was severely retarded in *ipd3* mutants, with nodule formation blocked at an early primordial stage. Occasionally, we observed more developed nodules in *ipd3-1* mutants and not in *ipd3-2* mutants but these more-developed nodules never showed bacterial release or

Fig. 4. A to L, *IPD3* and *CYCLOPS* restore the nodulation phenotype of *ipd3-1*. *IPD3* and *CYCLOPS* genes were introduced into *ipd3-1* roots with *Agrobacterium rhizogenes* and transformed roots were inoculated with *Sinorhizobium meliloti* 1021 carrying the *lacZ* gene. Complementation was assessed by the restoration of the typical zonation of the indeterminate nodules characteristic for *Medicago truncatula*. Transgenic roots were identified by green fluorescent protein fluorescence. Nodules on transgenic roots induced with either **A to C**, the empty vector or **D to F**, the vector containing the *IPD3* coding region driven by the 35S constitutive promoter did not show the typical zonation of indeterminate nodules. **G to I**, Complementation of the *ipd3-1* mutant was carried out with the construct containing 1.23 kb of the native *IPD3* promoter and the open reading frame of *IPD3*. **J to L**, Nodules on *ipd3-1* roots containing *LjCYCLOPS* gene showed infected cells in the invasion and nitrogen-fixing zones, indicating the restoration of the nodulation phenotype. **M to R**, Genetic complementation of *ipd3-2* was carried out with the construct containing the same *IPD3* insert as used for genetic complementation of *ipd3-1*. Transgenic roots were identified by DsRed fluorescence. Roots were inoculated with *S. meliloti* CSB357 (*nifH::uidA*). **M to O**, Nodules formed on *M. truncatula* R108 roots transformed with empty vector; **P to R**, nodules on *ipd3-2* roots transformed with the *pIPD3::IPD3* construct. Scale bar: **A, B, D E, G, H, J, and K** = 1 mm; **M, N, P, and Q** = 0.5 mm; **C, F, I, and L** = 200 μ m; and **O and R** = 100 μ m.

symbiosome formation. Early nodulin gene expression was defective in the *ipd3* mutants, and this correlates well with the defects we observed in nodule development. The detailed transcriptome analysis of the *Mtsym1* mutant (the *ipd3-3* allele) showed similar defects in gene expression in a recent study by Maunoury and associates (2010), whereby a majority of nod-

ule-specific genes were not induced in *Mtsym1* but a small number of genes, several of them supposed to function in primordium initiation, were activated at a lower level. *IPD3* is also necessary for the symbiotic association with mycorrhizal fungi, with *ipd3* mutants showing reduced fungal colonization. The severity of the mycorrhizal defect appears to

Fig. 5. A, The *ipd3-1* mutant shows normal calcium spiking response to Nod factor (NF). The shape and the frequency of calcium spiking traces detected on *ipd3-1* root hairs show similar pattern to wild-type root hairs, indicating that the calcium spiking response is intact in *ipd3-1* mutant plants. Both traces are from single representative root hairs monitored for 25 min following the application of 1 nM NF. The inset table summarizes the total number of monitored and responsive root hair cells. **B,** NF-induced *ENOD11::β-glucuronidase* (*GUS*) expression is reduced in *ipd3-1* mutant. One-week-old wild-type and *ipd3-1* mutant plants were continuously treated with 1 nM NF for 24 h and afterward stained for *GUS* activity. Mutant roots showed reduced *GUS* activity compared with the wild type. Nonsymbiotic *GUS* activity was detected in both the wild-type and mutant root caps. Scale bar: 1 mm. **C,** The *ipd3* mutants show defects in NF- and rhizobia-induced gene expression. Relative transcript levels of *ENOD11*, *HAP2*, and *NIN* genes in wild-type and mutant plants were calculated in relation to nontreated wild-type plants or 1-day-old inoculated wild-type plants. Values of relative transcript levels are the mean of three to six biological and three technical replicates. Error bars represent standard error; dpi = days postinoculation.

be dependent on the genetic background in which the *ipd3* mutation resides: the *ipd3-1* allele, that is in a Jemalong background, developed normal internal fungal infection structures, with apparently wild-type arbuscules, although at a reduced frequency. In the accompanying article, Ovchinnikova and associates (2011) report a similar mycorrhizal phenotype in the *ipd3-3* allele, also in a Jemalong background. In contrast, the *ipd3-2* allele, that is in a R108 (*M. truncatula* subsp. *tricycla*) background, shows a complete absence of arbuscules and the accumulation of phenolic compounds. From the analysis of both ecotypes, we interpret that *IPD3* is necessary to facilitate fungal colonization, particularly the invasion of inner cortical cells to form arbuscules.

Our analysis reveals that *IPD3* is orthologous to *L. japonicus* *CYCLOPS*. Despite this orthology, we see differences in the severity of the mutant phenotypes between *cyclops* and *ipd3-1*: nodulation is more developed in *ipd3-1* and mycorrhizal fungi are able to colonize *ipd3-1*, leading to arbuscule formation. These differences are unlikely to be the result of subtleties of mutant allele action, because *ipd3-1* and *ipd3-3* show very similar mutant phenotypes (Bénaben et al. 1995) and both are likely to be null mutants. This is also not the result of subtleties in protein action between *IPD3* and *CYCLOPS* because *CYCLOPS* can complement *ipd3-1*. The *ipd3-2* mutation is more analogous to *cyclops* mutants: both show severe defects in rhizobial infection and nodule formation and both show an absence of arbuscule development. This implies that the genetic background in which the *ipd3/cyclops* mutation resides dictates the severity of the mutant phenotype. Thus, genetic components present in *M. truncatula* subsp. *truncatula* Jemalong (where *ipd3-1* and *ipd3-3* reside) can partially compensate for the absence of *IPD3* and these components are less

active or absent in *M. truncatula* subsp. *tricycla* R108 and *L. japonicus*.

IPD3/CYCLOPS functions in symbiosis signaling downstream of calcium spiking but upstream of NF induced gene expression, at an analogous position to *CCaMK*. It was previ-

Fig. 7. Dissection of *IPD3*–calcium/calmodulin-dependent protein kinase (*CCaMK*) interactions using yeast two-hybrid analysis. *CCaMK* interacts with *IPD3* but not with itself. The kinase domain of *CCaMK* alone does not interact with *IPD3* but the constitutively active *CCaMK*-T271D mutant does. BD-*IPD3* auto-activates the yeast-two-hybrid system and the auto-activating region was mapped by serial deletions. BD-*IPD3* 385-513 does not auto-activate the system and retains interaction with *IPD3* but not with *CCaMK*. AD = activation domain of *GAL4*; BD = binding domain of *GAL4*. Selection media were LW, Leu- Trp-; LWH, Leu- Trp- His-; and LWHA, Leu- Trp- His- Ade-.

Fig. 6. Arbuscular mycorrhizal (AM) colonization of roots shows distinct defects in *ipd3* mutant plants. **A to D**, Two-day-old wild-type and *ipd3-1* seedlings were inoculated with purified *Glomus intraradices* spores. Root samples were stained with ink 6 weeks after inoculation to investigate colonization levels. Ink-stained fungal structures are shown for **A and B**, wild-type and **C and D**, *ipd3-1* plants. **D**, The *ipd3-1* plants displayed normal arbuscule development, as shown in the higher magnification images on the right. **E**, AM colonization levels in wild-type (wt) and *ipd3-1* plants. As a negative control for mycorrhization, the calcium/calmodulin-dependent protein kinase (*ccamk*) (*dmi3-1*) mutant was included. Error bars represent standard errors. **F to I**, The *ipd3-2* mutant showed more severe defects in establishing AM symbiosis; **G**, arbuscules, intraradical hyphae, and vesicles developed on wild-type plants. **I**, The *ipd3-2* roots inoculated with AM, showing the presence of intraradical and extraradical hyphae and pigmented cells (arrows) but no arbuscules. Images of noninoculated root sections with AM for **F**, wild type and **H**, *ipd3-2*; a = arbuscules, v = vesicles, h = hyphae, and ih = intraradical hyphae. Scale bars: **A to D** = 30 μ m; **F to I** = 100 μ m.

ously demonstrated that the mutation in the autophosphorylated threonine residue (T271D or T271A in *M. truncatula* in the equivalent position T265D in *L. japonicus*) results in a gain-of function activity of CCaMK and spontaneous nodule formation (Gleason et al. 2006; Tirichine et al 2006). Epistatic analysis in *L. japonicus* using gain-of-function mutations of *CCaMK* and loss-of-function alleles of symbiotic signaling genes revealed the central regulatory function of CCaMK in the coordination of nodule organogenesis and rhizobial infection (Hayashi et al. 2010; Madsen et al. 2010). IPD3/CYCLOPS interacts with CCaMK and CYCLOPS has been shown to be a target of CCaMK phosphorylation (Messinese et al. 2007; Yano et al. 2008). Thus, IPD3/CYCLOPS appears to function either in a complex with CCaMK or downstream of CCaMK, between perception of calcium oscillations by CCaMK and the activation of the transcription factors NSP1 and NSP2. In addition, the failure of infection in the spontaneous nodules induced by the autoactive form of CCaMK in *cyclops* mutants indicated the role of CYCLOPS in the CCaMK-mediated cross-signaling from organogenic to the IT pathway (Hayashi et al. 2010; Madsen et al. 2010). Considering this central role for IPD3/CYCLOPS in symbiosis signaling, it is surprising that mutations of *IPD3/CYCLOPS* do not phenocopy *ccamk* mutants. Yano and associates (2008) interpreted their data to suggest that *CYCLOPS* represents a branch point between nodule organogenesis and bacterial infection, with *CYCLOPS* functioning only in a pathway leading to bacterial infection. Based on these results, it was suggested that the arrest of infection in the epidermis disturbs the coordination between the infection and organogenic program affecting the progression of nodule organogenesis. However, Capoen and Oldroyd (2008) suggested an alternative explanation, that the IPD3/CCaMK complex is required directly for complete nodule organogenesis and the weak effect of the *cyclops* mutants on nodule organogenesis may simply be a result of the penetrance of the *cyclops* mutations. The work described here supports the latter interpretation, because the genetic background significantly impacts the severity of *ipd3* mutations. A partial genetic redundancy for *IPD3/CYCLOPS* function leading to incomplete penetrance of *ipd3/cyclops* mutations is supported by the differences in effect of *ipd3* in different *M. truncatula* backgrounds, and this may explain why *cyclops* mutants show less severe phenotypes to *ccamk* mutants despite both genes functioning at similar positions in symbiosis signaling. This genetic redundancy is not necessarily the result of the action of *CYCLOPS* homologs but, rather, could be the result of additional proteins phosphorylated by CCaMK.

It was previously found that, in addition to the mutation in the autophosphorylated threonine residue (T271D or T271A), the truncated form of CCaMK containing only the kinase domain (CCaMK 1-311) also results in a gain-of function activity of CCaMK and spontaneous nodule formation (Gleason et al. 2006; Tirichine et al 2006). Although CCaMK 1-311 caused spontaneous nodules, it was not able to complement a *ccamk* mutant for bacterial infection. In contrast, CCaMK T271D was able to complement *ccamk* mutants of *L. japonicus* and *M. truncatula* (Gleason et al. 2006; Hayashi et al. 2010; Tirichine et al 2006), indicating that *CCaMK* T271D encoded a fully functional gene product. The difference in activity of these two gain-of-function constructs of *CCaMK* has yet to be explained. Here, we show that CCaMK T271D interacts with IPD3 whereas CCaMK 1-311 failed to interact with IPD3. Therefore, it seems likely that the absence of rhizobial infection in *ccamk* mutants transformed with CCaMK 1-311 is due to the inability of CCaMK 1-311 to interact with IPD3. We have already suggested that additional genetic components exist that partially compensate for the lack of *IPD3/CYCLOPS*. Our data

suggest that CCaMK 1-311 interacts with these additional genetic factors and, through this activation, induces spontaneous nodulation. In addition, these additional genetic components can compensate the rhizobial and mycorrhizal infection phenotype to some extent, depending on the genetic background. However, rhizobial infection requires the complete action of CCaMK, including the phosphorylation of IPD3/CYCLOPS.

Our work highlights a role for *IPD3* in symbiosis signaling. Likely functions for IPD3/CYCLOPS action are the modulation of CCaMK to allow full activity of the protein or the transduction of the signal downstream of CCaMK. The mechanism by which CCaMK activates the transcription factors NSP1 and NSP2 remains unknown. It is likely that IPD3/CYCLOPS allows the appropriate activation of these transcription factors following calcium recognition by CCaMK. This could either be through facilitating CCaMK modification of NSP1/NSP2, direct modulation of these transcription factors by IPD3/CYCLOPS, or the modification of additional components that regulate NSP1/NSP2. Our data imply that alternative mechanisms exist in addition to IPD3/CYCLOPS for the activation of these transcription factors and, ultimately, the promotion of nodule organogenesis and bacterial infection.

MATERIALS AND METHODS

Plant growth and DNA isolation.

M. truncatula subsp. *truncatula* (Jemalong) and *M. truncatula* subsp. *tricycla* (R108) genotypes were used as wild-type control for genetic experiments and phenotypic and expression analyses of *ipd3-1* and *ipd3-2* mutants, respectively. Seed were chemically scarified with sulfuric acid and sterilized as described in the *M. truncatula* handbook. Sterilized *ipd3-1* and wild-type seed were germinated with overnight incubation in darkness at room temperature on inverted agar plates (0.8% water agar) following a 4-day-long cold treatment at 4°C. For phenotypic characterization, wild-type and *ipd3-1* mutant plants were grown on square plates containing Gibson (Gibson and Nutman 1960) or buffered nodulation media (BNM) media supplemented with 0.1 µM L-α-(2-aminoethoxyvinyl)-Gly (AVG) (Sigma-Aldrich, St. Louis) to enhance root growth and nodulation. Plates were placed in growth chambers and kept under photoperiods of 16 h of light and 8 h of darkness at 24 and 20°C, respectively. The seed of R108 and *Tnt1* transposon-tagged insertion lines were prepared similarly as described above and germinated on agar plates supplemented with 1 µM gibberellic acid (GA₃) at 4°C for 2 days followed by overnight incubation at room temperature. Germinated seedlings were further grown on Fahraeus medium.

For genetic mapping, the individuals of the F2 segregating population were grown and characterized for symbiotic phenotype in separate tubes containing Gibson media. F2 plants grown in tubes were placed in growth chambers under the above-described conditions and the symbiotic phenotype was scored 18 to 21 dpi with *S. meliloti* strain B1. The genomic DNA from *M. truncatula* plants was isolated using the ZenoGene40 plant DNA purification kit (Zenon Bio, Szeged, Hungary).

Nodulation, mycorrhization, and GUS assays.

Four-day-old *M. truncatula* seedlings grown for phenotypic characterization were inoculated with *S. meliloti* 1021 carrying pXLGD4 plasmid (Penmettsa and Cook 1997) expressing the *lacZ* reporter gene under the control of the *hema* promoter. *S. meliloti* strains were grown overnight in liquid TA medium in the presence of streptomycin at 100 µg/ml. Bacteria were pelleted and resuspended in liquid Gibson or BNM medium in a final dilution of 1:50. The number of rhizobial microcolonies was counted following *lacZ* staining and the infected region

was determined by measuring the root length between the first- and the last-detected infection event. Nodules were sectioned using a Thermo Scientific Microm HM 650V vibration microtome, and histochemical staining for lacZ activity was carried out as reported by Boivin and associates (1990).

For the mycorrhization assay, *G. intraradices* aseptic spores were produced as previously described by St-Arnaud and associates (1996). For each line, 12 2-day-old seedlings were transferred on 1:1 Terragreen (Oil-dri UK Ltd., Wisbech, U.K.) and low-nutrient seed compost (Levington Horticulture Ltd., Ipswich, U.K.) medium, and 250 purified spores per seedling were applied directly to their roots. Six weeks after inoculation, root samples were taken and ink stained following the protocol reported by Vierheilig and associates (1998), with some modifications. Roots were cleared in 10% KOH at 95°C for 10 min and then stained for 2.5 min at 95°C in a 5% ink and 5% acetic acid solution. Destaining was performed by repeated washes in deionized water. Colonization levels were measured using the grid-line intersection method as described by Giovannetti and Mosse (1980). For mycorrhization assays of transposon-tagged mutant and wild-type R108 lines, seedlings were transplanted into pots with sand and Turface and inoculated with 400 sterile *G. intraradices* spores (Premier Tech Biotechnologies, Rivière-du-Loup, Quebec, Canada). Plants were harvested 27 days after inoculation. Roots were washed and cleared with 10% KOH at 90°C for 10 min. Roots were rinsed with distilled water and stained with 0.01% (wt/vol) Trypan blue in lactoglycerol.

The *ipd3-1* mutant plants were assayed for early nodulin gene expression by histochemical staining for GUS activity. Wild-type plants carrying the *pMtENOD11::GUS* construct (Journet et al. 2001) were crossed to *ipd3-1* plants to introduce the construct into mutant background. F2 plants carrying the *pMtENOD11::GUS* construct and containing the *ipd3-1* deletion allele were identified by PCR and selected for multiplication. Wild-type and *ipd3-1* (*pMtENOD11::GUS*) plants were grown on plates of BNM supplemented with 0.1 µM AVG to enhance root growth for 7 days. Plants were then transferred into liquid BNM media containing 1 nM NF and treated for 24 h. Roots were washed with 0.1 M potassium phosphate buffer (pH 7.0) and immersed and incubated in the dark in staining solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 50 mM sodium phosphate buffer, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 20% methanol, and Triton X-100 at 0.1 ml/100 ml) overnight at 37°C. After rinsing in phosphate buffer and cleaning in 70% ethanol, the roots were analyzed and imaged using an Olympus SZX12 stereo microscope.

Identification of novel *ipd3* alleles.

The *ipd3-1* mutant was isolated in the same forward genetic screen in which the *nin-1* mutant was identified (Marsh et al. 2007). Genetic mapping of the *ipd3-1* mutant locus was carried out on an F2 segregating population of the cross of *ipd3-1* and A20 *M. truncatula* lines. The rough map position of the *IPD3* locus was determined by analyzing the genotypes of 38 F2 plants determined for a microsatellite core marker set of the *M. truncatula* genome (Mun et al. 2006). Fine mapping was carried out using an additional 420 F2 plants and chromosomal walking experiments were done with analyzing the phenotype and genotypes of a subset of F2 individuals preselected with flanking genetic markers ENOD40 and MtB93 (Mun et al. 2006). Genetic markers GTP and BRL identifying polymorphism between A20 and the Jemalong background were used to delimit the region for the *IPD3* locus.

Primers of genetic markers GTP and BRL are listed in Supplementary Table S1. PCR amplifications for genetic mapping were carried out at 94°C for 30 s; 50, 55, or 60°C, depending on the primer pairs, for 30 s; and 72°C for 1 min per cycle.

The insertion mutant allele of *ipd3* was isolated in a PCR-based screen of a *Tnt1*-insertional population of *M. truncatula* based on the R108 genotype (Tadege et al. 2008). The screen was carried out using nested PCR (IPD3 forward primers IPD3-fw1 and IPD3-fw2 with *Tnt1*-specific primers LTR3 and LTR4) with ExTaq DNA Polymerase (TaKaRa, Shiga, Japan) in deconvoluting DNA pools of 500, 100, and 10 to reach single plants. In all, 1 independent mutant line (NF5939) was selected out of 7,000 independent mutant lines. A PCR-amplified fragment was sequenced to identify the exact insertion site of the 5.3-kb *Tnt1* retrotransposon. PCR analyses were carried out on a segregating population of NF5939 (*ipd3-2*) to identify homozygous mutants using gene-specific primers IPD3-fw3 and IPD3-rv2 and the *Tnt1*-specific primer LTR6 (Fig. 3).

Microscopic analysis of nodules and calcium spiking experiments.

Confocal laser scanning microscopy was performed using an Olympus Fluoview FV1000 confocal laser scanning microscope (Olympus Life Science Europa GmbH, Hamburg, Germany). Microscope configuration was the following: objective lenses, UPLSAPO ×20 (dry, NA:0.75), UPLFLN ×40 (oil, NA:1.3), and UPLSAPO ×60 (oil, NA:1.35); sampling speed, 4 µs/pixel; line averaging, 2×; scanning mode, sequential unidirectional; excitation, 488 nm (SYTO13); laser transmissivity, 5%; main dichroic beamsplitter, DM405/488; intermediate dichroic beamsplitter, SDM 490; and emission filter, 505 to 530 nm. For fluorescence stereo microscopy, an Olympus SZX12 stereo microscope with ×0.5 and ×1 objectives was used. GFP and DsRed images were captured with the “green” and “red” filter sets, respectively, as follows: GFP: excitation, 460 to 490 nm; dichroic beam splitter, 505 nm, and emission, 510 to 550 nm; and DsRed: excitation, 530 to 560 nm, dichroic beam splitter, 570 nm, and emission, 590 to 650 nm. For bright-field images, a white LED light source (Photonic Optics, Vienna) was used. Photos were captured using an Olympus Camedia C7070 digital camera. Calcium spiking experiments were performed as described by Ding and associates (2008).

Gene expression analyses.

For real-time qRT-PCR experiments, plants were grown on plates containing BNM medium. Five-day-old A17 and mutant seedlings were inoculated with *S. meliloti* strain 1021 by flooding the roots on plate. Bacteria were pelleted and resuspended in liquid Gibson medium in a final dilution of 1:50 (optical density at 600 nm = 0.05 to 0.1). For NF treatment, 5-day-old seedlings were transferred into beakers and incubated for 16 h in liquid BNM medium supplemented with 10 nM NF. For qRT-PCR, roots were harvested and RNA was extracted by TRIzol reagent (Life Technologies Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's protocol. The RNA was treated with DNase I, amplification grade (Invitrogen) according to the manufacturer's instructions. RNA were cleaned up by RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), then genomic DNA free total RNA was quantified using a spectrophotometer (Nanodrop-1000[®]; Thermo Fisher Scientific, Inc. NanoDrop Technologies, Wilmington, DE, U.S.A.) and checked for quality by gel electrophoresis. Complementary DNA was prepared from 1 µg of total RNA with SuperScript III first-strand synthesis system for RT-PCR (Life Technologies, Gaithersburg, MD, U.S.A.) using oligo-dT primers according to the manufacturer's instructions. Real-time qRT-PCR was performed on a MiniOpticon System (Bio-Rad, Hercules, CA, U.S.A.) using Bio-Rad CFX Manager software 1.5. SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) and Maxima SYBR Green Master Mix (Thermo Fisher Scientific, Inc., Fermentas, Vilnius, Lithuania) were used to monitor double-

stranded DNA synthesis in a 48-well plate, and the final primer concentration of each gene-specific primer was 100 nM. The initial denaturation step of 94°C for 2 min (JumpStart) or 10 min (Maxima) was followed by 45 to 50 cycles of 94°C for 15 s, 60°C for 20 s, and 72°C for 20 s to perform PCR amplification. After each PCR amplification, melting curves were analyzed to check nonspecific and nonrequired PCR products. Primer-dimer formations were estimated by running control amplifications without cDNA template. Ubiquitin was used as a reference gene and ubiquitin intron for checking genomic DNA contamination in cDNA samples (Kakar et al. 2008). Results were represented as a threshold cycle (C_T) value, which were averaged from three replicate reactions. For normalization, the reference gene C_T value was subtracted from the C_T value of the gene of interest (ΔC_T). $\Delta\Delta C_T$ value was calculated by subtracting ΔC_T of the 0-h (experiments with NF induction) or 1-dpi (experiments with *S. meliloti* inoculation) sample from the ΔC_T value of the samples from other time points. The qRT-PCR analysis was repeated three times with the cDNA mix of three to six independent biological repeats. Fold induction ($2^{\Delta\Delta C_T}$) of the repeats were averaged and plotted using SE.

Complementation experiments accomplished by *A. rhizogenes* transformation.

The complementation of mutant alleles of *IPD3* was carried out by generating and testing transgenic hairy roots for nodulation as previously described by Boisson-Dernier and associates (2001). Constructs for complementation experiments were generated with both the 35S constitutive promoter and the native promoter of the *IPD3* gene using the Gateway cloning technology (Invitrogen). The coding sequence of the *IPD3* gene was inserted into vector pK7WG2D (Plant Systems Biology, VIB-Ghent University, Gent, Belgium) to create the construct under the control of the 35S promoter. In order to create the construct driven by the native promoter, the 1,233 bp of the promoter region and the 566 bp of the terminal sequence of the *IPD3* gene were assembled with *IPD3* coding sequence. The promoter and terminal sequences were PCR amplified with the primer pairs of *IPD3*prom_GWfor/*IPD3*_R10 and *IPD3*_F6/*IPD3*_3'UTR_Gwrev using Jemalong genomic DNA and the amplified fragments were assembled with the coding sequence of *IPD3* using the restriction sites *Nco*I in exon 1 and *Xag*I in exon10. The construct was recombined into vector pKGWD,0, (Plant Systems Biology), pK7FWG2-RR (Wang et al. 2010) and pKGW-R (provided by P. Smit; Wageningen University). The constructs were introduced into either the ARqual or MSU440 strain of *A. rhizogenes* in the case of *ipd3-1* and *ipd3-2* mutants, respectively, and used for hairy root transformation as described by (Boisson-Dernier et al. 2001). The vectors contained either the GFP or the DsRed reporter gene to identify transgenic hairy roots. Transformed plants were inoculated with *S. meliloti* 1021 (mutant in Jemalong background) or 2011 (mutant in R108 background) carrying the *lacZ* marker gene (pXLGD4) following transfer into pouches (Mega International, St. Paul, MN, U.S.A.) or zeolite substrate (Geoprodukt Kft., Mád, Hungary), and nodules were stained for LacZ activity similarly to wild-type and mutant nodules after 4 weeks of transferring.

Yeast two-hybrid analysis.

Yeast two-hybrid analysis was carried out using the Matchmaker GAL4 two-hybrid system 3 (Clontech, Palo Alto, CA, U.S.A.) as described by Messinese and associates (2007). The transformed AH109 yeast strains were selected on synthetic dropout (SD) plates lacking Leu and Trp (LW). The expression of *HIS3* or *HIS3* and *ADE2* reporter genes was assessed by colony growth of yeast strains transformed with the full-length

or mutated open reading frame of *CCaMK* and *IPD3* genes on SD-LWH (Leu-, Trp-, and His-) and SD-LWHA (Leu-, Trp-, His-, and Ade-) plates. The liquid β -galactosidase assay was carried out according to the Yeast Protocols Handbook PT3024-1 (Clontech).

ACKNOWLEDGMENTS

This work was supported by the Országos Tudományos Kutatási Alap 67576 grant of the Hungarian Research Scientific Fund, the Biotechnology and Biological Sciences Research Council, U.K., the United States Department of Agriculture Cooperative State Research Education and Extension Service grant number 05-562-35319, and by the National Science Foundation (NSF) Plant Genome Program (DBI 0703285). Microscopy performed at the University of Wisconsin-Madison Plant Imaging Center was supported by the NSF (DBI-0421266). We thank I. Szivós and M. Komláti for skillful technical assistance and K. Findlay and S. Bunewell for carrying out sample preparation and transmission electron microscopy imaging at the Department of Cell and Developmental Biology, John Innes Centre.

LITERATURE CITED

- Akiyama, K., Matsuzaki, K., and Hayashi, H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435:824-827.
- Ané, J. M., Kiss, G. B., Riely, B. K., Penmetsa, R. V., Oldroyd, G. E. D., Ayax, C., Lévy, J., Debellé, F., Baek, J. M., Kaló, P., Rosenberg, C., Roe, B. A., Long, S. R., Dénarié, J., and Cook, D. R. 2004. *Medicago truncatula* *DMI1* required for bacterial and fungal symbioses in legumes. *Science* 303:1364-1367.
- Auriac, M. C., and Timmers, A. C. J. 2007. Nodulation studies in the model legume *Medicago truncatula*: Advantages of using the constitutive EF1 alpha promoter and limitations in detecting fluorescent reporter proteins in nodule tissues. *Mol. Plant-Microbe Interact.* 20:1040-1047.
- Benaben, V., Duc, G., Lefebvre, V., and Huguet, T. 1995. TE7, an inefficient symbiotic mutant of *Medicago truncatula* Gaertn. cv. Jemalong. *Plant Physiol.* 107:53-62.
- Boisson-Dernier, A., Chabaud, M., Garcia, F., Bécard, G., Rosenberg, C., and Barker, D. 2001. Agrobacterium rhizogenes-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Mol. Plant-Microbe Interact.* 14:695-700.
- Boivin, C., Camut, S., Malpica, C., Truchet, G., and Rosenberg, C. 1990. *Rhizobium meliloti* genes encoding catabolism of trigonelline are induced under symbiotic conditions. *Plant Cell* 2:1157-1170.
- Bright, L. J., Liang, Y., Mitchell, D. M., and Harris, J. M. 2005. The *LATD* gene of *Medicago truncatula* is required for both nodule and root development. *Mol. Plant-Microbe Interact.* 18:521-532.
- Capoen, W., and Oldroyd, G. 2008. How CYCLOPS keeps an eye on plant symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 105:20053-20054.
- Capoen, W., Goormachtig, S., De Rycke, R., Schroevers, K., and Holsters, M. 2005. SrSymRK, a plant receptor essential for symbiosome formation. *Proc. Natl. Acad. Sci. U.S.A.* 102:10369-10374.
- Catoira, R., Galera, C., de Billy, F., Penmetsa, R. V., Journet, E. P., Maillet, F., Rosenberg, C., Cook, D., Gough, C., and Dénarié, J. 2000. Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. *Plant Cell* 12:1647-1665.
- Combiér, J. P., Frugier, F., de Billy, F., Boualem, A., El-Yahyaoui, F., Moreau, S., Vernie, T., Ott, T., Gamas, P., Crespi, M., and Niebel, A. 2006. *MtHAP2-1* is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. *Genes Dev.* 20:3084-3088.
- Ding, Y. L., Kaló, P., Yendrek, C., Sun, J. H., Liang, Y., Marsh, J. F., Harris, J. M., and Oldroyd, G. E. D. 2008. Abscissic acid coordinates Nod factor and cytokinin signaling during the regulation of nodulation in *Medicago truncatula*. *Plant Cell* 20:2681-2695.
- Ehrhardt, D. W., Wais, R., and Long, S. R. 1996. Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. *Cell* 85:673-681.
- Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kalo, P., and Kiss, G. B. 2002. A receptor kinase gene regulating symbiotic nodule development. *Nature* 417:962-966.
- Felle, H. H., Kondorosi, E., Kondorosi, A., and Schultze, M. 1998. The role of ion fluxes in Nod factor signalling in *Medicago sativa*. *Plant J.* 13:455-463.
- Gibson, A. H., and Nutman, P. S. 1960. Studies on the physiology of nodule formation. VII. A reappraisal of the effect of preplanting. *Ann. Bot.* 24:420-433.

- Giovannetti, M., and Mosse, B. 1980. Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol.* 84:489-500.
- Gleason, C., Chaudhuri, S., Yang, T. B., Munoz, A., Poovaiah, B. W., and Oldroyd, G. E. D. 2006. Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. *Nature* 441:1149-1152.
- Godfroy, O., Debellé, F., Timmers, T., and Rosenberg, C. 2006. A rice calcium- and calmodulin-dependent protein kinase restores nodulation to a legume mutant. *Mol. Plant-Microbe Interact.* 19:495-501.
- Groth, M., Takeda, N., Perry, J., Uchida, H., Draxl, S., Brachmann, A., Sato, S., Tabata, S., Kawaguchi, M., Wang, T. L., and Parniske, M. 2010. *NENA*, a *Lotus japonicus* homolog of Sec13, is required for rhizodermal infection by arbuscular mycorrhizal fungi and rhizobia but dispensable for cortical endosymbiotic development. *Plant Cell* 22:2509-2526.
- Hayashi, T., Banba, M., Shimoda, Y., Kouchi, H., Hayashi, M., and Imaizumi-Anraku, H. 2010. A dominant function of CCaMK in intracellular accommodation of bacterial and fungal endosymbionts. *Plant J.* 63:141-154.
- Haynes, J., Czymmek, K., Carlson, C., Veereshlingam, H., Dickstein, R., and Sherrier, D. 2004. Rapid analysis of legume root nodule development using confocal microscopy. *New Phytol.* 163:661-668.
- Imaizumi-Anraku, H., Takeda, N., Charpentier, M., Perry, J., Miwa, H., Umehara, Y., Kouchi, H., Murakami, Y., Mulder, L., Vickers, K., Pike, J., Downie, J., Wang, T., Sato, S., Asamizu, E., Tabata, S., Yoshikawa, M., Murooka, Y., Wu, G., Kawaguchi, M., Kawasaki, S., Parniske, M., and Hayashi, M. 2005. Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. *Nature* 433:527-531.
- Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., and Walker, G. C. 2007. How rhizobial symbionts invade plants: The *Sinorhizobium-Medicago* model. *Nat. Rev. Microbiol.* 5:619-633.
- Journet, E. P., Pichon, M., Dedieu, A., de Billy, F., Truchet, G., and Barker, D. G. 1994. *Rhizobium meliloti* Nod factors elicit cell-specific transcription of the *ENOD12* gene in transgenic alfalfa. *Plant J.* 6:241-249.
- Journet, E. P., El-Gachtouli, N., Vernoud, V., de Billy, F., Pichon, M., Dedieu, A., Arnould, C., Morandi, D., Barker, D. G., and Gianinazzi-Pearson, V. 2001. *Medicago truncatula ENOD11*: A novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. *Mol. Plant-Microbe Interact.* 14:737-748.
- Kakar, K., Wandrey, M., Czechowski, T., Gaertner, T., Scheible, W., Stitt, M., Torres-Jerez, I., Xiao, Y., Redman, J., Wu, H., Cheung, F., Town, C., and Udvardi, M. 2008. A community resource for high-throughput quantitative RT-PCR analysis of transcription factor gene expression in *Medicago truncatula*. *Plant Methods* 4:18. Online publication. doi:10.1186/1746-4811-4-18
- Kaló, P., Gleason, C., Edwards, A., Marsh, J., Mitra, R. M., Hirsch, S., Jakab, J., Sims, S., Long, S. R., Rogers, J., Kiss, G. B., Downie, J. A., and Oldroyd, G. E. D. 2005. Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. *Science* 308:1786-1789.
- Kanamori, N., Madsen, L. H., Radutoiu, S., Frantescu, M., Quistgaard, E. M. H., Miwa, H., Downie, J. A., James, E. K., Felle, H. H., Haaning, L. L., Jensen, T. H., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. 2006. A nucleoporin is required for induction of Ca²⁺ spiking in legume nodule development and essential for rhizobial and fungal symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 103:359-364.
- Kiss, E., Oláh, B., Kaló, P., Morales, M., Heckmann, A., Borbóla, A., Loza, A., Kontar, K., Middleton, P., Downie, J., Oldroyd, G., and Endre, G. 2009. LIN, a novel type of U-Box/WD40 protein, controls early infection by rhizobia in legumes. *Plant Physiol.* 151:1239-1249.
- Kosuta, S., Chabaud, M., Lounon, G., Gough, C., Dénarié, J., Barker, D. G., and Bécard, G. 2003. A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific *MtENOD11* expression in roots of *Medicago truncatula*. *Plant Physiol.* 131:952-962.
- Kosuta, S., Hazledine, S., Sun, J., Miwa, H., Morris, R., Downie, J., and Oldroyd, G. 2008. Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. *Proc. Natl. Acad. Sci. U.S.A.* 105:9823-9828.
- Kuhn, H., Kuster, H., and Requena, N. 2010. Membrane steroid-binding protein 1 induced by a diffusible fungal signal is critical for mycorrhization in *Medicago truncatula*. *New Phytol.* 185:716-733.
- Lévy, J., Bres, C., Geurts, R., Chalhou, B., Kulikova, O., Duc, G., Journet, E. P., Ané, J. M., Lauber, E., Bisseling, T., Dénarié, J., Rosenberg, C., and Debellé, F. 2004. A putative Ca²⁺ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* 303:1361-1364.
- Lima, P. T., Faria, V. G., Patraquim, P., Ramos, A. C., Feijo, J. A., and Sucena, E. 2009. Plant-microbe symbioses: New insights into common roots. *Bioessays* 31:1233-1244.
- Limpens, E., Mirabella, R., Fedorova, E., Franken, C., Franssen, H., Bisseling, T., and Geurts, R. 2005. Formation of organelle-like N-2-fixing symbiosomes in legume root nodules is controlled by *DMI2*. *Proc. Natl. Acad. Sci. U.S.A.* 102:10375-10380.
- Lodwig, E., and Poole, P. 2003. Metabolism of *Rhizobium* bacteroids. *Crit. Rev. Plant Sci.* 22:37-78.
- Madsen, L. H., Tirichine, L., Jurkiewicz, A., Sullivan, J. T., Heckmann, A. B., Bek, A. S., Ronson, C. W., James, E. K., and Stougaard, J. 2010. The molecular network governing nodule organogenesis and infection in the model legume *Lotus japonicus*. *Nat. Commun.* 1:10.
- Maillet, F., Poinot, V., André, Puech-Pagès, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E. A., Driguez, H., Bécard, G., and Dénarié, D. 2011. Fungal lipochitoooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* 469:58-64.
- Marsh, J. F., Rakocevic, A., Mitra, R. M., Brocard, L., Sun, J., Eschstruth, A., Long, S. R., Schultze, M., Ratet, P., and Oldroyd, G. E. D. 2007. *Medicago truncatula NIN* is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiol.* 144:324-335.
- Maunoury, N., Redondo-Nieto, M., Bourcy, M., Van de Velde, W., Alunni, B., Laporte, P., Durand, P., Agier, N., Marisa, L., Vaubert, D., Delacroix, H., Duc, G., Ratet, P., Aggerbeck, L., Kondorosi, E., and Mergaert, P. 2010. Differentiation of symbiotic cells and endosymbionts in *Medicago truncatula* nodulation are coupled to two transcriptome-switches. *PLOS ONE* 5:e9519. doi:10.1371/journal.pone.0009519. Published online.
- Messinese, E., Mun, J. H., Yeun, L. H., Jayaraman, D., Rouge, P., Barre, A., Lounon, G., Schornack, S., Bono, J. J., Cook, D. R., and Ané, J. M. 2007. A novel nuclear protein interacts with the symbiotic DMI3 calcium- and calmodulin-dependent protein kinase of *Medicago truncatula*. *Mol. Plant-Microbe Interact.* 20:912-921.
- Mitra, R. M., Gleason, C. A., Edwards, A., Hadfield, J., Downie, J. A., Oldroyd, G. E. D., and Long, S. R. 2004. A Ca²⁺/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. *Proc. Natl. Acad. Sci. U.S.A.* 101:4701-4705.
- Mun, J. H., Kim, D. J., Choi, H. K., Gish, J., Debellé, F., Mudge, J., Denny, R., Endre, G., Saurat, O., Ducez, A. M., Kiss, G. B., Roe, B., Young, N. D., and Cook, D. R. 2006. Distribution of microsatellites in the genome of *Medicago truncatula*: A resource of genetic markers that integrate genetic and physical maps. *Genetics* 172:2541-2555.
- Navazio, L., Moscatello, R., Genre, A., Novero, M., Baldan, B., Bonfante, P., and Mariani, P. 2007. A diffusible signal from arbuscular mycorrhizal fungi elicits a transient cytosolic calcium elevation in host plant cells. *Plant Physiol.* 144:673-681.
- Oláh, B., Brière, C., Bécard, G., Dénarié, J., and Gough, C. 2005. Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in *Medicago truncatula* via the DMI1/DMI2 signalling pathway. *Plant J.* 44:195-207.
- Oldroyd, G. E. D., and Downie, J. A. 2006. Nuclear calcium changes at the core of symbiosis signalling. *Curr. Opin. Plant Biol.* 9:351-357.
- Oldroyd, G. E. D., and Downie, J. A. 2008. Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annu. Rev. Plant Biol.* 59:519-546.
- Oldroyd, G. E. D., and Long, S. R. 2003. Identification and characterization of *nodule-signaling pathway 2*, a gene of *Medicago truncatula* involved in Nod factor signaling. *Plant Physiol.* 131:1027-1032.
- Ovchinnikova, E., Journet, E. P., Chabaud, M., Cosson, V., Ratet, P., Duc, G., Fedorova, E., Liu, W., Op den Camp, R., Zhukov, V., Tikhonovich, I., Borisov, A., Bisseling, T., and Limpens, E. 2011. IPD3 controls the formation of nitrogen-fixing symbiosomes in pea and *Medicago*. *Mol. Plant Microbe Interact.* 24:1333-1344.
- Parniske, M. 2004. Molecular genetics of the arbuscular mycorrhizal symbiosis. *Curr. Opin. Plant Biol.* 7:414-421.
- Parniske, M. 2008. Arbuscular mycorrhiza: The mother of plant root endosymbioses. *Nat. Rev. Microbiol.* 6:763-775.
- Penmetsa, R. V., and Cook, D. R. 1997. A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science* 275:527-530.
- Riely, B. K., Mun, J. H., and Ané, J. M. 2006. Unravelling the molecular basis for symbiotic signal transduction in legumes. *Mol. Plant Pathol.* 7:197-207.
- Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H., Umehara, Y., Kouchi, H., Murooka, Y., Szczygłowski, K., Downie, J., Parniske, M., Hayashi, M., and Kawaguchi, M. 2007. NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in *Lotus japonicus*. *Plant Cell* 19:610-624.
- Smith, S. E., Barker, S. J., and Zhu, Y. G. 2006. Fast moves in arbuscular mycorrhizal symbiotic signalling. *Trends Plant Sci.* 11:369-371.
- StArnaud, M., Hamel, C., Vimard, B., Caron, M., and Fortin, J. 1996. En-

- hanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. *Mycol. Res.* 100:328-332.
- Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Szczyglowski, K., and Parniske, M. 2002. A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* 417:959-962.
- Tadege, M., Wen, J. Q., He, J., Tu, H. D., Kwak, Y., Eschstruth, A., Cayrel, A., Endre, G., Zhao, P. X., Chabaud, M., Ratet, P., and Mysore, K. S. 2008. Large-scale insertional mutagenesis using the *Tnt1* retrotransposon in the model legume *Medicago truncatula*. *Plant J.* 54:335-347.
- Timmers, A. C. J., Auriac, M. C., and Truchet, G. 1999. Refined analysis of early symbiotic steps of the *Rhizobium-Medicago* interaction in relationship with microtubular cytoskeleton rearrangements. *Development* 126:3617-3628.
- Tirichine, L., James, E. K., Sandal, N., and Stougaard, J. 2006. Spontaneous root-nodule formation in the model legume *Lotus japonicus*: A novel class of mutants nodulates in the absence of rhizobia. *Mol. Plant-Microbe Interact.* 19:373-382.
- Tirichine, L., Sandal, N., Madsen, L. H., Radutoiu, S., Albrechtsen, A. S., Sato, S., Asamizu, E., Tabata, S., and Stougaard, J. 2007. A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. *Science* 315:104-107.
- Vierheilig, H., Coughlan, A., Wyss, U., and Piche, Y. 1998. Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Appl. Environ. Microbiol.* 64:5004-5007.
- Wais, R. J., Galera, C., Oldroyd, G., Catoira, R., Penmetsa, R. V., Cook, D., Gough, C., Denarie, J., and Long, S. R. 2000. Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. *Proc. Natl. Acad. Sci. U.S.A.* 97:13407-13412.
- Wang, B., Yeun, L., Xue, J., Liu, Y., Ané, J. M., and Qiu, Y. 2010. Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants. *New Phytol.* 186:514-525.
- Yano, K., Yoshida, S., Muller, J., Singh, S., Banba, M., Vickers, K., Markmann, K., White, C., Schuller, B., Sato, S., Asamizu, E., Tabata, S., Murooka, Y., Perry, J., Wang, T., Kawaguchi, M., Imaizumi-Anraku, H., Hayashi, M., and Parniske, M. 2008. CYCLOPS, a mediator of symbiotic intracellular accommodation. *Proc. Natl. Acad. Sci. U.S.A.* 105:20540-20545.

AUTHOR-RECOMMENDED INTERNET RESOURCE

Noble Foundation *M. truncatula* Handbook:
www.noble.org/MedicagoHandbook