IPD3 Controls the Formation of Nitrogen-Fixing Symbiosomes in Pea and *Medicago* Spp.

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A successful nitrogen-fixing symbiosis requires the accommodation of rhizobial bacteria as new organelle-like structures, called symbiosomes, inside the cells of their legume hosts. Two legume mutants that are most strongly impaired in their ability to form symbiosomes are *sym1/TE7* in *Medicago truncatula* and *sym33* in *Pisum sativum*. We have cloned both *MtSYM1* and *PsSYM33* and show that both encode the recently identified interacting protein of DMI3 (IPD3), an ortholog of *Lotus japonicus* (Lotus) CYCLOPS. IPD3 and CYCLOPS were shown to interact with DMI3/CCaMK, which encodes a calcium- and calmodulin-dependent kinase that is an essential component of the common symbiotic signaling pathway for both rhizobial and mycorrhizal symbioses. Our data reveal a novel, key role for IPD3 in symbiosome formation and development. We show that *MtIPD3* participates in but is not essential for infection thread formation and that *MtIPD3* also affects DMI3-induced spontaneous nodule formation upstream of cytokinin signaling. Further, *MtIPD3* appears to be required for the expression of a nodule-specific remorin, which controls proper infection thread growth and is essential for symbiosome formation.

During the *Rhizobium* spp.–legume symbiosis, *Rhizobium* bacteria are hosted inside the cells of a newly formed organ, the root nodule. There, they participate in the formation of new transient organelle-like structures, called symbiosomes, where they find the right conditions to fix atmospheric nitrogen. In legumes such as *Medicago truncatula* (Medicago) and *Pisum sativum* (pea), symbiosomes consist of individual bacteria that

are surrounded by a plant-derived membrane, called the peribacteroid or symbiosome membrane. Identifying the underlying mechanisms to accommodate the bacteria as new, organelle-like structures inside the plant cells is a key question in symbiosis.

Medicago and pea form so-called undetermined nodules that have a persistent meristem, and symbiosome formation continuously occurs in a few cell layers just below this meristem. There, the bacteria, which are transported via tubular structures called infection threads, are taken up into the nodule cells to form symbiosomes (Brewin 2004; Jones et al. 2007). Symbiosome formation begins with the formation of an unwalled infection droplet at the otherwise cell-wall-bound infection thread. From there, the bacteria are individually taken up into the cell via an endocytosis-like process (Jones et al. 2007; Limpens et al. 2009; Verma and Hong 1996; Whitehead and Day 1997). After release from the infection thread, the symbiosomes divide and, subsequently, the symbiosomes terminally differentiate into their nitrogen-fixing form (Maunoury et al. 2010). Due to the activity of the meristem, nodules show a highly ordered zonation containing symbiosomes at subsequent stages of development (Vasse et al. 1990). The zone below the meristem where infection threads invade the cells and symbiosome formation and subsequent differentiation occur is called the infection zone. In the adjacent fixation zone, infected nodule cells contain fully differentiated symbiosomes where nitrogen fixation takes place.

Although symbiosome formation is a crucial step to establish a successful symbiosis in most legumes, the molecular basis of this process is far from understood. Nodule organogenesis and infection thread formation both require the activation of a signaling pathway by rhizobial signal molecules called Nod factors (Oldroyd and Downie 2008). Several recent studies suggest that symbiosome formation is also controlled by this Nod-factor-signaling pathway. All the Nod-factor-signaling genes appear to be expressed in the nodule, especially in the infection zone of the nodule where symbiosome formation takes place (Bersoult et al. 2005; Limpens et al. 2005; Riely et al. 2007). Nodule-specific knock-down of the leucine-rich repeat-containing receptor kinase DMI2 blocks the release of the bacteria from the infection threads (Capoen et al. 2005; Limpens et al. 2005). Signaling by DMI2 is thought to trigger a calcium-spiking response that is

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Nucleotide sequence data for the genes used in this article can be found in the GenBank and EMBL data libraries or TIGR Gene Indices under the following accession numbers: *MtIPD3*, EF117279; *PsIPD3*, EF569222; *MtGAPDH*, BT052418; *MtHAP2*, EF488826; *MtNIN*, FJ179774; *MtDMI3*, AY496049; *MtCRE1*, AC141922/TC109250; *MtERN*, EF396330; *MtEFD*, EU251063; *MtSYMREM*, TC116072; *MtFlotillin-2*, GU224279; *MtFlotillin-4*, GU224281; *MtRPG*, DQ854741, and *MtLIN*, EU926661.

*The e-Xtra logo stands for “electronic extra” and indicates that six supplementary figures are published online.

decoded in the nucleus by a calcium- and calmodulin-dependent kinase, DMI3, which activates specific transcription factors (Lévy et al. 2004; Mitra et al. 2004). Among transcription factors that are strongly upregulated during nodule formation and involved in symbiosome formation are the CCAAT transcription factor HAP2 and the AP2/ERF transcription factor EFD (Combiér et al. 2006; Vernié et al. 2008). Knock-down of *HAP2* expression causes a block of release of the bacteria from the infection threads (Combiér et al. 2006), while EFD is involved in the control of both release and symbiosome differentiation (Vernié et al. 2008). One of the genes that is upregulated during nodule formation and essential for symbiosome formation encodes a recently identified remorin, called SYMREM. This remorin was shown to interact with DMI2 as well as Nod factor receptors, and knock-down of this remorin blocked the release of the bacteria from the infection threads in the nodule (Lefebvre et al. 2010). Both DMI2 and DMI3 are part of a common symbiotic signaling module that is also essential for the establishment of an arbuscular mycorrhizal (AM) symbiosis (Capoen and Oldroyd 2008; Catoira et al. 2000). Recently, an interacting protein of DMI3, called IPD3 in Medicago and CYCLOPS in *Lotus japonicus* (Lotus), was identified that was shown to be essential for infection thread formation as well as mycorrhizal arbuscule formation in Lotus (Messinese et al. 2007; Yano et al. 2008).

To get additional insight into the mechanism of symbiosome formation and how Nod factor signaling might control this process, we focused on two mutants, *sym1* (TE7) in Medicago and *sym33* in pea, that are most strongly impaired at the first step of symbiosome formation (i.e., the release of the bacteria from the infection thread) (Bénaben et al. 1995; Tsyganov et al. 1998; Voroshilova et al. 2009).

Mtsym1 (TE7) was reported to make two types of nodules: small nodules (primordia) (type 1) with infection threads arrested at the outer cortical cells of the root or the cortex of the nodule, and elongated nodules (type 2) filled with an extensive network of infection threads with thick cell walls (Bénaben et al. 1995). Occasionally, some release of bacteria was observed in these type 2 nodules but subsequent symbiosome differentiation did not take place and these plant cells underwent early senescence. The lack of (proper) symbiosome formation is accompanied by a lack of endoreduplication in *Mtsym1* nodule cells, which has been postulated to be a prerequisite for symbiosome development (Maunoury et al. 2010). Similar to *Mtsym1*, pea *sym33* also shows impaired invasion of the nodule primordium at early stages (Voroshilova et al. 2009). At later time points, two types of nodule structures are formed: white nodules with an extensive network of “locked” infection

threads, with thick cell walls that do not release bacteria, and nodules containing undifferentiated symbiosomes that undergo premature senescence (Tsyganov et al. 1998; Voroshilova et al. 2001, 2009). Both mutants show signs of host defense responses such as extensive deposition of cell wall material on the infection threads and accumulation of polyphenolics. In addition to the nodulation phenotypes, *sym33* was also reported to show decreased root colonization by AM fungi, although arbuscules can still be formed (Jacobi et al. 2003a and b).

Here, we describe the cloning of *MtSYM1* and *PsSYM33* and show that both encode the interacting protein of DMI3 (IPD3). Our data demonstrate that the common symbiotic signaling module in pea and Medicago has been recruited to facilitate symbiosome formation (i.e., the release of the bacteria from the infection threads) as well as subsequent symbiosome development. We propose a model in which IPD3 controls the activity or stability of a DMI3 complex to allow successful symbiosis.

RESULTS

Cloning of *MtSYM1*.

A map-based cloning strategy was undertaken to clone the *MtSYM1* gene. A segregating population (99 F2 plants) of a cross between *Mtsym1*(TE7) and A20 was used to map the location of *Mtsym1* to the upper arm of chromosome V, between markers 19c21a and CU469(B). Sequence analysis of the corresponding approximately 1-Mb BAC contig (Supplementary Fig. 1) identified several promising *MtSYM1* candidates because they are thought to play a role in the symbiotic signaling pathway. These include HMGR1, an interactor of DMI2 (Kevei et al. 2007), and IPD3, an interactor of DMI3 (Messinese et al. 2007). Sequence analysis of the genomic regions of these candidate genes identified a mutation in *MtIPD3*: a deletion of G at position 1,266 in the *MtIPD3* mRNA coding sequence resulting in a frame shift and premature stop leading to a truncated protein that lacks the second nuclear localization signal (NLS) and the predicted C-terminal conserved coiled-coil motif (Fig. 1A and B; Table 1). To confirm that *MtSYM1* encodes MtIPD3, we complemented the mutant using the *MtIPD3* open reading frame under the control of its putative promoter (1-kb region upstream of the start codon). Two weeks after inoculation, pink nitrogen-fixing nodules were formed on *IPD3p:MtIPD3*-transformed *Mtsym1* roots containing normally developed symbiosomes (Fig. 2A and B). As a control, *Mtsym1* was transformed with an empty vector, which resulted in only type 1 and type 2 nodules (Fig. 2C). This confirms that *MtSYM1* encodes MtIPD3. This allele was named *Mtsym1-1*.

Fig. 1. Interacting protein of DMI3 (*IPD3*) gene and protein structure. **A**, Schematic representation of the exon-intron structure of the *IPD3* gene. Identified mutations in the corresponding *Medicago truncatula sym1* and pea *sym33* mutants are indicated. The open box after exon (black rectangles) 3 marks intron 3, which is not spliced in *Psym33-1*. **B**, Schematic representation of the domain structure of IPD3 protein. The conserved C-terminal coiled-coil domain, two identified phosphorylation sites (Grimsrud et al. 2010), as well as two predicted nuclear localization sites (NLS) are indicated.

Fig. 2. Complementation of *Mtsym1* with MtIPD3. **A**, Brightfield image of an *IPD3p:IPD3* transformed *Mtsym1-1* plant, showing a transgenic root (white arrow) with a pink nitrogen-fixing nodule and a nontransgenic root (arrowhead) with white ineffective nodules. **B**, Confocal image of an *IPD3p:IPD3* expressing *Mtsym1-1* nodule, showing a wild-type cytology with well developed symbiosomes (s). Rhizobia are expressing green fluorescent protein (GFP). **C**, Confocal image of a *Mtsym1-1* type 2 nodule transformed with an empty vector. Rhizobia are expressing GFP. Note the presence of infection threads in the central tissue of the nodule, but no symbiosomes. **D** and **E**, Confocal image of an *IPD3p:GFP-IPD3* expressing *Mtsym1-1* nodule showing GFP-IPD3 localization in the nucleus of cells in the **D**, infection zone and **E**, fixation zone. Rhizobia are expressing mRFP. **F**, Confocal close-up of GFP-IPD3 localizing to the nucleus of an infected cell in the nodule fixation zone. Note the full complementation of the mutant phenotype, showing well-developed symbiosomes. S = symbiosome, It = infection thread. Bars: B, D, and E = 20 μ m; C = 50 μ m; F = 10 μ m.

Additionally, we identified a *Tnt1*/Mere-transposon tagged line (76A-24), which showed a phenotype comparable with *Mtsym1* and that segregated as a monogenic recessive trait (data not shown). After two back-crosses (BC), BC2 lines formed both type 1 and type 2 Fix⁻ nodules similar to *Mtsym1* (Table 1; data not shown). Crossing 76A-24 to *Mtsym1* showed that the mutants are allelic. IPD3-*Tnt1*/Mere transposon junctions in 76A-24 were determined and it turned out that one single copy of *Mere1-1* (Rakocevic et al. 2009) is inserted after base 5 of exon 9, resulting in a premature stop (*sym1-3*) (Table 1). Genotyping of 87 mutant and three wild-type (WT) 76A-24 BC lines showed that the presence of a homozygous Mere insertion in IPD3 is necessary for the mutant nodulation phenotype (data not shown). Previous allelism tests with Medicago Fix⁻ mutants had shown that *Mtsym1* is allelic to the previously identified complementation group, termed *Mtsym17* (Morandi et al. 2005; Sagan et al. 1998). Mutations in *MtIPD3* were confirmed in six *Mtsym17* lines by sequencing the IPD3 genomic region (Table 1). TRV15 (*sym1-2*) contains a 4-bp deletion leading to a frame shift introducing 13 different amino acids and a premature stop, thus deleting 180 amino acids (including both NLS sequences) at the C terminus. The *Mtsym17* mutants TR3, TR9, TR13, TR62, and TR69 all contain the same mutation as *Mtsym1-1* (Fig. 1A; Table 1). All *Mtsym1* alleles, including back-crossed lines, show a very similar infection and nodulation phenotype forming two types of nodules (Table 1), although the ratio between the two nodule types varied between lines and experiments.

IPD3 is an ortholog of the recently identified CYCLOPS protein from Lotus and is highly conserved in angiosperms that are able to establish an AM symbiosis (Chen et al. 2008; Messinese et al. 2007; Yano et al. 2008). It contains a highly conserved C-terminal coiled-coil domain and a functional NLS. Further, two phosphorylation sites have been identified (Fig. 1B) (Grimsrud et al. 2010; Yano et al. 2008).

Syntenic-based cloning of PsSYM33.

The pea *Pssym33* mutants, of which three alleles (RisFixU, SGE-Fix-2, and SGE-Fix-5) have been identified, have a strikingly similar phenotype as *Mtsym1*: the formation of white nodules with an extensive network of thick-walled infection threads but no release of bacteria (Tsyganov et al. 1998; Voroshilova et al. 2001, 2009). One of the *sym33* alleles, SGE-Fix-2, has been shown to occasionally make pinkish nodules in which bacteria are released but symbiosomes do not develop properly; such nodules are referred to as type 3 nodules (Table 1) (Tsyganov et al. 1998). SGE-Fix-5, as reported for RisFixU (Voroshilova et al. 2001), does not show type 3 nodules but

makes mostly type 2 nodules, where release of the bacteria from the infection threads does not occur (Supplementary Fig. 2). *SYM33* was mapped to a region on linkage group I in pea, which is highly syntenic with the upper arm of chromosome V in Medicago (Aubert et al. 2006; Tsyganov et al. 2006). Additional mapping using a cross between WT10584 and SGE-Fix-2 (95 F2 plants) positioned *SYM33* within a 14-centimorgan region between the markers Gst and Alat-C on pea linkage group I, corresponding to the syntenic location of *SYM1* in Medicago (Supplementary Fig. 3). *PsIPD3* was converted into a genetic marker and showed complete co-segregation with the mutant phenotype, suggesting that *PsSYM33* is the ortholog of *MtIPD3*. To test this, we sequenced IPD3 from pea in the three available *sym33* mutants; RisFixU, SGE-Fix-2, and SGE-Fix-5 (Fig. 1A; Table 1). RisFixU (background Finale) showed a mutation in the 5' splice-site (G>A) of intron 3. Reverse-transcription polymerase chain reaction (RT-PCR) analysis confirmed that this mutation impairs the splicing of intron 3 (Supplementary Fig. 4), by which a stop codon is introduced leading to a truncated protein of 390 amino acids. SGE-Fix-5 (background SGE) shows a C319T mutation in the *PsIPD3* coding sequence, introducing an early stop at amino acid 107. SGE-Fix-2 contains a C1357T mutation causing a premature stop, eliminating the final 60 amino acids. SGE-Fix-5 and RisFixU do not show the occasionally occurring nodules as in SGE-Fix-2, where release of bacteria from the infection threads occurs. This suggests that the phenotype of SGE-Fix-2 may be somewhat leaky, which fits with C-terminal location of the mutation in the protein. Nevertheless, it indicates an important role for the highly conserved 60 amino acids in the C-terminal coiled-coil domain for the function of IPD3. The comparable phenotypes, similar map location, and mutations in *PsIPD3* strongly indicate that *SYM33* is the ortholog of *MtSYM1*, and we conclude that *PsSYM33* encodes *PsIPD3*. Together, the data from pea and Medicago reveal a novel, key function for IPD3 in symbiosome formation and development.

IPD3 localizes to the nucleus throughout the nodule infection and fixation zone.

DMI3 is most strongly expressed in the infection zone of the nodule, where it localizes to the nucleus (Limpens et al. 2005; Smit et al. 2005). Promoter- β -glucuronidase analyses were reported to show that *IPD3* is maximally expressed in the fixation zone of the nodule (Messinese et al. 2007). Significant lower levels were observed in the distal infection zone, where maximal *DMI3* expression is observed. To test whether IPD3 localizes to the nucleus in cells of the distal infection zone in which symbiosome formation starts and

Table 1. *MtSYM1* and *PsSYM33* mutant alleles identified in this study

Allele	Original mutant	Background ^b	Reference	Mutation		Phenotype ^a	
				cDNA	Protein ^c	ITs	Nodule
<i>sym1-1</i>	<i>Mtsym1</i> /TE7	<i>Mt</i> J5	Bénaben et al. 1995	Deletion G1266	+17 aa after K421	+	1 and 2
<i>sym1-1</i>	TR3, TR9, TR13, TR62, TR69	<i>Mt</i> J5	Sagan et al. 1998; Morandi et al. 2005	Deletion G1266	+17 aa after K421	+	1 and 2
<i>sym1-2</i>	TRV15	<i>Mt</i> J5	Sagan et al. 1998; Morandi et al. 2005	4-bp deletion from T1000	+ 13 aa after A333	+	1 and 2
<i>sym1-3</i>	T00076A-24	<i>Mt</i> Jemalong H2A	This work	Mere insertion after C1245	+1 aa after G415	+	1 and 2
<i>sym33-1</i>	RisFixU	<i>Ps</i> Finale	Voroshilova et al. 2001	5' splice-site G>A mutation intron 3	+1 aa after I123	+	1 and 2
<i>sym33-2</i>	SGEFix-5	<i>Ps</i> SGE	This work	C319T	Q107 stop	+	1 and 2
<i>sym33-3</i>	SGEFix-2	<i>Ps</i> SGE	Tsyganov et al. 1998	C1357T	R453 stop	+	2 and 3

^a IT = infection thread. Nodule = nodule types: type 1 = small nodules with infection threads limited to the outer cortex, type 2 = elongated nodules with an extensive network of infection threads but no release of bacteria, and type 3 = elongated nodules with undifferentiated symbiosomes that undergo premature senescence.

^b Genotypic background. *Mt* = *Medicago truncatula* and *Ps* = *Pisum sativum*.

^c Abbreviation: aa = amino acids.

DMI3 is present, a *IPD3p:GFP-IPD3* construct was used to complement *Mtsym1-1*. Nodules formed on these transgenic roots were pink and showed mature, elongated symbiosomes (Fig. 2F). Green fluorescent protein (GFP)-IPD3 localized to the nucleus of cells throughout the infection zone as well as in the fixation zone (Fig. 2D to F). No or very low signal was observed in the nodule meristem. In contrast to the full-length IPD3 construct, we were not able to visualize the *Mtsym1-1*-encoded truncated IPD3 protein fused to GFP. This indicates that the truncated GFP-IPD3 protein is not synthesized or rapidly degraded.

DMI3 was shown to be present throughout the infection zone but not in the cells of the fixation zone that contain mature nitrogen-fixing symbiosomes (Smit et al. 2005). This shows that IPD3 is present in cells that express DMI3 at the time of symbiosome formation and co-localizes with DMI3 in the nucleus. The presence of IPD3 in the fixation zone in the absence of DMI3 suggests that IPD3 has an additional role during these later stages.

IPD3 controls specific genes required for symbiosome formation.

The presence of IPD3 in the nucleus and the reported interaction with DMI3 (Messinese et al. 2007; Yano et al. 2008) suggest that IPD3 controls the activation of genes that are required for symbiosome formation. Several genes have recently been identified that show DMI3-dependent induction during nodulation and are required for proper infection thread invasion of the nodule primordium or release of the bacteria from the infection threads in the nodule (Marsh et al. 2007). To determine whether some of these genes require a functional IPD3 to be induced in nodules, we compared their expression in WT, type 1, and type 2 *Mtsym1-1* nodules by quantitative RT-PCR. These genes include the coiled-coil protein *RPG* (Arrighi et al. 2008), the U-box containing E3 ubiquitin-ligase *LIN* (Kiss et al. 2009), *Flotillin-2* and *Flotillin-4* (Haney and Long 2010), a nodule-specific remorin *SYMREM* (Lefebvre et al. 2010), and the transcription factors *ERN* (Middleton et al. 2007), *EFD*

(Vernié et al. 2008) and *HAP2* (Combiér et al. 2006). Mutants of *RPG*, *LIN*, and *ERN* show impaired growth of infection threads that fail to penetrate the nodule primordium, comparable with *Mtsym1* type 1 nodules (Arrighi et al. 2008; Kiss et al. 2009; Middleton et al. 2007). Mutants of *SYMREM* and *HAP2* show the formation of nodules that are invaded by infection threads but subsequent release of bacteria from the infection threads appears blocked, similar to *Mtsym1* type 2 nodules (Combiér et al. 2006; Lefebvre et al. 2010). Similarly, *EFD* mutants show delayed release of bacteria and subsequent impaired symbiosome development (Vernié et al. 2008). *Flotillin-2* and *Flotillin-4* were shown to be required for proper infection thread growth and nodule formation, although the exact nodule phenotype has not yet been described (Haney and Long 2010). Additionally, we examined the expression of *NIN*, an early Nod-factor-induced gene that controls both infection and nodule organogenesis (Marsh et al. 2007).

Type 1 and type 2 nodules were excised from *Mtsym1-1* roots 12 days postinoculation (dpi). The use of GFP-expressing rhizobia allowed the selection of type 1 and type 2 nodules based on the penetration of infection threads into the nodules. At 12 dpi, WT nodules formed pink nitrogen-fixing nodules, containing fully developed symbiosomes. *NIN*, *LIN*, *HAP2*, and *Flotillin-2* and *Flotillin-4* show a more or less similar expression level (less than twofold change) in type 1 and type 2 *Mtsym1* nodules compared with WT nodules (Fig. 3). The expression level of *ERN* was approximately three times higher in *Mtsym1* type 1 and type 2 nodules compared with WT nodules (Fig. 3). *RPG* showed an approximately eight-times higher expression in *Mtsym1* type 1 nodules, whereas type 2 nodules showed an expression level comparable with WT nodules (Fig. 3). Therefore, MtIPD3 does not appear to be essential for the induction of these genes. On the other hand, expression of *EFD* and *SYMREM* was significantly reduced in *Mtsym1-1* type 1 and type 2 nodules (approximately 7 and >100 times, respectively) compared with WT nodules (Fig. 3). This indicates that MtIPD3 is required for the induction of a specific subset of genes that are required for symbiosome formation.

Fig. 3. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) expression analyses in *Mtsym1*. qRT-PCR analysis of *ERN*, *NIN*, *HAP2*, *SYMREM*, *EFD*, *RPG*, *LIN*, and *Flotillin-2* and *-4* in wild-type control (J5) = black bars; *Mtsym1-1* type 1 = light gray bars; and *Mtsym1-1* type 2 nodules = dark gray bars 12 days postinoculation. The expression level of the corresponding genes in the wild-type control nodules is set to 1. The relative expression level corresponds to the fold change compared with the wild-type control.

IPD3 also controls infection thread growth.

In Lotus, the IPD3 ortholog CYCLOPS was shown to be essential for infection thread formation (Yano et al. 2008). Because DMI3 is required for infection thread formation and, also, SYMREM controls proper infection thread growth (Godfroy et al. 2006; Lefebvre et al. 2010), we reinvestigated whether, in Medicago, IPD3 already affects infection thread formation in the root hairs. In WT control roots, most (>80%) infection threads have a tubular structure of one to two rhizobia wide (Fig. 4A; Table 2) (Limpens et al. 2003). In contrast, many infection events in *Mtsym1-1* are blocked at the microcolony stage, forming sac-like structures or infection threads that grow with sac-like structures along their length and abort in the epidermis (Fig. 4B and C; Table 2). This shows that IPD3 also controls infection thread growth already in the root hairs.

Infection thread growth is Nod-factor-structure dependent and likely depends on the activity of Nod-factor-signaling output via DMI3, which might depend on IPD3 (Ardourel et al. 1994; Limpens et al. 2003; Smit et al. 2007). Therefore, we tested whether infection and nodule formation in *Mtsym1* would be more affected by rhizobia that make aberrant Nod factors. Therefore, we inoculated plants with GFP-expressing $\Delta nodL$ mutant rhizobia, which make aberrant Nod factors that lack an acetate substitution at the non-reducing terminal glucosamine (Ardourel et al. 1994). In WT roots, $\Delta nodL$ rhizobia show impaired infection thread growth in root hairs; most infection threads (approximately 80%) develop sac-like structures that are nevertheless able to reach and penetrate the primordium and form WT numbers of N_2 -fixing nodules (Limpens et al. 2003). In contrast, in *Mtsym1-1*, the majority of infection events by $\Delta nodL$ mutant rhizobia got arrested at the microcolony stage

Fig. 4. *Mtsym1* infection thread and nodule phenotypes. **A**, Tubular (wild-type) infection thread in a *Mtsym1-1* root hair. **B**, Infection thread with sac-like structures (arrowhead) along its length in a *Mtsym1-1* root hair. **C**, Sac-like infection arrested at the microcolony stage in a *Mtsym1-1* root hair. Bacteria in A–C are expressing green fluorescent protein. Red color of the root hairs results from autofluorescence. **D**, Semithin section through a *Mtsym1-1* type 1 nodule. **E**, Semithin section through a *Mtsym1-1* type 2 nodule. It = infection thread. Bars: A to C = 10 μ m; D and E = 100 μ m.

(Table 2). The infection threads that were formed all contained sac-like structures (Table 2). This resulted in the formation of only type 1 nodules, whereas WT bacteria form approximately 10 to 25% type 2 nodules (on average, two per root) (Fig. 4D and E; Table 2). Therefore, the infection thread growth defect associated with *ΔnodL* rhizobia and in *Mtsym1-1* both appear to be exaggerated in this combination, suggesting interference between the two mutant phenotypes.

IPD3 affects DMI3-induced spontaneous nodule formation.

The formation of nodules in *Mtsym1* indicates that IPD3 is not essential for nodule organogenesis. In contrast, DMI3 is also essential for nodule organogenesis and introduction of an always-active DMI3/CCaMK gene product, lacking the autoinhibitory domain or containing an autophosphorylation-mimick mutation, is sufficient to trigger the formation of spontaneous nodules in the absence of rhizobia (Gleason et al. 2006; Tirichine et al. 2006). Strikingly, when we introduced an always-active DMI3/CCaMK construct lacking the autoinhibitory domain (35S:DMI3*[1-311]) into *Mtsym1-1* roots, no spontaneous nodules were formed (0/65 transgenic roots), whereas introduction of 35S:DMI3* in WT control roots resulted in the formation of, on average, 5 spontaneous nodules/transgenic root ($n = 40$) (Supplementary Fig. 5A). To rule out secondary effects on spontaneous nodule formation, we made use of a gain-of-function mutation in the cytokinin receptor MtCRE1. A gain-of-function mutation [L266F] in the extracellular receiving domain of this receptor histidine kinase also triggers spontaneous nodule formation (Gonzalez-Rizzo et al. 2006; Hayashi et al. 2010; Murray et al. 2007; Tirichine et al. 2007). Introduction of 35S:CRE1*[L267F] did trigger the formation of spontaneous nodules on *Mtsym1-1* roots (18 spontaneous nodules per 28 roots) with the same efficiency as on WT control roots (16 spontaneous nodules per 20 roots). These data suggest that IPD3 affects the stability or activity of a DMI3 signaling complex upstream of cytokinin signaling.

MtIPD3 is required for efficient mycorrhizal colonization but not arbuscule formation.

DMI3 is part of the common signaling cascade that is essential for both rhizobial as well as AM symbioses. In Lotus, the IPD3 ortholog CYCLOPS was shown to be essential for arbuscule formation (Yano et al. 2008). However, the pea IPD3 mutant (*Pssym33-3*, *SGE-Fix-2*) was reported to show decreased mycorrhizal colonization but with increased arbuscule abundance (Jacobi et al. 2003a and b). Therefore, we examined the AM phenotype in Medicago *Mtsym1-1* according to Trouvelot and associates (1986), using the following parameters: the intensity of AM colonization (M%) and the arbuscule abundance (a%) at 18 and 62 dpi. *Mtsym1-1* showed decreased AM colonization at 18 dpi (M%, $P \geq 0.95$) but increased arbuscule abundance (a%, $P \geq 0.95$) at 62 dpi (Supplementary Fig. 6) similar to the reported AM phenotype of *Pssym33-3* (Jacobi et

al. 2003a and b). These data suggest that IPD3 is not essential for arbuscule formation in Medicago and pea.

DISCUSSION

Here, we cloned *SYM1* from Medicago and *SYM33* from pea and show that both encode IPD3. From the phenotypes of the respective mutants, we reveal a novel and essential function for IPD3 in symbiosome formation and development.

One of the striking phenotypes of *Mtsym1* and *Pssym33* is the block of release of rhizobia from the infection threads into the nodule cells. A similar block was found when the essential Nod-factor-signaling protein DMI2 was knocked down specifically in the nodule (Capoen et al. 2005; Limpens et al. 2005) or when a non-legume DMI3 ortholog from rice was introduced into the Medicago *dmi3* mutant (Godfroy et al. 2006). The identification of a knock-out mutation in IPD3 now clearly establishes an essential role for the common symbiotic signaling module DMI2-DMI3-IPD3 in symbiosome formation. In the orthologous *cyclops* mutant from Lotus, infection thread formation does not occur by which later roles for CYCLOPS in symbiosome formation could not be observed (Yano et al. 2008).

The presence of IPD3 in the nucleus and its interaction and likely phosphorylation by DMI3 suggest that IPD3 is required to activate specific genes to allow symbiosome formation. By analyzing the expression of a selected set of genes that are induced during nodule formation and required for nodule invasion or symbiosome formation, we show that IPD3 is only essential for a subset of induced genes. Interestingly, the expression of the nodule-specific remorin, *SYMREM*, was strongly reduced in *Mtsym1* nodules. Similar to *Mtsym1*, *SYMREM* mutants form nodules that are invaded by infection threads but subsequent symbiosome formation does not occur (Lefebvre et al. 2010). Therefore, the impaired expression of *SYMREM* in *Mtsym1* can be sufficient to explain the observed *Mtsym1* phenotypes. Interestingly, this remorin protein interacts with the symbiotic receptors NFP, LYK3, and DMI2 that are essential for perception and signal transduction of rhizobial Nod factors (Lefebvre et al. 2010). IPD3-dependent induction of *SYMREM* may represent a feedback mechanism to regulate the function of these symbiotic receptors during rhizobial infection. However, the reported presence of *SYMREM* on mature symbiosomes indicates additional roles for this protein in symbiosome development (Lefebvre et al. 2010). In contrast, the expression of *ERN*, *RPG*, *LIN*, *HAP2*, and *Flotillin-2* and *Flotillin-4* does not require MtIPD3 because the expression of these genes in *Mtsym1* nodules was either comparable with or higher than their expression level in WT nodules 12 dpi (Arrighi et al. 2008; Combier et al. 2006; Haney and Long 2010; Kiss et al. 2009; Middleton et al. 2007; Vernié et al. 2008). The transcription factor *ERN* and nuclear protein *RPG* showed an approximately three and eight times higher expression level, respectively, in *Mtsym1* type 1 (and type 2 in the case of *ERN*) nodules compared with the WT, which might

Table 2. Infection thread (IT) and nodule phenotypes of *Mtsym1-1* inoculated with different *Sinorhizobium meliloti* (Sm) 2011 strains^a

Bacterial strain ^b	Plant	Percent nodule phenotype			Percent IT phenotype		
		WT	Type 1	Type 2	Sac-like infection ^c	IT with sac ^d	Tubular IT
Sm 2011-GFP	WT (J5)	100 (9)	...	...	...	12 (3)	88 (22)
Sm 2011-GFP	<i>Mtsym1</i>	...	75 (6)	25 (2)	56 (35)	40 (25)	4 (3)
Sm 2011 Δ nodL-GFP	WT (J5)	100 (4)	...	...	12 (3)	80 (20)	8 (2)
Sm 2011 Δ nodL-GFP	<i>Mtsym1</i>	...	100 (4)	...	81 (46)	19 (11)	...

^a Percentage of nodule or infection thread types is given per root; numbers in parenthesis are average number of nodules or infection threads per root. WT = wild type.

^b GFP = green fluorescent protein.

^c Sac-like infection arrested at the microcolony stage.

^d Infection thread with sac-like structure.

reflect a developmental block at early stages of nodule development.

The block in symbiosome formation in *Mtsym1* and *Pssym33* appears to be correlated with the extensive deposition of cell wall material in the infection threads and accumulation of polyphenolic compounds (Bénaben et al. 1995; Tsyganov et al. 1998). This indicates that a more severe defense response is triggered that may block the progression of infection thread growth and release of bacteria. Extensive cell wall depositions were also reported on infection threads in *DMI2* knock-down nodules in *Sesbania rostrata*, where release was blocked (Capoen et al. 2005). The extensive deposition of cell wall material somewhat resembles the formation of cell wall appositions that block the invasion of plant cells by pathogenic fungi during basal defense responses (Schulze-Lefert 2004). Therefore, IPD3 might be required to suppress or control basal defense responses to allow a successful symbiosis.

In addition to release of the bacteria from the infection threads, IPD3 is also required for subsequent symbiosome development. The pea *sym33* (SGEFix-2) mutant occasionally forms nodules where release of rhizobia from the infection threads takes place (Tsyganov et al. 1998). Similarly, although we did not observe any release of bacteria from the infection threads in *Mtsym1*, it has been reported that occasional release can occur in *Mtsym1* (Bénaben et al. 1995; Maunoury et al. 2010). In such cases, the symbiosomes remain morphologically undifferentiated and the nodule cells show premature senescence (Tsyganov et al. 1998). A role for IPD3 during later stages of symbiosome development fits with its observed presence in the nucleus of infected cells in the infection zone as well as in the fixation zone. The presence of IPD3 in the fixation zone, where DMI3 is not present, raises the possibility that, in these cells, IPD3 interacts with components other than DMI3 to control late steps of symbiosome development.

IPD3 also controls the progression of infection threads through the root outer cortex and regulates infection thread growth already in the root hairs. Although normal tubular infection threads are formed, a significant number of infection events appear to be blocked at the microcolony stage and form sac-like structures. A more severe block of infection thread formation, as reported for *Lotus cyclops* mutants (Yano et al. 2008), became apparent when $\Delta nodL$ mutant rhizobia that make different Nod-factor structures (Ardourel et al. 1994) were used to inoculate *Mtsym1*. Almost all infection threads got arrested at the microcolony stage. This indicates an additive effect of impaired Nod-factor signal input and IPD3-dependent signaling in *Mtsym1*. The less severe effect on infection thread growth observed in *Medicago* (and pea) compared with *Lotus* cannot be explained through less severe mutations in IPD3. *Mtsym1-1* shows a mutation nearly identical to that found in the *Ljyclops-5* allele (Yano et al. 2008); the resulting truncated peptide sequence is missing the second NLS and the coiled-coil domain, and it is likely that the protein is unstable or not properly targeted to the nucleus (our data). Moreover, the different alleles show similar phenotypes within each species (*Medicago*, pea, and *Lotus*), suggesting they all represent loss-of-function alleles (Table 1). In the accompanying article Horváth and associates (2011) identified a *Tnt1* transposon tagged insertion mutant allele of IPD3 in the *Medicago* 'R108' genetic background. Strikingly, this mutant shows a phenotype comparable with *Lotus cyclops*, which indicates that the genetic background of the plant ('Jemalong' versus R108) may influence the infection phenotype. Given the fact that, in *Medicago*, the ratio of type 1 and type 2 nodules is bacterial strain dependent, it is likely that the specific combination of plant and bacteria determines the IPD3 mutant phenotype.

In addition to infection phenotypes, we found that IPD3 is also required for the induction of spontaneous nodules by an always-active truncated DMI3 construct. In *Lotus cyclops* mutants, an autophosphorylation-mimick always-active form of DMI3 was still able to trigger spontaneous nodules, although the numbers were severely reduced compared with WT plants (Capoen and Oldroyd 2008; Yano et al. 2008). Introduction of an always-active or more sensitive CRE1 cytokinin receptor was able to trigger spontaneous nodules efficiently in *Mtsym1*, indicating that activation of cytokinin signaling bypasses IPD3 requirement for nodule organogenesis. Similarly, in *Lotus*, it was recently shown that the action of the cytokinin receptor is downstream of DMI3/CCaMK (Hayashi et al. 2010; Tirichine et al. 2007). DMI3 likely forms a multimeric signaling complex with other proteins in addition to IPD3 that trigger transcriptional responses. Loss of IPD3 could destabilize such a DMI3-dependent protein complex and, thereby, affect DMI3-dependent gene expression. Different responses might require different levels of DMI3 complex activity or stability. This model implies that the truncated DMI3 protein forms a complex that is less stable or active than the WT form, and an additional mutation in IPD3 renders this complex unable to trigger spontaneous nodule formation. Such a mechanism could also explain the additive mutant effects observed when inoculating *Mtsym1* with $\Delta nodL$ bacteria; the less efficient $\Delta nodL$ Nod factors (lacking the acetate substitution) would activate DMI3 less efficiently in a *Mtsym1* background that is already reduced for the activity or stability of the DMI3 complex.

IPD3 and DMI3 are widely conserved from monocots to eudicots in plants that are able to establish an AM symbiosis, indicating an ancient role in symbiosis (Messinese et al. 2007; Yano et al. 2008). The rhizobial symbiosis is thought to have recruited part of this mycorrhizal symbiosis pathway (Capoen and Oldroyd 2008; Catoira et al. 2000). The recent identification of a single LysM-type receptor, orthologous to a Nod-factor receptor, in the nonlegume *Parasponia* sp. that controls the accommodation of both rhizobia and arbuscular mycorrhiza suggests a conserved ancient function for this pathway in the intracellular accommodation of symbionts (Op den Camp et al. 2010). In the pea *sym33-3* (SGE-Fix-2) and *Medicago sym1-1* mutants, mycorrhizal colonization is decreased; however, arbuscules can still efficiently be formed (Jacobi et al. 2003a and b). In contrast, an IPD3 knock-out mutation in the *Medicago* R108 ecotype blocks arbuscule formation similarly to *Lotus cyclops* mutants, which indicates that the effects on the AM interaction are also influenced by the genetic background of the plant (Horváth et al. 2011; Yano et al. 2008). In conclusion, in pea and *Medicago*, the DMI3-IPD3 module is essential for the accommodation of rhizobia in new organelle-like membrane compartments in analogy to the intracellular accommodation of AM fungi.

MATERIALS AND METHODS

Plant lines, bacterial and fungal strains, and growth conditions.

The *M. truncatula* 'Jemalong' lines J5 and A17 were used as WT reference lines. *Mtsym1-1/TE7* (Bénaben et al. 1995) was crossed to *M. truncatula* accession A20 and selfed to generate a segregating F2 population. The *Tnt1*/Mere-transposon-tagged line 76A-24 (T00076A self-descent numbered 24) was identified in a collection of *Tnt1*-tagged *M. truncatula* Jemalong lines generated at LIPM Toulouse (Iantcheva et al. 2009) and screened for nodulation mutant phenotypes. Phenotypic analysis was performed after two BC (BC2 lines). 76A-24 was crossed to *Mtsym1/TE7* to show allelism. *Mtsym1-1* was further crossed to TR3 (Sagan et al. 1998) to study allelism. TR3, TR9, TR13, TR62, TR69, and TRV15 were previously shown

to be allelic (Sagan et al. 1998). M4 and F2 of BC1 seed were used for phenotypic analysis of these alleles. For analysis of nodule phenotypes, plants were grown in either perlite (Limpens et al. 2004) or aeroponic caissons (Barker et al. 2006). *Sinorhizobium meliloti* strain Sm2011 was used for inoculation. Additionally, Sm2011-*GFP* (Limpens et al. 2003), Sm2011-*mRFP* (Smit et al. 2005), and Sm2011 Δ *nodL::Tn5-GFP* (Limpens et al. 2003) were used.

Three previously identified *Pssym33* alleles were analyzed: RisFixU (genetic background Finale) (Voroshilova et al. 2001), SGEFix-2 (Tsyganov et al. 1998), and SGEFix-5, with genetic background SGE. Finale and SGE were used as respective WT references. Pea plants were inoculated with *Rhizobium leguminosarum* bv. *viciae* CIAM1026 in quartz sand according to Borisov and associates (1997).

Agrobacterium rhizogenes-mediated root transformations were done according to Limpens and associates (2004) using strain MSU440 (Sonti et al. 1995). The *Escherichia coli* strain DH5 α was used for cloning and strain DB3.1 (Invitrogen, Carlsbad, CA, U.S.A.) for propagation of GATEWAY compatible vectors containing the *ccdB* gene.

Glomus intraradices N. C. Schenck & G. S. Sm., isolate CIAM8 (Muromtsev et al. 1989) from the Collection of the All-Russia Institute for Agricultural Microbiology (registered in the European Bank of Glomales as isolate BEG144), was used for inoculation of Medicago plants. To inoculate plants, a living culture of *G. intraradices* was obtained in an inoculation system with a nurse plant (Rosewarne et al. 1997). *Allium schoenoprasum* L. was used as a nurse plant, as described by Demchenko and associates (2004), except that phosphates were not included in the nutrient solution.

AM phenotype analysis.

Plants were collected at 18 and 62 days. Root fragments were collected individually from 6 to 10 plants per treatment combination. To visualize fungal structures in the root samples, ink staining was performed as described by Vierheilig and associates (1998). Roots were cut in fragments, with a total length up to 30 cm per plant, and mounted on glass slides in glycerol. AM development in the roots was estimated according to Trouvelot and associates (1986) using an Opton 35 (Opton, Germany, Oberkochen, Germany) light microscope for two parameters: intensity of mycorrhizal colonization in the root system (M%) and arbuscule abundance in mycorrhizal root fragments (a%). The data were compared by Student's *t* test with the help of SigmaStat 2.3 for Windows (SPSS Inc., Chicago).

Constructs.

The inferred MtIPD3 promoter, 1,048 bp upstream of the IPD3 start codon, was PCR amplified with Phusion high-fidelity *Taq* polymerase (New England Biolabs, Beverly, MA, U.S.A.) from genomic DNA using primers MtIPD3pF (5'-CACCAA GCTTAAGTGGAGTCAAAGAAATAGTTTATATG-3') and MtIPD3pR (5'-AAAGGCGCGCCGAAACACTTGAATGATG CTTGAACCTATT-3'). The promoter fragment was cloned into pENTR-p4p1r using *HindIII* and *AscI* (underlined), resulting in pENTR4-1-MtIPD3p. Additionally, this promoter fragment was *HindIII*-*AscI* cloned into pENTR-p4p1r-MCS-GFP (containing a multiple cloning site in front of eGFP to make N-terminal GFP fusions) to generate pENTR4-1-MtIPD3p-GFP. The MtIPD3 cds was PCR amplified from 14-day-old nodule cDNA using primers MtIPD3-F (5'-CACCA TGGAAGGGAGAGGATTTCTGGT-3') and MtIPD-R (5'-T CAAATCTTTCCAGTTTCTGATAGA-3'). The resulting 1,546-bp fragment was directionally cloned into pENTR-D-TOPO (Invitrogen), resulting in pENTR-MtIPD3. Next, a multisite GATEWAY reaction with LR Clonase II plus (Invitrogen) was

performed using pENTR4-1-MtIPD3p, pENTR-MtIPD3, pENTR-p2rp3-Stop-T35S, and pKGW-RR-MGW (a modified multisite GATEWAY-compatible binary vector containing *AtUbiquitin10p::DsRED1* to select transgenic roots) (Limpens et al. 2009) to generate MtIPD3p:IPD3. A multisite GATEWAY reaction using pENTR-MtIPD3, pENTR4-1-MtIPD3p-GFP, pENTR-p2rp3-Stop-T35S, and pKGW-RRMGW was performed to generate MtIPD3p:GFP-MtIPD3. To construct UBQ3:GFP-IPD3, pENTR-MtIPD3 was recombined into UBQ3-pK7WGF2-RR (Limpens et al. 2009) using LR Clonase II (Invitrogen). Primers MtIPD3-F and MtIPD3-R were further used to amplify the MtIPD3 genomic fragment from *Mtsym1/TE7* genomic DNA using Phusion *Taq* polymerase. The resulting 3,835-bp fragment was directionally cloned into pENTR-D-TOPO and subsequently recombined into UBQ3-pK7WGF2-RR to generate UBQ3:GFP-IPD3tr.

For the 35S:DMI3*[1-311] construct, an enhanced *Cauliflower mosaic virus* (CaMV)35S promoter (ep35S) was first PCR amplified from pCAMBIA1300 using primers P35S_t (5'-GGGGGCGCGCCATGGTGGAGCACGACACTC-3') and P35S_b (5'-GGCCCCGGGAGAGATAGATTTGTAGAGAGAG ACTGGTGA-3'). It was inserted into pENTR-p2rp3-MCS-Stop-T35S using *AscI* and *SmaI* restriction enzymes. A 933-bp DMI3 cDNA fragment was PCR amplified from A17 root cDNA using primers cMtDMI3_t (5'-GGGGCCCCGGGATG GGATATGGAACAAGAAAAC-3') and tMtDMI3_b (5'-GG GGTACCGGCTTTCTCACCTTTGACC-3') and cloned into pENTR-p2rp3-ep35S-MCS-Stop-T35s using *SmaI* and *KpnI*. Finally, it was recombined into pKGW-RR-MGW in a multiple GATEWAY reaction using Clonase II plus (Invitrogen), resulting in 35S:DMI3*[1-311].

For the 35S:CRE1*[L267F] construct, MtCRE1 was PCR amplified from *M. truncatula* genomic DNA in two parts, using the primers 5'-CACCATGGGCTTCTCTTGAAGATG AA-3' and 5'-TAGGCACTTACTGATACTCAAACCA-3' for part one and 5'-CACCTTAATT-GCTGATGAAGAGTTT-3' and 5'-TCATGAATCTACTGAAGTAGGTTTGT-3' for part two. The two parts were then cloned into pENTR-D-Topo vector (Invitrogen), creating pENTR1-2_MtCRE1-part1 and pENTR1-2_MtCRE1-part2. The L267F mutation was introduced in pENTR1-2_MtCRE1-part1 using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) with primers 5'-GTCACTTGTGGAAATTTAATTGGTCAA CTTG-CTGGTCATC-3' and 5'-GATGACCAGCAAGTTGAC CAAATAAATTTTCCAC-AAGTGAC-3', creating pENTR1-2_MtCRE1L267F-part1*. Next, part1* and part2 were combined by overlap PCR, using the primers 5'-ggggGCGGCCGCATG G-GTCTTCTCTTGAAGATGAA-3', 5'-ttgaccaccattagtccaac TAGGCACTTAC-TGATACTCAAACCA-3', 5'-GTAAGTGC CTAGttgaactaatgggtgtgtaaa-3', and 5'-ggggGCGGCCGCCTCA TGAATCTACTGAAGTAGGTTTGT-3', and the product was ligated using *NotI* and *AscI* (underlined) into a pENTR-D-Topo vector, creating pENTR1-2_MtCRE1L267F. The CaMV35S promoter and terminator were cloned into pENTR4-1 and pENTR2-3 (Invitrogen), thereby creating two modified pENTR clones: pENTR4-1_p35S and pENTR2-3_T35S. All three ENTR vectors were combined into the binary destination vector pKGW-RR-MGW by a multisite gateway reaction (Invitrogen), resulting in 35S:CRE1*[L267F].

Map-based cloning.

A segregating F2 population (99 plants) of a cross between *Mtsym1/TE7* and A20 containing 23 homozygote mutants was used for mapping. All plants showing crossovers between markers 002C10 and 18M20 were phenotyped in the next generation (20 F3 plants per F2 plant) to determine the *Mtsym1* genotype of the corresponding F2 plants. DNA was extracted using

the Qiagen DNeasy plant mini kit. The following simple-sequence repeat (or cleaved amplified polymorphic sequence [CAPS]) markers were used for mapping on *Medicago Linkage Group 5*; PCR products were analyzed on a 3.5% agarose gel: MtB60, MtB146, MtB23, and MtB255 (Mun et al. 2006): 18M20, TCAGATCGACTGCAACTCCA and TGAGCACATGATCCTTTTATACTTG; 8D15, TAATTGGGGATGAAAATC TG and CCTCATGTCACTCATCATCA; 34B21, TTCAGAA AAACCCAAACCAAG and ACATGAGAGCATCCGACC CA; CU4695, TGCCACCAAAGCTAACAACTC and TGG GTTCATCATGGACCTTTTAC; CU469(B), CCACAAAGCG TCCACCATAAAC and CAACCAAAGAAGAAGAACTCC AAACA; CU914, GTGTCATTAATGAACCTTCCAACCTT and GTCCACATCAAACACATCGTAAGA; CU469, GGACCTAT GCAAAATGACCTTCTACAA and CTTTAATGGCCGCAAT CTTACTT; 81G16 (CAPS enzyme *HindIII*), CCACGCGATT TTGGGACTA and CAAAAAGCCTTATGAACCTAACCT; CU137, GAGGAAGGAGAAGGGAAAATAATC and CAAC ATAATGGGAAATCAAACAAC; CT573, TAAATGTGACC GGGGGAGTATG and GCTCATGGATTAGGAGGTGGTCA; 19c21a, AAATGGACCCTGATGATTG and GGGGACATGG ATACTAGAGGG; 002C10, GGGAGTTAAGCATTTCCCA and TGGTTTGTGGCATTCAATTG; 001B12, TGATCCTTTC CAAGAAGCG and CGCTAATTGCTGGCTTCAAA

The MtIPD3 gene was PCR amplified from genomic DNA of the different mutant lines and the corresponding WT reference lines using primers MtIPD3-F and MtIPD3-R and examined by sequence analysis.

Sym33 mapping was done using a F2 population (95 plants) of a cross between WT10584 (Swiecicki and Irzykowska 1998) and SGEFix-2(*sym33*) (Tsyganov et al. 1994). DNA was extracted using the standard cetyltrimethylammonium bromide method (Rogers and Bendich 1985). The following cross-species CAPS markers were used: *Gst* (restriction-enzyme *Tail*) forward (F), CAA TTG AAC ACT CTT ACT CAG and reverse (R), AGA TGC AAA GCT TCC TTG CT; *IPD3* (restriction-enzyme *PfeI*) F, TGC AAA ATA CTC AAG CAC CA and R, TGT TTC CAT GAG CTA TCA CAA AG; *Alat-C* F, CTG ACC TTC CCT CGC CAG (restriction-enzyme *FspBI*), and R, CCT TGC TCG CAC CAT CAG T; and *Enod40* F, TGA AGG TTG TCT GGT GTC TA (WT10584-allele-specific PCR) and R, TTG AAG AAA AGA AAC AGG AAT TA.

The PsIPD3 gene was PCR amplified from genomic DNA of the mutant lines and the corresponding WT reference lines using primers PsIPD3-F (5'-ATGGAAGGGAGAGGATTTTC TG-3') and PsIPD3-R (5'-TTAAATCTTTCCAGTTTCTGATA AAAG-3') and examined by sequence analyses.

Microscopy.

Transgenic roots and nodules were selected based on GFP or DsRED1 expression using a Leica MZFLIII binocular fitted with HQ470/40, HQ525/50, HQ553/30, and HQ620/60 optical filters (Leica Microsystems, Rijswijk, The Netherlands). Transgenic nodules were hand sectioned using double-razor blades and mounted on microscope slides in 0.1 M phosphate buffer, pH 7.4, containing sucrose at 25 mg/ml. Transgenic roots and sectioned nodules were further analyzed on a Zeiss LSM 510 confocal laser scanning microscope (Carl-Zeiss Axiovert [Oberkochen, Germany] 100 M equipped with a LSM510, an argon laser with a 488-nm laser line, a helium-neon laser with a 543-nm laser line); 488 nm (GFP) and 543 nm (DsRED1/mRFP); GFP emission was selectively detected using a 505- to 530-nm band-pass filter; DsRED1/mRFP emission was detected using a 560- to 615-nm band-pass or 560-nm long-pass filter. Infection threads phenotypes were examined using a Nikon Optiphot-2 coupled to a mercury lamp using an optical filter to detect GFP fluorescence (GFP-expressing Sm2011 strains).

Quantitative RT-PCR.

Quantitative RT-PCR was conducted on RNA isolated from 12 day-old *Medicago* WT nodules as well as on RNA from 12 day-old *Mtsym1* type 1 and type 2 nodules of plants grown in perlite (Limpens et al. 2009). Total RNA was isolated and DNase treated using the Plant RNeasy kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA using the Taqman Gold RT-PCR kit (Perkin-Elmer Applied Biosystems) in a total volume of 50 µl using random hexamer primers (10 min at 25°C, 30 min at 48°C, and 5 min at 95°C). Quantitative PCR reactions were performed in (technical) triplicate on 1 µl of cDNA using the Quantitative PCR Core kit for SYBR Green I (Eurogentec), and real-time detection was performed on a MyiQ (Bio-Rad) (40 cycles of 95°C for 10 s and 60°C for 1 min) followed by a heat dissociation step (from 65 to 95°C). Expression data were obtained from two independent biological repetitions. Primers were used at a final concentration of 300 nM. GAPDH was used as reference gene. qPCR primers used were MtIPD3: F, GCGCTCAAGAAAAATGGCTGA AGC and R, GCTTTAGTGATCGAAGCTTCTTCTCAAGG; HAP2: F, AAACATTGGTGGCCTACAGC and R, CATGACG ATACCGTGTCCAG; NIN: F, GGGAGAAAGTCCGGGGAC AA and R, GACACACACCGATGCTCTTTGTC; GAPDH: F, CTTCTGTTGCTTGATGAGGCTGAG and R, AGAACTGT GTCTCGGCGAAATC; ERN: F, AGGTTCAATCCAACA TTTC and R, TCAAATTCTGAAAGCTCCAAGC; EFD: F, GTCACATGGCTTCTCTTTCTCTTCA and R, CAAACCCA ACACATATTACCTTCAT; SYMREM: F, ACAAGAGCG TGTCGTGATG and R, TCGGTCAACTGAGTCCTTTGT; Flotillin-2: F, CAAGAGCTTTTCTCAGATAAGGC and R, ACTGATGGTGGCATGGGTATG; Flotillin-4: F, TGCCTCT GCTAATGCTTTCTGTG and R, CCGAAGTTGAGGCTGCC AAAG; RPG: F, AAAGGCGTTGACTCATTTGG and R, CTC ACCATTTGCCATCTGAA; and LIN: F, ATCAAGGTATGGG ACGCAAG and R, CCAATTTAGCATTGGCTGCT.

RT-PCR analysis of *PsIPD3* in RisFixU and Finale was performed using RNA isolated from 3-day-old seedling roots using Trizol (Invitrogen). RNA (1 µg) was used to make cDNA using an oligo(dT) primer, followed by a PCR reaction (35 cycles) using primers PsIPD3-3 GTTAATGTGTCTAATCA GCAAC and PsIPD3-4 GGTATTTGCGACATTGAAAGGTA ATC.

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