Medicago truncatula NIN Is Essential for Rhizobial-Independent Nodule Organogenesis Induced by Autoactive Calcium/Calmodulin-Dependent Protein Kinase


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The symbiotic association between legumes and nitrogen-fixing bacteria collectively known as rhizobia results in the formation of a unique plant root organ called the nodule. This process is initiated following the perception of rhizobial nodulation factors by the host plant. Nod factor (NF)-stimulated plant responses, including nodulation-specific gene expression, is mediated by the NF signaling pathway. Plant mutants in this pathway are unable to nodulate. We describe here the cloning and characterization of two mutant alleles of the Medicago truncatula ortholog of the Lotus japonicus and pea (Pisum sativum) NIN gene. The Minin mutants undergo excessive root hair curling but are impaired in infection and fail to form nodules following inoculation with Sinorhizobium meliloti. Our investigation of early NF-induced gene expression using the reporter fusion ENOD11::GUS in the Mtinin-1 mutant demonstrates that MnIN is not essential for early NF signaling but may negatively regulate the spatial pattern of ENOD11 expression. It was recently shown that an autoactive form of a nodulation-specific calcium/calmodulin-dependent protein kinase is sufficient to induce nodule organogenesis in the absence of rhizobia. We show here that MnIN is essential for autoactive calcium/calmodulin-dependent protein kinase-induced nodule organogenesis. The non-nodulating hcl mutant has a similar phenotype to Mtinin, but we demonstrate that HCL is not required in this process. Based on our data, we suggest that MtNIN functions downstream of the early NF signaling pathway to coordinate and regulate the correct temporal and spatial formation of root nodules.

Legumes are unusual among flowering plants in their ability to form mutually beneficial root-microbe associations with both arbuscular mycorrhizal (AM) fungi and the genera of bacteria collectively known as rhizobia (Doyle, 1998). The AM symbiosis is the more ancient of the two symbioses and may have provided the basic genetic framework onto which the rhizobial endosymbiosis was elaborated (Remy et al., 1994; Gianinazzi-Pearson, 1996; Redecker et al., 2000). The combination of rhizobial and AM symbioses for the legume is significant, because it provides the plant with nitrogen and phosphorous, which represent two of the three essential macronutrients for normal plant growth and development (Govindaraju et al., 2005; Harrison, 2005). Rhizobia provide nitrogen to the plant via the formation of specialized root organs called nodules that form de novo upon colonization of the root by the bacteria. The nodule forms a specialized microenvironment in which the bacteria fix atmospheric nitrogen that is transported to portions of the plants where nitrogen demand is highest. This allows legumes to be grown in relatively nutrient poor conditions and their ability to naturally enhance fixed nitrogen levels in soils makes them indispensable for sustainable agricultural systems.

The process of nodulation is the result of tightly regulated biochemical and molecular interactions between the two symbionts (Schultze and Kondorosi, 1998). Plants release flavonoids into the rhizosphere, which stimulate the production of lipochito-oligosaccharide Nod factors (NF) by rhizobia (Dénarié et al., 1996; Long,
Perception of NF and rhizobia by the plant root leads to the formation of a characteristic root hair curl or shepherd’s crook, which entraps a bacterial microcolony. From this microcolony develops an infection thread that penetrates the root hair and continues advancing toward the cortical cells of the root that have begun to divide to form the nodule primordia. Bacteria are released from the infection thread into the developing nodule where they differentiate into nitrogen-fixing bacteroids. Nodule formation is negatively regulated by high nitrate levels (Streeter, 1988), ethylene (Pennetsa and Cook, 1997), and feedback inhibition of nascent nodule primordia by established nodules (Pierce and Bauer, 1983; Kossak and Bohlool, 1984; Caetano-Anollés and Bauer, 1988; Caetano-Anollés and Gresshoff, 1991). These mechanisms restrict nodule numbers, ensuring the plant invests in nodulation only to an extent that is sufficient to meet its nitrogen needs.

Under normal conditions, the formation of nitrogen-fixing nodules is dependent on NF activation of the early NF signaling pathway and is a prerequisite for the subsequent processes of bacterial entry and nodule morphogenesis. NF stimulation of this signal transduction cascade elicits the earliest measurable plant responses in root hair cells: ion flux (Ehrhardt et al., 1992), calcium oscillations (Ehrhardt et al., 1996; Harris et al., 2003), cytoskeletal changes (Van Brussel et al., 1992; de Ruijter et al., 1999), root hair deformation (Lerouge et al., 1990), and the expression of NF-dependent genes (early nodulins or ENODs; Horvath et al., 1993). In the model legume Medicago truncatula, the early nodulins ENOD11 and KIP1 have been valuable markers of early NF signaling (Cook et al., 1995; Journet et al., 2001). While the precise function of these nodulins in the rhizobial symbiosis is unknown, expression is induced within hours of NF application, and continued expression is associated with bacterial entry, nodule formation, and nodule function (Cook et al., 1995; Journet et al., 2001). The activation of ENODs is associated with calcium spiking that most likely functions as a mechanism of signal transduction linking NF perception to gene expression changes (Felle et al., 1999; Engstrom et al., 2002; Charron et al., 2004). The fact that mutations and inhibitors that abolish NF-induced calcium spiking also inhibit ENOD expression provides strong evidence for a causal link between calcium spiking and ENOD induction (Walker et al., 2000; Wais et al., 2002; Ben Amor et al., 2003; Radutoiu et al., 2003; Charron et al., 2004; Miwa et al., 2006).

Although many of the cellular and physiological changes that the plant undergoes have been described in detail, it is only recently that we are beginning to understand the genetics that underpin nodulation. This advance in our knowledge has come primarily from the use of forward genetic screens in model legumes for non-nodulating plant mutants (Oldroyd and Downie, 2004, 2006). This approach has lead to the identification of six genes that currently delineate the early NF signaling pathway in M. truncatula: NFP, DMI1, DMI2, DMI3, NSP1, and NSP2. Initial perception of NF is believed to be the function of the genes NFP in M. truncatula and NFR1/NFR5 in Lotus japonicus, which encode predicted sugar-binding receptor-like kinases (Ben Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Between the perception of NF and the activation of calcium spiking are a number of genes that are common to both NF signaling and the establishment of the AM symbiosis (Catoira et al., 2000; Marsh and Schulze, 2001; Parniske, 2004; Kistner et al., 2005). The M. truncatula gene DMI2 and L. japonicus SymRK encode Leu-rich repeat receptor-like kinases (Endre et al., 2002; Stracke et al., 2002), while M. truncatula DMI1 and L. japonicus CASTOR/POLLUX are predicted to encode ion channels (Ané et al., 2004; Imaizumi-Anraku et al., 2005). Additionally, Sym24 and a nucleoparin, Nup133, are required for the common signaling pathway in L. japonicus, but no orthologous genes have yet been identified in M. truncatula (Kistner et al., 2005; Kanamori et al., 2006). Mutations in any of these genes eliminate NF-dependent calcium spiking, indicating that these proteins play an essential role in generating the calcium signal.

Two nodulation-specific GRAS family transcriptional regulators, NSP1 and NSP2, are required downstream of the calcium-spiking response (Catoira et al., 2000; Oldroyd and Long, 2003; Kaló et al., 2005; Smit et al., 2005; Heckmann et al., 2006).

Perception of the NF-generated calcium-spiking signal and transduction of the information it encodes is believed to be accomplished through a plant-specific calcium/calmodulin-dependent protein kinase (CCaMK) encoded by DMI3 in M. truncatula and Sym15 in L. japonicus (Lévy et al., 2004; Mitra et al., 2004a; Tirichine et al., 2006). This gene is also required for nodulation and mycorrhization. We have recently demonstrated that an autoactive CCaMK comprised solely of the kinase domain is sufficient to induce expression of the early nodulin ENOD11 and nodule formation in the absence of Sinorhizobium meliloti (Gleason et al., 2006). Mutations in L. japonicus CCaMK were also shown to be responsible for the spontaneous nodulation phenotype of snf1 mutants (Tirichine et al., 2006). These results clearly indicate that calcium is central to nodulation and that early NF signaling leading to nodule organogenesis can be differentiated from the process of bacterial entry.

For many years, it has been proposed that nodulation is the result of two coordinated but distinct NF-dependent processes: early signaling and bacterial entry (Ardourel et al., 1994; Geurts and Franssen, 1996; Walker and Downie, 2000). This model was based on both plant and bacterial mutants that differentially activate early epidermal responses versus bacterial entry and infection thread growth. Two plant mutants in particular have phenotypes consistent with a defect in bacterial entry: Ljnln (sym35 in pea [Pisum sativum]) and hcl (Schauer et al., 1999; Catoira et al., 2001; Borisov et al., 2003). Both Ljnln and hcl mutants
are non-nodulating but undergo excessive root hair curling in response to rhizobia in the absence of bacterial entry. The HCL gene encodes the LysM receptor-like kinase LYK3 (P. Smit and T. Bisseling, personal communication). Suppression of LYK3 expression by RNA interference inhibits bacterial entry (Limpens et al., 2003). Furthermore, root hair deformations prior to shepherd’s crook formation and expression of MtENOD11, MtENOD12, and RIP1 are normal in the hcl mutant, indicating this gene is unlikely to function in the early epidermal NF signaling cascade. Also consistent with this hypothesis is the fact that both NF and S. meliloti induce cortical cell divisions in hcl, although at reduced levels relative to wild type. It has been demonstrated that NF treatment alone, presumably functioning through the early NF signaling cascade, is sufficient to stimulate cortical cell divisions (Truchet et al., 1991). The cortical cell division phenotype of hcl contrasts with both Ljin and all mutants in the NF signaling cascade in which no cortical cell divisions can be observed. Consequently, it would appear that the Ljin mutant displays characteristics of mutations in both the genes that are essential for early NF signaling and those required for bacterial entry. Based on the mutant phenotype, it has been suggested that the primary function of NIN is in the entry process, along with HCL (Geurts et al., 2005), and not in early NF signaling (Schau er et al., 1999). However, with the exception of the recent analysis of calcium spiking in L. japonicus nodulation mutants (Miwa et al., 2006), the precise relationship of NIN to the early NF signaling and bacterial entry has not been rigorously investigated. Therefore, we have used a combination of physiological and gene expression markers and the autoactive CCaMK to address the question of MtNIN function relative to early NF signaling.

In this article, we present evidence that NIN is not essential for early NF signaling but is required for bacterial entry and autoactive CCaMK-induced nodule organogenesis. We have isolated two Mtchin mutant alleles, cloned the M. truncatula ortholog of L. japonicus and pea NIN, and demonstrate that rapid induction of the early nodulin reporter ENOD11::GUS is independent of MtNIN. Our data suggests that MtNIN also plays a role in restricting the spatial pattern of nodulin gene expression and this role may be related to control of nodule number. Critically, we show that spontaneous nodulation induced by the autoactive CCaMK is dependent on MtNIN. In contrast, HCL is dispensable for this form of CCaMK-induced nodule formation. Taken together, these findings suggest that NIN functions downstream of the early NF signaling pathway leading to nodule organogenesis. NIN also appears to play an important role in bacterial entry. Our work highlights the distinct and yet coordinated roles of the components controlling NF perception, nodule organogenesis, bacterial invasion, and control of nodule number and suggests that NIN may be a key integrator of these processes.

RESULTS

Phenotype of the Non-Nodulating Mutants 12S and Tnt148

The 12S and Tnt148 mutants were isolated in separate forward genetic screens of fast neutron mutagenized Jemalong and Tnt1 transposon-tagged R108 (d’Erfurth et al., 2003) populations, respectively, based on their inability to form nodules (Nod−; Fig. 1A). Closer inspection revealed that S. meliloti did not stimulate cortical cell divisions in these mutants, even at a time point in which wild-type plants had fully formed nodules (Fig. 1, B and C). The mutants also exhibited the characteristic nin mutant phenotype of excessive root hair curling in response to S. meliloti (Fig. 1E; Schau er et al., 1999; Borisov et al., 2003). The wild-type root hair response is normally complete with the formation of a single curl or shepherd’s crook and concomitant entrapment of bacteria (Fig. 1D). In

Figure 1. Wild type and the non-nodulating mutant phenotypes of 12S and Tnt148. All images are of plants cultivated in vitro with S. meliloti 1021 pXLG D4 for 14 d. A, Wild-type nodulating root on the right and the non-nodulating 12S root on the left. B, Spot inoculation of a wild-type root produces cortical cell division leading to nodule formation. C, Spot inoculation of 12S does not induce cortical cell divisions. The black mark indicates site of S. meliloti application. D, The formation of a shepherd’s crook and an infection focus in the center of the crook in wild type. E, Root hairs of 12S demonstrating the characteristic nin phenotype. Arrows indicate root hairs undergoing excessive curling. F, A root hair of Tnt148 that has formed an infection focus and a limited infection thread. These infection events are infrequent in Tnt148 and absent in 12S. Scale bars = 10 mm (A), 1 mm (B and C), 40 μm (D and E), and 20 μm (F).
contrast, 12S and Tnt148 continued to form multiple curls in a single root hair. Occasional infection foci and/or limited infection thread development was observed in Tnt148, but infection threads did not penetrate beyond the root hair cell (Fig. 1F). The Tnt148 mutant formed 0.2 ± 0.2 foci/cm of root compared with 1.9 ± 1.2 foci/cm for wild type. Neither the formation of infection foci nor threads was observed in the mutant 12S. Both mutants were identified in screens in which plants were cocultivated with *S. meliloti* and the AM fungus *Glomus hoi*. In primary and subsequent screening, the mutants formed normal mycorrhizas (data not shown). Taken together, these observations are consistent with the phenotypes previously reported for the *L. japonicus* and pea *nin* mutants (Schauser et al., 1999; Borisov et al., 2003).

For reasons described in the following section, we have renamed these mutants *Mtnin* (12S) and *Mtnin* (Tnt148). Crosses between the *Mtnin* and all of the NF signaling mutants (and *hcl*) produced wild-type F1 plants, indicating that *Mtnin-1* defines a novel complementation group in *M. truncatula* (Table I). The F1 progeny of reciprocal crosses between *Mtnin-1* and *Mtnin-2* were nod− demonstrating allelism (Table I). From our data, we conclude that *Mtnin-1* expresses the stronger phenotype of the two alleles and that the occasional infection foci and infection threads formed in *Mtnin-2* indicates that this is a weaker allele.

### Cloning and Sequence Analysis of MtNIN

Systematic sequencing of over 50 genomic DNA sequences 5' and 3' of Tnt1 insertion sites in *Mtnin-2* identified three insertions that cosegregated with the nod− phenotype. Genetic markers physically linked to the sequences bordering two of these insertion sites positioned the insertions in the top arm of linkage group 5 of *M. truncatula*. The syntenic genomic region of pea within linkage group 1 contains *PsNIN*. Therefore, we used the *PsNIN* sequence to identify the orthologous gene, *MtNIN*, which is contained within a sequenced *M. truncatula* bacterial artificial chromosome (CR936325). Using transposon-specific primers and primers designed against the *MtNIN* sequence, we showed that one of the three Tnt1 insertions was in the *MtNIN* locus (Fig. 2A) 20 bp upstream of the predicted translational start site of *MtNIN* (data not shown). Sequencing of the second allele, *Mtnin-1*, also revealed a mutation at the *MtNIN* locus: an 11-bp deletion starting at position 1,850. To further demonstrate that the *Mtnin-2::Tnt1* insertion is responsible for the nod− phenotype, a mutant *Mtnin-2* T1 plant was backcrossed to the R108 parental line. The F2 progeny from three F1 lines were tested for their symbiotic phenotype, as well as for the presence of the transposable element. Of the F2 progeny tested, 28 were nod− and 77 nod+, as expected for a monogenic recessive mutation. Primers specific for the wild-type *MtNIN* sequence were designed that flank the *Tnt1* insertion site so that amplification of the *MtNIN* gene is not possible when the locus is homozygous for the *Mtnin-2::Tnt1* insertion (Fig. 2A).

Using the *MtNIN*-specific primers, the wild-type locus was never amplified in nod− plants but was always amplified in nod+ plants, demonstrating that the *Mtnin-2::Tnt1* insertion cosegregated with the nod− phenotype (Fig. 2A). Amplification from the same nod− plants with a combination of *MtNIN* and *Mtnin-2::Tnt1*-specific primers confirmed the presence of the transposon (Fig. 2A).

The *M. truncatula NIN* gene is predicted to encode a protein of 933 amino acids, which shares 53% and 72% overall identity with *L. japonicus* and pea NIN proteins.

### Table 1. Allelism tests

Nodulation phenotypes were determined in the F1 generation 4 weeks postinoculation with the rhizobial strain B1.

<table>
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<th>Male</th>
<th>Female</th>
<th>No. of Crosses</th>
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<td>A17</td>
<td>A17</td>
<td>na*</td>
<td>3</td>
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<tr>
<td>Mtnin-1</td>
<td>Mtnin-1</td>
<td>na*</td>
<td>0</td>
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<tr>
<td>Mtnin-2</td>
<td>Mtnin-2</td>
<td>na*</td>
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<tr>
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<td>Mtnin-1</td>
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<td>dmi2</td>
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<td>Mtnin-1</td>
<td>dmi3</td>
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<td>dmi3</td>
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<td>Mtnin-1</td>
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<td>nsp2</td>
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<td>nsp2</td>
<td>Mtnin-1</td>
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*Plants were not crossed but allowed to self-pollinate. na, Not available.*
respectively (Fig. 2B). Despite the relatively low overall amino acid homology, all three NIN proteins have six highly homologous domains in common (I–VI) that have been previously defined in the analysis of PsNIN (Borisov et al., 2003). Domains I to III have unknown function but share greater than 87% identity between M. truncatula and pea and greater than 73% identity between M. truncatula and L. japonicus. Domains IV to VI are predicted to contain transmembrane/hydrophobic structures and DNA-binding motifs (RWP-RK), as well as a protein–protein interacting region at the C terminus (PB1). These domains show a high degree of homology between pea, M. truncatula, and L. japonicus (Fig. 2B). The interdomain regions show only limited homology. With respect to gene structure, the position of introns in MtNIN is comparable to that of L. japonicus and pea, in which three introns are contained within the coding region and a fourth is located immediately 5′ to the translational start codon (Schauer et al., 1999; Borisov et al., 2003).

**The NF Signaling Pathway Is Required for the Induction of MtNIN Expression**

In wild-type L. japonicus, an increase in the steady-state levels of LjNIN transcript is detectable within several hours, and strong expression within days, of either NF or Mesorhizobium loti treatment (Schauer et al., 1999). We observed a similar increase in the steady-state transcript levels of MtNIN in wild-type plants (Fig. 3A). An increase in transcript level was detected within 2 d, and by 10 d after inoculation with S. meliloti, the transcript level of MtNIN had increased significantly over un inoculated levels. Compared to wild-type expression levels, Mtnin-2 showed greatly reduced NIN expression. Consistent with the weaker Mtnin-2 phenotype, we detected a low-level increase of transcript 2 d after S. meliloti inoculation in this allele (Fig. 3A). In agreement with the result obtained by reverse transcription (RT)-PCR, Affymetrix analysis of MtNIN expression in wild type at 1 d after inoculation with S. meliloti showed an 8.1-fold increase over un inoculated plants (Fig. 3B). Similar Affymetrix analysis comparing MtNIN induction by S. meliloti in the NF signaling mutants indicated that all of the genes essential for early NF signal transduction, NFP, DMI1, DMI2, DMI3, NSP1, and NSP2, were also required for the increase in the steady-state levels of NIN transcript 1 d after treatment (Fig. 3B). In contrast, HCL is not required for NIN induction. It has been shown that NIN transcription in L. japonicus is dependent on the NF signaling genes NFR1, NFR5, and SymRK (Radutoiu et al., 2003). MtNIN Is Not Required for Early NF Signaling

Rapid physiological and gene expression changes in the root epidermis are associated with NF treatment. One of the earliest measurable M. truncatula responses to NF is calcium spiking (Wais et al., 2000; Shaw and Long, 2003). We have recently demonstrated that NF treatment induces normal calcium spiking in Ljnin (Miwa et al., 2006). Consistent with this, Mtnin-1 also exhibited a normal calcium oscillatory response following NF treatment (Fig. 4). Both the frequency
and shape of the spikes is comparable between wild-type and \textit{Mtnin-1} plants. To further assess the relationship between \textit{MtNIN} and NF signal transduction, we analyzed NF induction of ENOD11 using a promoter GUS fusion (ENOD11::GUS; Journet et al., 2001). This fusion is a useful reporter for the activation of early NF signaling (Journet et al., 2001). NF treatment of \textit{Mtnin-1} plants homozygous for ENOD11::GUS induced a pattern of GUS expression identical to wild-type plants at 6 or 12 h after treatment (Fig. 5, A and B). These results suggest that \textit{MtNIN} is not required for NF-induced gene expression. Taken together with the wild-type calcium response in \textit{Mtnin-1}, we conclude that NIN must function downstream of the early NF signaling cascade in which the calcium-spiking response is predicted to play an essential role.

\textbf{ENOD11 Spatial Expression Is Dramatically Altered in Mtnin-1}

NF and \textit{S. meliloti} can induce strong ENOD11::GUS expression in the epidermis of \textit{M. truncatula} roots in an approximately 10-mm NF-responsive zone located behind the growing root tip within 6 h of treatment (Fig. 5A). As bacterial colonization progresses, the pattern of ENOD11::GUS expression becomes restricted to a limited number of cells containing infection threads and cells associated with successful infection sites where nodules form (Journet et al., 2001). The restriction of nodulation-specific gene expression to infected cells may reflect the function of the mechanisms that regulate nodule number. Following 6 h of NF treatment, strong expression of ENOD11::GUS occurred in both wild type and \textit{Mtnin-1} and was restricted to the NF- and \textit{S. meliloti}-responsive zone (Fig. 5A). However, whereas the ENOD11::GUS expression in wild type remained restricted to this zone 24 h after treatment, the expression pattern in \textit{Mtnin-1} was greatly expanded toward the root tip, so much so that strong ENOD11::GUS expression could be detected within millimeters of the root cap (Fig. 5A). Following 4 d of continuous NF treatment, the pattern of greatly expanded and contiguous ENOD11::GUS expression continued in \textit{Mtnin-1}, while in the wild type, the expression pattern became focused into a small number of epidermal cells confined to the responsive zone and was mostly absent in the vast majority of root cells (Fig. 5A). These results suggest that \textit{MtNIN} plays an active role in restricting ENOD11 expression.

Sequence homology with the nitrogen-responsive MId proteins of Chlamydomonas suggests that \textit{LjNIN} may be involved in the regulation of nodulation by nitrogen (Schauer et al., 1999). It has been known for many years that nodulation can be suppressed by high nitrate levels and that this phenomenon is determined in large part by the host plant genome (Carroll et al., 1985; Delves et al., 1986; Streeter, 1988). We have tested the NF-induced expression of GUS under the control of ENOD11 in plants grown in the presence or absence of nitrate (Fig. 5B). Both wild-type and \textit{Mtnin-1} plants showed markedly reduced ENOD11::GUS expression levels when grown in the presence of nitrate, indicating that \textit{MtNIN} retains wild-type responsiveness to nitrogen status under these experimental conditions.

\textbf{Nodule Organogenesis Requires NIN But Not HCL}

In both \textit{M. truncatula} and \textit{L. japonicus}, mutant forms of CCaMK, a central component of the early NF signaling

![Figure 3](image-url)  
**Figure 3.** NIN expression requires the early NF signaling pathway. A, In wild-type plants, steady-state transcript levels of \textit{MtNIN} increased from 2 d to 10 d after inoculation with \textit{S. meliloti}. In \textit{Mtnin-2}, little or no transcript was detectable during the same time course. The barely detectable increase in \textit{Mtnin-2} is consistent with the weaker Nod phenotype in this allele. B, The steady-state expression levels of NIN, ENOD11, and RIP1 were compared between mock-inoculated and \textit{S. meliloti} 1021-inoculated wild type and the indicated mutants using the Affymetrix \textit{M. truncatula} oligonucleotide microarray. Each square represents the log\textsuperscript{2} fold change of inoculated versus uninoculated samples in triplicate, 1 d postinoculation. Expression levels above zero are depicted in magenta, and expression levels below zero are depicted in green. The non-nodulating mutants \textit{nfp}, \textit{dnm1}, \textit{dnm2}, \textit{dnm3}, \textit{rsp1}, and \textit{rsp2} are defective in NF signaling, resulting in the abolishment of NIN, ENOD11, and RIP1 expression compared to wild type and \textit{hcl} in which these genes are expressed normally. The expression profiles of ENOD11 and RIP1 were previously published (Mitra et al., 2004).

![Figure 4](image-url)  
**Figure 4.** NF-induced calcium spiking is normal in \textit{Mtnin-1}. Cytosolic free calcium levels were monitored over approximately 30 min in wild-type and \textit{Mtnin-1} root hairs following 1-nM NF treatment (black vertical bar). Both traces are from single representative root hairs stimulated with NF. Inset table summarizes the total number of root hairs treated compared to the number that generated the spiking response.
cascade, can induce spontaneous nodulation in the absence of rhizobia (Gleason et al., 2006; Tirichine et al., 2006). These experiments show that formation of the nodule is distinct from invasion of the root by rhizobia. In M. truncatula, transgenic expression of an autoactive CCaMK (DMI31–311), comprised of the kinase domain alone, is capable of inducing spontaneous nodules and has been useful in determining the order of gene product function within the early NF signaling pathway relative to CCaMK (Gleason et al., 2006). Components of the pathway predicted to act before CCaMK form nodules when DMI31–311 is introduced, while gene products predicted to act later than CCaMK in the signaling cascade will not form spontaneous nodules. We have applied this analysis to infer the position of NIN and HCL relative to CCaMK. Spontaneous nodules formed on both dmi3-1 and hcl roots transformed with the DMI31–311 construct, but none developed on transformed roots of Mtnin-1 (Fig. 6). Out of more than 85 independently transformed Mtnin-1 composite plants, none formed spontaneous nodules. Transformation of Mtnin-1 with DMI31–311 was confirmed by PCR (data not shown). In contrast, 25% of transformed dmi3-1 and 33% of transformed hcl composite plants formed spontaneous nodules (Table II). This result clearly demonstrates that Mtnin is essential for the process of nodule organogenesis mediated by the autoactive CCaMK. The fact that hcl forms autoactive CCaMK-dependent nodules suggests either that HCL functions upstream of CCaMK in the NF-signaling pathway or that HCL does not play a significant role in nodule organogenesis. The latter hypothesis is consistent with the hcl phenotype and the proposed role of HCL in the process of bacterial entry (Catoira et al., 2001; Geurts et al., 2005).

**DISCUSSION**

**Mtnin Is Required for Nodule Organogenesis**

We have isolated and characterized two Mtnin mutants. Consistent with the phenotype observed for L. japonicus and PsNIN, Mtnin mutations completely abolish nodule formation (Schauer et al., 1999; Borisov et al., 2003). It was recently shown in both M. truncatula and L. japonicus that the activated CCaMK is sufficient to induce the formation of spontaneous nodules in the absence of S. meliloti (Gleason et al., 2006; Tirichine et al., 2006). In M. truncatula, this was also shown to depend on the GRAS family transcriptional regulators NSP1 and NSP2 (Gleason et al., 2006). The data presented here clearly demonstrate that autoactive CCaMK-induced nodule formation is also dependent on Mtnin, suggesting that Mtnin functions downstream of CCaMK, leading to nodule morphogenesis. In addition, we have demonstrated that HCL is not required for autoactive CCaMK-induced nodule formation. This places HCL either upstream of CCaMK in the NF signaling pathway or in a separate pathway. It has been previously suggested that HCL functions on a pathway required for bacterial entry (Catoira et al., 2001; Geurts et al., 2005). Our data is consistent with this model.

**Is Mtnin a Component of the Early NF Signaling Pathway?**

We have used physiological and gene expression markers of early NF signaling to assess whether Mtnin is a component of this pathway downstream of CCaMK. Based on our analysis of the NF-stimulated calcium-spiking response and NF-induced GUS expression driven by the ENOD11 promoter, we conclude that it is very unlikely that Mtnin participates in early NF signaling. One of the earliest physiological markers for activation of the NF signaling pathway is the NF-dependent calcium-spiking response (Ehrhardt et al., 1996; Harris et al., 2003; Oldroyd and Downie, 2006). We have previously demonstrated that this response is wild type in Ljnin (Miwa et al., 2006). We have now shown that Mtnin is also not required. Normal NF-induced spiking in Mtnin-1 confirms that NIN acts downstream of CCaMK in the process of nodule morphogenesis and the abolishment of nodulation in Mtnin is not the result of a modification of the
calcium-spiking response. Also consistent with this interpretation is the fact that DMI3 is required for the induction of MtNIN expression, as are NSP1 and NSP2.

NF-induced ENOD11::GUS expression is also informative. Six hours post-NF treatment, GUS expression in the MtNIN mutant background is completely wild type. This is in contrast to all of the early NF signaling mutants in which nodulin gene expression is completely lost. Wild-type ENOD11::GUS expression in the Mtnin-1 background strongly suggests that MtNIN is not a component of early NF signaling, although it is clearly required in addition to the early NF signaling pathway for CCaMK-mediated nodule organogenesis.

The probable role of endogenous plant hormones in nodule organogenesis has been postulated for some time. In support of this, the localized production of cytokinin was shown to phenocopy nodule morphogenesis and cytokinins activate the expression of early nodulin genes (Dehio and Debruijn, 1992; Cooper and Long, 1994; Bauer et al., 1996; Fang and Hirsch, 1998; Mathesius et al., 2000). The autoactive CCaMK also induces nodule formation in the absence of S. meliloti, raising the possibility that NF-induced nodulation and cytokinin-induced nodulation are mechanistically related. Very recently, it has been shown that silencing of a predicted M. truncatula cytokinin receptor strongly reduces nodule formation and that the probable ortholog in L. japonicus, LHK1, is necessary and sufficient for nodule organogenesis (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007). Furthermore, LHK1 function is dependent on LjNIN (as well as LjNSP2). Taken together with our data, this suggests that NIN may be playing a significant role in both epidermal (infection and gene expression) and cortical cell responses (nodule organogenesis) to NF and rhizobia.

Based on our data and recently published results from other groups, we propose a model relating NIN to NF perception and downstream events (Fig. 7). Our model emphasizes the central role NIN plays in nodule organogenesis and bacterial entry, including a possible role in coordinating cytokinin and NF signaling. Normal calcium spiking and wild-type ENOD11::GUS induction place NIN downstream of the early NF signaling pathway. The dependence of the induction of NIN expression on NFP, DMI1, DMI2, DMI3, NSP1, and NSP2 is consistent with this assertion. The order of function of NIN and ENOD11 cannot currently be resolved, and we have therefore placed them at an equivalent position in the signaling process. However, NIN appears to negatively regulate the root epidermal response to NF, and this is reflected in the active suppression of ENOD11::GUS expression. Formally, our results cannot rule out the possibility that NIN functions in the NF signaling pathway at a parallel position to NSP1 and NSP2 on a branch point that does not regulate ENOD11 induction, but the lack of NIN expression in nsp1 and nsp2 does not support this interpretation.

Does MtNIN Play a Role in the Regulation of Nodule Number?

Nodule number and position on the plant root is tightly controlled (Bhuvaneswari et al., 1980; Pierce and Bauer, 1983; Kossak and Bohlool, 1984). Plants that have lost this control form excessive numbers of nodules (Carroll et al., 1985; Delves et al., 1986; Sagan and Duc, 1996; Schauser et al., 1998; Szczyglowski et al., 1998; Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003). Two nodule number mutants, sumn

![Figure 6. MtNIN is required for nodule organogenesis induced by autoactive CCaMK. A, Rhizobia induced nodules in wild-type transformed roots. B, Spontaneous nodules induced by the autoactive CCaMK (DMI31–311) construct are present on hcl mutants but absent in Mtnin-1 (C). Wild-type roots (A) were inoculated with S. meliloti, but mutants (B and C) were grown in the absence of S. meliloti. Scale bar = 5 mm.](image)
and the ethylene-insensitive mutant sickle, have been characterized in *M. truncatula* (Pennmets and Cook, 1997; Oldroyd et al., 2001; Penmetsa et al., 2003; Schnabel et al., 2005). Although these two mutants appear to define separate mechanisms, both form 10-fold more nodules than wild type and express abnormally persistent and high steady-state levels of the early nodulins RIP1 and ENOD40 compared to wild type (Penmetsa et al., 2003).

We have observed that the loss of *Mtnin* results in the spatial expansion of *GUS* expression driven by the ENOD11 promoter following treatment with NF or *S. meliloti*. Although *GUS* expression in *Mtnin-1* plants is indistinguishable from wild type 6 to 12 h after treatment, 24 h and 4 d after treatment, the *GUS* expression extends well outside the normal zone of responsiveness and within millimeters of the root tip. In *L. japonicus*, it was also noted that the zone of responsiveness defined by root hair curling is expanded approximately 4-fold in *NIN* mutants (Schauser et al., 1999). A similar expansion occurs in *Psinin* (Borisov et al., 2003). These observations suggest the possibility that NIN is involved in the numerical and/or spatial regulation of nodulation. Based on our results, we would hypothesize that NIN plays a negative regulatory role, repressing nodulation outside of the zone of responsiveness.

In addition to the regulation of nodule number and patterning by endogenous signals, nitrate is also known to be a potent inhibitor of nodulation. Homology between *LjNIN* and the nitrogen-regulated Mid proteins of Chlamydomonas raises the possibility that NIN plays a role in nitrogen sensing. Our results indicate that ENOD11 expression is regulated by nitrate in *Mtnin-1*, suggesting that if NIN is involved in regulating nodule number, then it is unlikely to do so at the point that integrates plant nitrogen status.

**How Does NIN Function?**

Taken together, our results demonstrate a positive regulatory role for NIN in both nodule organogenesis and bacterial entry. The data also suggest a possible negative regulatory role for NIN in the spatial pattern of NF-specific gene expression. Clues to how NIN might accomplish both positive and negative regulation of nodulation may be gleaned from a previous suggestion (Schauser et al., 1999) that NIN may share functional homology with proteins, like the Notch receptor (Schweisguth, 2004), that undergo regulated intramembrane proteolysis (Brown et al., 2000). This proteolytic mechanism can generate both cytosolic and extracellular peptides from a single precursor. In Notch signaling, translocation of the cytosolic peptide to the nucleus coregulates Notch target genes. The extracellular peptide(s) generated in the process can also have signaling function. In *Enterococcus faecalis*, a two-step proteolytic mechanism releases a small extracellular peptide that functions as a potent pheromone, inducing mating-specific gene expression in other enterococci (Dunny and Leonard, 1997). If NIN is a membrane-anchored transcriptional coactivator that undergoes this type of regulated proteolysis, then we can propose that an unknown ligand or ligand-bound coregulator stimulates the cleavage of NIN, releasing cytosolic transcriptional regulator(s) and/or extracellular peptides. We would predict that the requirement for NIN in the process of nodule morphogenesis and bacterial invasion is the consequence of transcriptional activation by a nuclear localized intracellular NIN peptide. These two processes could be differentially activated by the presence or absence in different cell

### Table II. Spontaneous nodulation in mutant backgrounds

<table>
<thead>
<tr>
<th>Composite Plant</th>
<th>Construct</th>
<th>Total No. of Plants</th>
<th>Phenotypea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nod1</td>
</tr>
<tr>
<td><em>dmi3</em></td>
<td>1-311</td>
<td>45</td>
<td>13</td>
</tr>
<tr>
<td><em>hcl</em></td>
<td>1-311</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td><em>Mtnin</em></td>
<td>1-311</td>
<td>86</td>
<td>0</td>
</tr>
</tbody>
</table>

*a*Nodulation phenotype refers to number of composite plants, not number of nodules per plant. Nodule numbers between plants was comparable for all lines tested.
types (i.e., epidermal versus cortical) of different ligands, ligand-bound coregulators, or specific transcriptional coactivators. Control of the pattern of gene expression, on the other hand, might be accomplished through repression of gene induction by a cytosolic peptide and/or proteolytic production of extracellular peptides or a NIN-generated signal.

We have presented data to demonstrate that MnNIN is required for nodule organogenesis, bacterial entry, and, possibly, the control of nodule number. Although MnNIN is essential for autoactive CCAK-induced nodule organogenesis, based on our results we conclude that MnNIN is not essential for early NF signaling. Very recently it has been shown that MnNIN may also play a significant role in NF-related cytokinin signaling. These many proposed functions raise the possibility that NIN is a key coordinator between nodule organogenesis and bacterial entry and may also integrate nutritional, hormonal, or other endogenous/exogenous signals into the nodulation process. The prediction that NIN mediates signaling through regulated intramembrane proteolysis provides a mechanism by which a single protein can give rise to many different outcomes via the production of multiple peptides or a NIN-generated signal.

MATERIALS AND METHODS

**Plant Growth and Bacterial Strains**

*Medicago truncatula* ‘Jemalong’ A17 or R108 were used as the wild-type control for all experiments involving *Mtnin-1* or *Mtnin-2*, respectively. GUS-expressing *Mtnin-*1 plants were generated by crossing the ENOD11::GUS (Journet et al., 2001) line into *Mtnin-1* and selecting plants homozygous for both (Nod/GUS) in the F2 and F3 progeny. Prior to in vitro growth, all seed were prepared by scarification with sandpaper and sterilization in bleach for 3 min followed by multiple rinses with sterile distilled water. Seeds were then imbibed for 3 to 5 h in water and germinated on damp filter paper in inverted plates for 2 to 4 d at 4°C followed by overnight incubation at room temperature. Unless otherwise indicated, plants were grown on plates of buffered nitrogen-free Nod/GUS medium (BNM) supplemented with 0.1 μM 1-α-(2-aminoethoxyvinyl)-Gly according to Oldroyd and Long (2003). Rhizobia used in this study are *Sinorhizobium meliloti* B1. Composite plants were generated according to the protocol of Boisson-Dernier et al. (2001) using the *Mtnin-2* transposon-tagged population of *Jemalong*. Primary and secondary divisions were revealed by 1 to 2 h staining in Lugol (Sigma).

**Calcium-Spiking Experiments**

Analysis of calcium spiking was performed as described by Wais et al. (2000) with slight modifications. Wild-type A17 and the *Mtnin-1* mutant were grown overnight on BNM agar containing 0.1 μM 1-α-(2-aminoethoxyvinyl)-Gly. Micropipettes were pulled from filamented capillaries on a pipette puller (model 773, Camdren Instruments). These were loaded with Oregon Green 488 1.2-bis(2-aminophenyl)ethane-N,N,N’,N’-tetraacetic acid-1-dextran 10,000 molecular weight and Texas Red-dextran 10,000 molecular weight (Invitrogen) and diluted 10-fold with current solutions. Calcium spiking was performed from currents generated from a cell amplifier (model Intra 767, World Precision Instruments) equipped with a stimulus generator made to our specifications (World Precision Instruments). Cells were analyzed on an inverted epifluorescence microscope (model TE2000, Nikon) using a monochromator (model optoscan, Cairn Research) to generate specific wavelengths of light. Images were captured with a CCD camera (model ORCA-ER) and fluorescent data analyzed using Metafoller (Metallor Devices).

**Nodulation Assays and Staining**

Bacterial inoculations were carried out with 1:50-ml dilutions in BNM of either *S. meliloti* 1021 pXLD4 or B1 grown overnight in TY medium at 28°C. For root hair curling and ENOD11::GUS analysis, plates were flooded with *S. meliloti* 1021 pXLD4 suspension. Nodulation of A17 composite plants in growth pouches was accomplished by inoculation with 1 mL B1 suspension. Infection foci and threads were visualized using an Axiohot (Zeiss) light microscope following LacZ staining. The GUS activity of ENOD11::GUS roots was visualized following 6 to 8 h incubation with the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Melford) on a Nikon SMZ1500 (Nikon) stereo-dissecting microscope. All staining for reporter-fusion expression and spot inoculations with *S. meliloti* 1021 pXLD4 suspensions was carried out according to the protocols of Oldroyd and Long (2003). Root cortical cell divisions were revealed by 1 to 2 h staining in Lugol (Sigma).

**Affymetrix and PCR Analyses**

Affymetrix hybridization and data analysis were performed as described in Mitra et al. (2004b). For each plant line, the fold change in the expression of the indicated genes is generated from a comparison of hybridization intensities between buffer (anoinoculated) and *S. meliloti* 1021 (inoculated) seedling roots. Color coding of the log2 fold change values was carried out using the MultiExperiment Viewer module of the TM4 microarray software suite (Saeed et al., 2003).

Total RNA for RT-PCR analysis was prepared from *M. truncatula* roots using the RNeasy Mini kit (QIAGEN). Residual genomic DNA was removed with RNase-free DNase (QIAGEN). From 2 μg total RNA, cDNA was generated by Superscript reverse transcriptase (GibcoBRL/Life Technologies). The *NIN*-specific primer pair used was 5'-CATTTGGCAGCTTCACACACATCA-3' and 5'-GCAATGTGGGGATTTAGAGATT-3'. Primers designed against the constitutively expressed *M. truncatula* elongation factor gene (*MtEF1a*), EF1a (5'-AGTCTCTCTTCGTGCCTAG-3') and EF1b (5'-CGATTTTCATGATGATG-3') were used in control amplifications. Genomic DNA for segregation analysis of the *Mtnin-2*:Tnt1 locus was prepared using the DNeasy Mini Kit (QIAGEN). Primers used for this analysis were NIN-3' (5'-GCCCTGACGCTCAACACACATCA-3'), NIN-4' (5'-ACACCTGCATCACGAAAAGAT-3'), and LTR (5'-TACGCTATCCTGCTGCTACA-3'). For both RT-PCR and segregation analysis, 30 cycles of PCR were carried out at 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s per cycle. Amplification products were analyzed by 0.7% (w/v) agarose gel electrophoresis.

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We are grateful to the many individuals who helped us isolate symbiotic mutants (including *Mtnin-1*) during the summer of 2003 at the John Innes

**Isolation of *Mtnin* Alleles**

The two *Mtnin* mutants were identified in separate forward genetic screens of plants co-inoculated with B1 and the mycorrhizal fungus *Glomus hoi*. The allele *Mtnin-2* was recovered from the screening of approximately 200 T2 families (12 seedlings/family) of a Tnt1 transposon-tagged population of R108. The allele *Mtnin-1* was identified during the large-scale screening of approximately 600 T2 pools (10–25 families/pool and 60 seedlings/family) of a fast-neutron generated population of Jemalong. Primary and secondary screening were carried out in environmentally controlled walk-in growth chambers at 25°C, 200 μmol/m2·s light intensity, and ambient relative humidity. Plants were grown in a 1:1 mixture of calcined clay (TerraGreen) and medium-fine grade silica sand for 4 to 6 weeks after B1 inoculation. Non-nodulating plants recovered from the primary and secondary screen were grown to seed in standard potting soil and greenhouse conditions.
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