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A Putative Ca^{2+} and Calmodulin-Dependent Protein Kinase Required for Bacterial and Fungal Symbioses

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Legumes can enter into symbiotic relationships with both nitrogen-fixing bacteria (rhizobia) and mycorrhizal fungi. Nodulation by rhizobia results from a signal transduction pathway induced in legume roots by rhizobial Nod factors. *DMI3*, a *Medicago truncatula* gene that acts immediately downstream of calcium spiking in this signaling pathway and is required for both nodulation and mycorrhizal infection, has high sequence similarity to genes encoding calcium and calmodulin-dependent protein kinases (CCaMKs). This indicates that calcium spiking is likely an essential component of the signaling cascade leading to nodule development and mycorrhizal infection, and sheds light on the biological role of plant CCaMKs.

The legume-rhizobia symbiosis fixes as much nitrogen worldwide as the chemical fertilizer industry, owing to the ability of rhizobial bacteria to induce the morphogenesis of a new plant organ, the legume root nodule, in which they fix nitrogen. Rhizobial signals, the lipochito-oligosaccharidic Nod factors, initiate symbiotic responses on the roots of legume hosts and are required for recognition, controlled infection, and nodule formation (*1*–*4*). These molecules induce a variety of responses, including rapid calcium influx and calcium spiking in root hair cells, specific gene induction, alterations in epidermal cell morphology, and cortical cell mitosis (*4*).

Genetic studies in the model legumes *Medicago truncatula* and *Lotus japonicus* have led to the identification of genes involved in the perception and transduction of Nod factors. Two types of transmembrane receptor–like serine/threonine kinases with putative extracellular regions containing LysM domains (LysM-RLKs), required for Nod factor responses and rhizobial infection, are hypothesized to form heterodimeric Nod factor receptors (*5*–*7*). In *M. truncatula*, three downstream genes, *DMI1*, *DMI2*, and *DMI3*, are required for both nodulation and the formation of arbuscular mycorrhizae (the AM symbiosis). The AM symbiosis occurs in most land plants (a notable exception is the Brassicaceae family, which includes *Arabidopsis thaliana*) and involves fungi of the order Glomales (*8*–*10*). Both the fungal and bacterial symbiosis partners trigger plant host genetic programs that permit controlled and localized infection. *DMI2* encodes a leucine-rich repeat receptor–like protein kinase (NORK) (*11*). Mutants of *DMI1*, *DMI2*, and *DMI3* are blocked for most responses to Nod factors: They do not exhibit induction of root hair branching, early nodulin gene expression, and cortical cell division (*3*). However, they differ in their ability to respond to Nod factors by the induction in root hair cells of sharp oscillations in the concentration of cytoplasmic calcium (calcium spiking), which is lost in *dmi1* and *dmi2* but not in *dmi3* mutants (*12*, *13*). *DMI3* thus seems to act immediately downstream of calcium spiking.

DMI3 maps to the south arm of chromosome 8 (*14*), between the two markers SDP1 and PU01, located 1 and 2 cM, respectively, from *DMI3*. These two markers were used to isolate primary bacterial artificial chromosomes (BACs), and an 800-kb contig of BACs that spanned the *DMI3* region was assembled using a combination of chromosome walking from BAC end sequences and restriction endonuclease fingerprinting of the clones (fig. S1). Genetic markers originating from the BAC contig were developed and recombination events were used to position the *DMI3* locus to a 190-kb interval. Two BACs encompassing this region were sequenced (GenBank accession numbers AY508218 and AY508219), which allowed the identification of candidate genes on the basis of sequence homologies. Among them we noted several genes coding for transcription factors (fig. S1). A putative calcium and calmodulin-dependent protein kinase (CCaMK) detected in the sequenced region appeared as a promising candidate for *DMI3* (Fig. 1A). Using specific primers, we amplified this gene from genomic DNA of two *dmi3* mutant alleles, TRV25 and T1-5, and sequenced it. The wild-type and TRV25 allele differed by a 14–base pair (bp) deletion in the predicted kinase domain of the mutant, leading to a premature stop codon, whereas the T1-5 allele exhibited a point mutation in the kinase domain, leading to a stop codon. Identification of mutations in two independent mutant alleles together with the physical and genetic location of *DMI3* provides strong evidence that this candidate gene is *DMI3*.

To test this hypothesis, we attempted to complement the Nod^- mutation in TRV25 using the wild-type genomic sequence of the candidate gene. Because the nodulation phenotype of TRV25 depends on the root genotype (*14*), we used *Agrobacterium rhizogenes* for root transformation. Roots appearing on TRV25 seedlings after *A. rhizogenes* infection were inoculated with the rhizobial symbiont *Sinorhizobium meliloti*. Eighty percent of plants transformed by *A. rhizogenes* carrying the candidate gene developed nodules (an average of five nodules per plant) (Fig. 2). Microscopic examination of a representative sample of mature nodules revealed the presence of bacteria within the central tissue. No nodules could be detected on roots of plants transformed by *A. rhizogenes* carrying the vector (*15*). This complementation, together with the above described genetic evidence,

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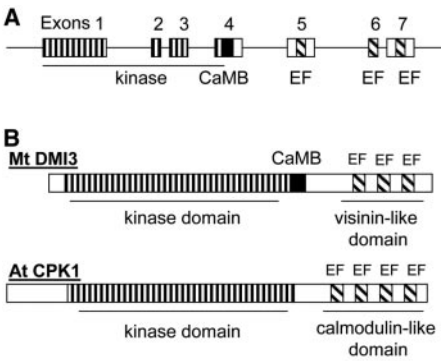

Fig. 1. Structure of the *M. truncatula DMI3* gene. (**A**) Intron-exon structure of *DMI3* and position of the predicted protein domains and motifs. (**B**) Comparison of the structural organization of two types of calcium sensor kinases: a putative CCaMK (*M. truncatula* DMI3) and a CDPK (*A. thaliana* CPK1). The kinase domain (vertical bars), calmodulin binding domain (black box), and calcium-binding EF hands (hatched boxes) are indicated.

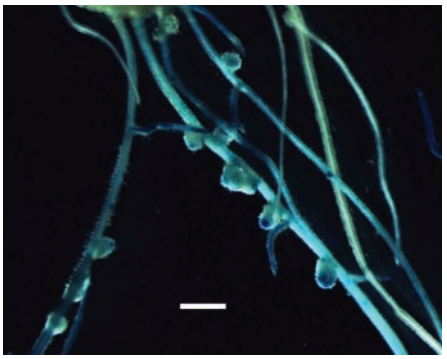

Fig. 2. Functional complementation of the *dmi3* mutation by the wild-type *DMI3* genomic sequence. Roots of seedlings of the TRV25 *dmi3* mutant, defective for nodulation, were transformed with an *Agrobacterium rhizogenes* strain, Arqua1, into which we introduced the pF5 plasmid that carries the wild-type *DMI3* gene. Ninety percent of plants exhibited transformed roots, as detected by GUS activity. A total of 45 plants transformed by pF5 were tested, and 36 of them nodulated with an average of five nodules per plant. X-Gal treatment of a representative sample of nodules revealed the presence of bacteria inside the nodules. No nodules were detected on 26 control plants transformed by the pCAMBIA2201 vector. Bar, 1 mm.

demonstrates that the candidate gene homologous to CCaMK is *DMI3*.

To further substantiate that this gene is required for nodulation and mycorrhizal infection phenotypes, we sequenced the orthologous gene in pea, a legume phylogenetically related to *Medicago* for which mutants with phenotypes and syntenic map positions similar to that of TRV25 have been described (*14*, *16*–*18*) (table S1). As *dmi3* mutants, pea mutants at the *sym9* locus are altered downstream of the calcium spiking response induced by treatment of root hairs by Nod factors and are defective for root hair curling, infection thread formation, nodulation, and mycorrhizal infection (*17*, *18*). Using a polymerase chain reaction (PCR) approach, we identified a pea gene that exhibited high overall sequence similarity (90% identity) to *M. truncatula DMI3* (GenBank accession numbers AY502067, AY502068, and AY502069). Each sequenced allele from eight *sym9* mutants of *P. sativum*, obtained in three different genetic backgrounds, exhibited single base-pair alterations in the predicted kinase domain that were likely to alter the protein function (Table 1 and fig. S2). The identification of mutations in eight alleles obtained from independent *sym9* mutants further supports a role of CCaMKs in nodulation and mycorrhizal infection. In addition, it allows the molecular characterization of a major gene controlling both nodulation and mycorrhizal infection in pea.

From the *M. truncatula* expressed sequence tag (EST) collection, we identified eight cDNAs from various libraries with sequences matching the *DMI3* genomic sequence. Alignment of these sequences defined a gene structure with seven exons. The full-length open reading frame of 1569 nucleotides encodes a protein of 523 amino acids with a predicted molecular mass of 58,600 daltons. Comparative sequence analysis indicated that DMI3 belongs to the CCaMK group of serine-threonine protein kinases, together with proteins encoded by genes previously characterized from tobacco, lily, rice, and the moss *Physcomitrella* (Figs. 1 and 3; fig. S2) (*19*, *20*). Like these proteins, DMI3 is predicted to share with the more common plant calcium-dependent protein kinases (CDPKs) an N-terminal kinase domain. However, it differs from CDPKs in the structure of its C-terminal calcium-binding regulatory domain, which has higher similarity to a mammalian visinin-like domain (with three calcium-binding EF hands) than to the calmodulin-like domain typical of CDPKs (with four EF hands) (Fig. 1B). Between the kinase and calcium-binding domains lies a calmodulin-binding domain that overlaps an autoregulatory domain. This structure allows regulation of the kinase activity by both calcium and calmodulin (*21*, *22*).

Electronic Northern analysis of the *M. truncatula* EST collection indicated that *DMI3* is preferentially expressed in root tissues (seven out of eight ESTs). This was confirmed by quantitative reverse transcription–polymerase chain reaction (RT-PCR), which showed that *DMI3* expression in roots was 10-fold as high as that in flowers, whereas no expression was detected in leaves or stems (Fig. 4). A minor up-regulation of *DMI3* was observed in nodules (Fig. 4). The expression of *DMI3* was similar to, although slightly lower than, that of *DMI2* (*NORK*), another *M. truncatula* gene required for nodulation and mycorrhizal infection.

Table 1. Summary of *M. truncatula dmi3* and *P. sativum sym9* alleles.

Genetic background	Allele	Mutation
Mt cv. Jemalong	TRV25	14-bp deletion: 198–203/stop
Mt cv. Jemalong A17 ENOD11-GUS	T1-5	CGA/TGA : R97/stop
Ps cv. Frisson	P1	CAA/TAA : Q230/stop
	P2	CAA/TAA : Q230/stop
	P3	CAA/TAA : Q230/stop
	P53	TGG/TGA : W240/stop
Ps cv. Sparkle	R72	TGT/TGA : S224/stop
Ps cv. Finale	DK6	CTT/C-T : L188/stop
	DK9	GGG/AGG : G202/R
	DK22	TCT/TTT : S24/F

On the basis of sequence homologies, we have identified DMI3 as a member of the small plant family of CCaMKs. DMI3 has high sequence similarity to rice, tobacco, and lily CCaMKs (73.5% identity between *Medicago* and lily CCaMKs). The latter protein has been studied in great detail at the biochemical level (*19*, *21*, *23*–*25*) and shown to undergo two steps of calcium regulation. First, interaction of Ca^{2+} with the C-terminal EF hands results in autophosphorylation of CCaMK, which leads to increased affinity for calmodulin. In a second step, Ca^{2+}/CaM binds to CCaMK, which allows substrate phosphorylation. In addition, autophosphorylation of CCaMK results in a time-dependent loss of enzyme activity (*25*). The structural features of DMI3 suggest that, like CCaMK, it might sense calcium directly through the three calcium-binding EF hands, and indirectly by binding a calcium-activated calmodulin. DMI3 might thus recognize complex calcium "signatures" such as sharp oscillations of cytoplasmic Ca^{2+} concentration. Proteins able to decode calcium oscillations have not yet been described in plants, but in animal cells Ca^{2+}/calmodulin-dependent protein kinases II can be activated in a Ca^{2+}-spike frequency-dependent manner (*26*). This property depends on their multimeric structure and ability to autophosphorylate (*26*).

Modulation of intracellular calcium concentration is associated with Nod factor signaling. Nod factors elicit two separable calcium responses in *M. truncatula* root hair cells: a rapid calcium influx that occurs within minutes, followed by the induction of calcium spiking about 10 min after application (*27*). Because these calcium responses are absent in different mutants unable to associate with rhizobia, they have been proposed to be part of the symbiotic signaling pathway. However, until now it has not been possible to rule out the possi-

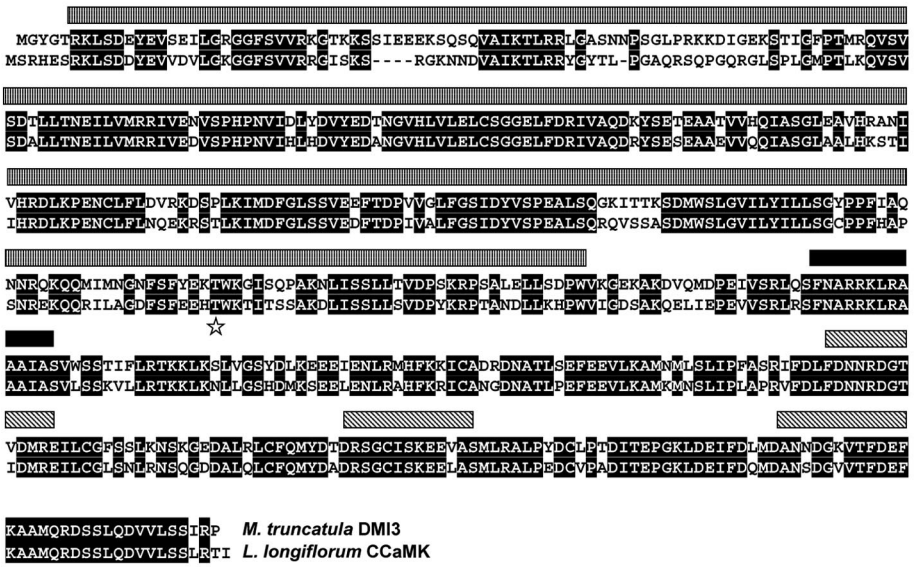

Fig. 3. Comparison of *M. truncatula* DMI3 (GenBank accession number AY502066) to the previously characterized *Lilium longiflorum* CCaMK (GenBank accession number U24188) (*19*). Conserved amino acids are shaded black. The serine/threonine kinase domain (vertical bars), calmodulin-binding site (black box), calcium-binding EF hand motifs (hatched bars), and autophosphorylation site (star) of CCaMK are indicated. DMI3 also has high sequence similarity to rice (GenBank accession number AK070533), tobacco (GenBank accession number AF087813), and *Physcomitrella* (GenBank accession number AY155462) putative CCaMKs (fig. S2).

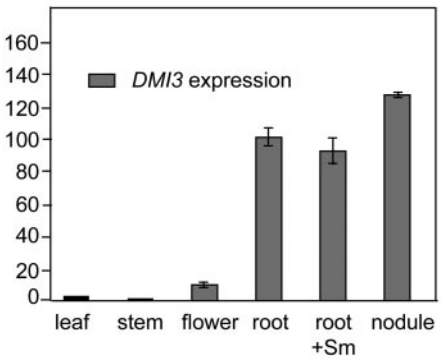

Fig. 4. Quantification of *DMI3* mRNA levels in leaves, stems, flowers, roots, roots 48 hours after inoculation with *S. meliloti*, and root nodules. Relative transcript abundance was determined by quantitative RT-PCR and normalized against *MtACTIN2* (*MtACT2*), which is constitutively expressed in all tissues tested.

bility that calcium responses are a side branch of the main Nod factor signaling pathway (*12*). The identification of DMI3 as a putative calcium-sensitive effector protein now confirms the role of calcium as an integral part of the Nod factor transduction pathway. Furthermore, mutants of *DMI1* and *DMI2* (*NORK*) genes, which are unable to form nodules and mycorrhizae (*3*), are defective for most responses to Nod factors, including the calcium spiking response, but are still able to generate the rapid calcium influx response (*12*, *27*). These results suggest that the calcium spiking, and not the rapid calcium influx, is the calcium signature recognized by DMI3, the role of which would be to translate this signal to various cellular components controlling nodulation and mycorrhizal infection responses. Future characterization of a calmodulin isoform that interacts with DMI3, together with the identification of targets of DMI3 kinase activity, should help to decipher the precise role of DMI3 in the Nod factor signaling pathway. In particular, although it is required for Nod factor activity, DMI3 is also proposed to be involved in negative regulation of this pathway (*28*). The requirement of DMI3 for mycorrhizal infection suggests that a signal transduction pathway with calcium as a second messenger controls the establishment of this fungal symbiosis. In this case, however, the nature of the signal(s) involved—putative Myc factors—is unknown, as is the type of calcium response(s) induced by this signal.

Little is known about the biological role of CCaMKs in plants. The preferential expression of the lily, tobacco, and rice CCaMKs in developing anthers and root tips (*24*, *29*) has led to the suggestion that they could play a role in mitosis and meiosis (*23*). Here, using mutants in both *M. truncatula* and *P. sativum*, we provide evidence for the role of a CCaMK in the signaling pathway leading to mycorrhizal infection and in the developmental process of nodulation.

Members of the CCaMK group have been described in a number of plants, ranging from the moss *Physcomitrella* to higher plants, both monocotyledonous and dicotyledonous. This type of calcium-sensitive effector protein, which seems to be restricted to plants, is thus likely to be of ancient origin. This conclusion fits with the hypothesis of an ancient origin for the widespread arbuscular mycorrhizal symbiosis, which could have accompanied colonization of the land by plants more than 400 million years ago (*8–10*). We hypothesize that a CCaMK was used by mycorrhized plants to interpret a complex calcium signature elicited in response to a mycorrhizal signal. Interestingly, the CCaMK family has no known member in the sequenced genome of *A. thaliana*, a plant that, like most members of the Brassicaceae family, is unable to establish symbiosis with mycorrhizal fungi (*20*, *23*).

In the legume family, genes involved in the signaling pathway of the mycorrhizal symbiosis that are homologous to *DMI1*, *DMI2*, and *DMI3* could have been recruited later in evolution (~70 million years ago), for establishing the signaling machinery of the rhizobial symbiosis. To determine whether in legumes DMI3 is interpreting the same calcium signature (a calcium spiking with defined frequency and amplitude) for both types of endosymbioses, or is recognizing two different calcium messages and informing the host cell of the presence of either the rhizobial or the mycorrhizal symbiont, is an exciting challenge for the future.

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Medicago truncatula DMI1 Required for Bacterial and Fungal Symbioses in Legumes

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Legumes form symbiotic associations with both mycorrhizal fungi and nitrogen-fixing soil bacteria called rhizobia. Several of the plant genes required for transduction of rhizobial signals, the Nod factors, are also necessary for mycorrhizal symbiosis. Here, we describe the cloning and characterization of one such gene from the legume *Medicago truncatula*. The *DMI1* (does not make infections) gene encodes a novel protein with low global similarity to a ligand-gated cation channel domain of archaea. The protein is highly conserved in angiosperms and ancestral to land plants. We suggest that DMI1 represents an ancient plant-specific innovation, potentially enabling mycorrhizal associations.

Mycorrhizal and rhizobial associations represent the two most important symbiotic relations between higher plants and microorganisms, providing access to otherwise limiting supplies of phosphate and nitrogen, respectively. Although better characterized at a molecular-genetic level, nitrogen fixation is the more recent of the two associations, primarily restricted to members of the Legume family and their close relatives (*1*). Mycorrhizal associations are considered to be ancestral to the lineage of land plants (*2*).

The initiation of symbiosis between leguminous plants and nitrogen-fixing bacteria is tightly controlled, with specificity determined by a molecular dialogue between the two partners. Legume roots exude metabolites, principally flavonoids, that induce the synthesis of lipo-chitooligosaccharidic signals (called "Nod factors") in the bacterial symbiont (*3*). Nod factors, in turn, elicit plant responses that culminate in infection by rhizobia and the development of the root nodule. Extremely low concentrations (10^{-9} to 10^{-12} M) of purified Nod factors trigger many of the same responses in the roots of legume hosts that are induced by symbiotic rhizobia, including specific ion fluxes, gene expression, and cell division (*4*). Genetic and molecular analyses in the model legumes *Medicago truncatula* and *Lotus japonicus* have identified multiple genes that are required for nodule development and also for early Nod factor responses (*5*–*12*). Phenotypic analyses have placed these genes into a hierarchy. *NFP* and *NFR1/NFR5* reside at the top of the cascade, as mutants of these genes lack all known responses to Nod factor. *DMI1* and *DMI2* genes are necessary for the induction of calcium spiking in root hairs and appear to function upstream of *DMI3*, *NSP1*, and *NSP2* (*13*). *DMI1*, *DMI2*, and *DMI3* mutants are also blocked for mycorrhizal symbiosis (Nod$^-$ Myc$^-$ mutants), which demonstrates that the signaling pathways of nodulation and mycorrhization share common components (*5*, *14*). *DMI2* encodes a receptor-like kinase homolog with leucine-rich repeats (*8*). The cloning of *DMI1* reported here, and an accompanying manuscript by Lévy *et al*. (*15*) that reports the cloning of *DMI3*, further characterize the known conserved elements for rhizobial and mycorrhizal associations.

DMI1 is located at one extremity of *M. truncatula* chromosome 2 (*16*). Genetic marker 1N1R, which defined the telomere end of linkage group 2 and was invariantly linked to the *DMI1* locus in an F_2 population of 499 Nod$^-$ individuals, was used to seed a chromosome walk toward *DMI1*. In parallel to physical mapping, we used knowledge of chromosomal synteny between *M. truncatula* and *M. sativa* (*17*) to systematically position new genetic markers between 1N1R and the telomere. Genetic mapping of 1N1R in *M. sativa* revealed a single genetic marker, U212D, that was on the telomeric side of 1N1R in both species (Fig. 1A). Using U212D as a probe, we identified additional bacterial artificial chromosome (BAC) clones of *M. truncatula* and completed a 1.2-Mb contig extending from genetic marker 88P13S to the adjacent telomere of chromosome 2 (Fig. 1).

Table 1. Summary of *M. truncatula dmi1* alleles. EMS, ethylmethane sulfonate; FNB, fast neutron bombardment.

Mutagenesis	*DMI1* mutant	Nature of mutations
EMS	B129	G1068A point mutation that results in a premature stop codon truncating the protein at amino acid position 356.
EMS	C71 (domi)	G1264A point mutation that occurs at the 5′ splice site of the third intron and that causes a missplicing event.
EMS	Y6	C913T point mutation that creates a premature stop codon truncating the protein at amino acid position 305.
FNB	GY15-3F-4	Large deletion of all the 5′ of the gene and the promoter.
FNB	GY15-1B-5.1	Large deletion of all the 5′ of the gene and the promoter.

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