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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).

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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.

Genotyping of >1500 individuals identified a single recombination event that delimited *DMI1* to a 550–kilobase pair (kbp) interval between genetic marker 57N18S and the telomere. The observation of suppressed recombination in the subtelomeric region of chromosome 2 prompted us to abandon further genetic analyses in favor of a candidate gene approach to identify the gene. We sequenced a minimum tiling path composed of five BAC clones (~550 kb in length) using a BAC sublibrary shotgun strategy. FGENESH (*18*) predicted 85 genes in the sequenced region, roughly half of which were supported by unigene assignments in the *M. truncatula* EST data. Oligonucleotide primers were designed for candidate genes, and the polymerase chain reaction was used to amplify both genomic DNA and cDNA isolated from independent *DMI1* alleles, including three ethylmethane sulfonate and two fast neutron bombardment mutants. A single unknown protein gene on BAC mth2-54A24 was altered in each of five independent alleles (Table 1), and the mutations were predicted to strongly affect the transcript or the conceptually translated protein structure. The ability of a wild-type *DMI1* transgene to complement the Nod$^-$ phenotype of *DMI1* alleles C71 and Y6 (Fig. 2) confirmed the identity of this gene as *DMI1*.

A full-length *DMI1* cDNA cloned from *M. truncatula* root mRNA encodes a 2649-bp open reading frame with a deduced protein of 883 amino acids. Predicted features of the protein include four transmembrane domains, the first two of which encompass a putative leucine zipper (LZ) motif, and a proline-rich domain that could facilitate protein–protein interactions (*19*). Other than these general features, the DMI1 protein lacks even moderate similarity to functionally characterized proteins or protein subdomains in plants, although the deduced protein is conserved throughout most lineages of land plants that have been sampled by EST and genome sequencing projects.

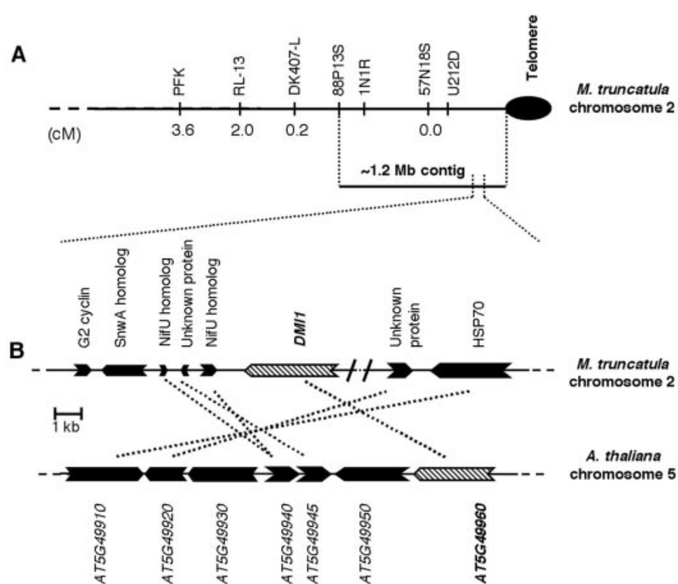


Fig. 1. Defining the *DMI1* locus. (**A**) Initial genetic mapping of the *DMI1* region indicated that the gene is located between the 88P13S marker and the telomere. We constructed a 1.2-Mb contig covering this region by means of BAC fingerprinting. From a new population consisting of 1500 individuals, we localized *DMI1* between the 57N18S marker and the telomere. We sequenced and annotated a minimum tiling path of five BAC clones covering this region. (**B**) The *Arabidopsis* and *M. truncatula* homologs, but not their rice counterpart (not shown on figure), reside in a region of conserved genome microsynteny, presumably indicative of the ancestral chromosomal context.

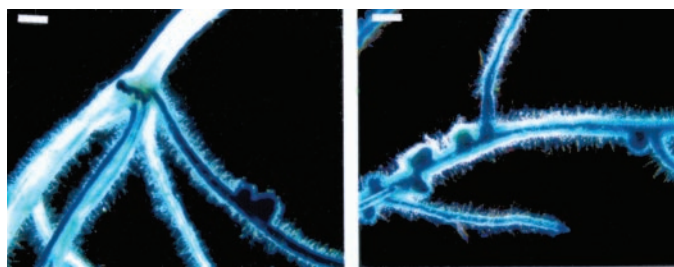


Fig. 2. Complementation of the *DMI1* phenotype by using *A. rhizogenes*–based transformation (*25*). Both panels show mutant *dmi1* roots complemented by the *DMI1* wild-type transgene. The full-length wild-type *DMI1* cDNA was amplified and cloned into the pCR2.1 vector (Invitrogen). Of the promoter sequence, 1.6 kb was also amplified from BAC mth2-54A24 and cloned upstream the cDNA. This construct was introduced into the pCAMBIA-1303 binary vector (clone pCAMBIA-DMI1). *dmi1* and wild-type roots were transformed with either pCAMBIA-DMI1 or pCAMBIA-1303 vector alone and inoculated with *Sinorhizobium meliloti*, then nodulation was scored visually after 15 days. Constitutively expressed *uidA* was used as a reporter to identify transformed tissues based on GUS staining. Among more than 80 independent *dmi1* transformants, only those transformed by the *DMI1* transgene were nodulated by *S. meliloti*. Transformation of wild-type plants with either *DMI1* or control constructs had no effect on nodulation. Scale bar, 1 mm.

DMI1 is constitutively expressed in roots, with lower levels of transcript detected in pods, flowers, leaves, and stems (Fig. 3A). Treatments known to activate expression of plant "nodulin genes," including inoculation with the compatible symbiotic bacterium *Sinorhizobium meliloti* (Fig. 3B) or 10^{-9} M cognate Nod factors, had no effect on *DMI1* transcript levels.

The signaling pathway leading to nodulation is highly conserved across the characterized legume species (*8–12*), and thus, diverse legume species are expected to contain orthologous signaling components. Southern blot experiments indicate that *DMI1* is a gene with a low copy number *in M. truncatula* and that homologs are present in many other leguminous plants, including *Medicago sativa*, *Melilotus alba*, *Vicia hirsuta*, *Lotus japonicus*, *Sesbania*, *Cassia*, *Trifolium*, *Desmodium*, *Vigna*, *Macroptilium*, and *Vigna radiata*. BLASTN analyses identified similar expressed sequence tags (ESTs) in soybean and *L. japonicus*, which indicated that the sequence homologs in these species represent expressed genes. The *sym8* mutations of pea (*Pisum sativum*), which are phenotypically similar to *DMI1* mutants of *Medicago truncatula*, map to a syntenic location between the two genomes (*16*), consistent with the possible identity of *sym8* as pea *DMI1*.

BLASTX and TBLASTN searches of the National Center for Biotechnology Information (NCBI) nonredundant and EST databases predicted similar proteins in 28 monocot and dicot species, as well as a more distant homolog in the nonvascular plant *Physcomitrella patens*. Related sequences were not identified in the fully sequenced genomes of animals, fungi, or green algae. Relatively close homologs were identified in only two eubacterial genera, namely the nitrogen-fixing symbiont *Mesorhizobium loti* and *Streptomyces* spp. Homologous genes were not evident in any other sequenced bacterial genome, including close relatives of *M. loti* such as *Sinorhizobium meliloti* and *Agrobacterium tumefaciens*. We used phylogenetic reconstruction based on parsimony analysis to infer the evolutionary history of this gene family. Analysis of several full-length deduced proteins from *M. truncatula*, *Arabidopsis*, rice, and bacterial species yielded three well-supported clades, corresponding to two paralogous groups of plant genes and a single group of bacterial genes (Fig. 4B).

The complete sequence of *DMI1* putative orthologous genes was available for the *A. thaliana* At5 g49960 and rice BAB64102 genes, corresponding to single-copy genes on chromosomes 5 and 1, respectively. The *Arabidopsis* and *M. truncatula* loci, but not their

rice counterpart, reside in a region of conserved genome microsynteny, presumably indicative of the ancestral chromosomal context (Fig. 1B). The *A. thaliana* and rice predicted proteins share 80% identity with *DMI1*, with the highest similarity found in the C-terminal region. Although ESTs corresponding to the *A. thaliana* gene were absent from the public databases, semiquantitative reverse transcription polymerase chain reaction (RT-PCR) analysis indicates that the *Arabidopsis* gene is expressed in roots but not in leaves (Fig. 3A).

Comparison of the deduced partial protein *Physcomitrella patens* with either group of angiosperm paralogs indicates that all three groups are roughly equally diverged from one another (i.e., 26 to 28% identity and 53 to 56% similarity), consistent with an origin in the nonvascular plants. Taken together with the absence of homologs in the fungal, animal, and green algal lineages, these results suggest that the DMI1 protein represents a plant-specific innovation that potentially arose near the base of the land plant lineage. The fact that the *M. loti* and *Streptomyces* sequences also branch from the base of the DMI1 lineage, near the inferred plant duplication, is suggestive of horizontal transfer from an ancient plant genome or genomes to a limited number of bacterial species.

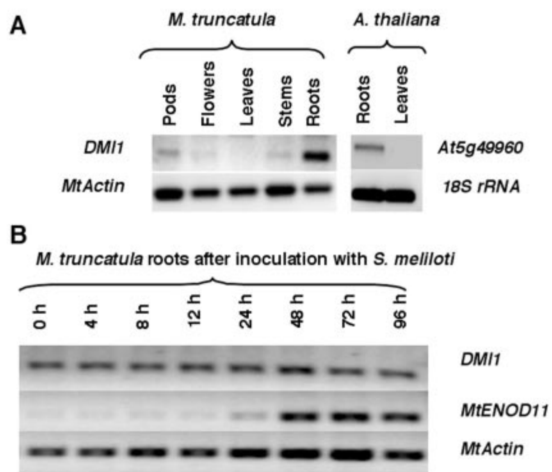
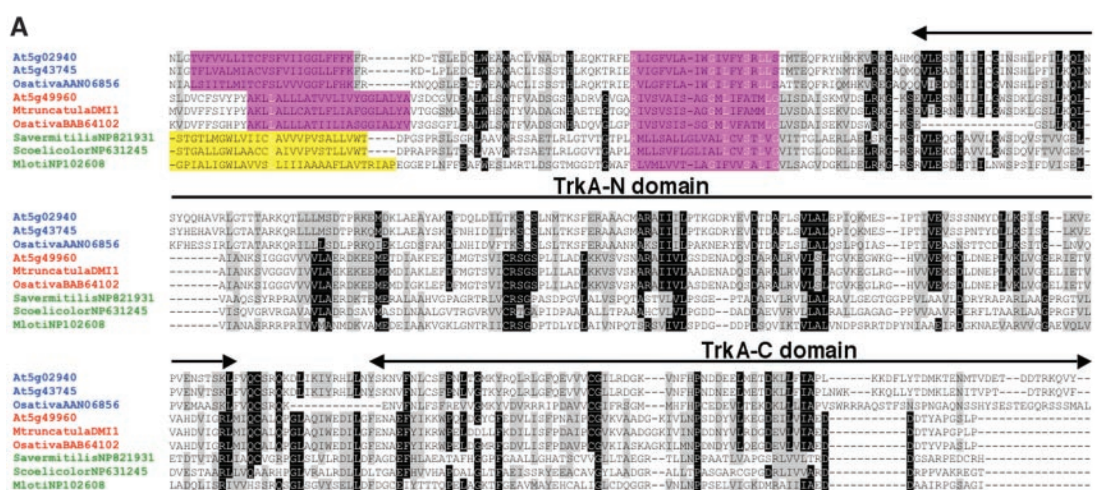


Fig. 3. Expression profiles of *DMI1* in *M. truncatula* and *A. thaliana*. (**A**) *M. truncatula DMI1* and *A. thaliana At5 g49960* cDNA analysis by semiquantitative RT-PCR in different tissues. (**B**) *M. truncatula DMI1* expression is not affected by inoculation with wild-type *S. meliloti* as compared with the nodulation-associated control gene *ENOD11* (*25*). Purified total RNA was normalized with the use of the Ribogreen RNA quantification kit (Molecular Probes) and analyzed using the PowerScript RT-PCR kit (Clontech Laboratories).

DMI1 is predicted to act in genetic proximity to two additional *M. truncatula* genes, *DMI2* and *DMI3*, which together make up the set of known genes common to both mycorrhizal and rhizobial symbioses (*5*). All three *DMI* genes are implicated in transduction of the Nod factor signal; DMI1 and DMI2 are required for Nod factor–induced calcium spiking, which by analogy to animal systems is predicted to have a causal role in the Nod factor signal transduction cascade. Despite the fact that DMI1 homologs have been identified in the genomes of many plant species, DMI1 represents the first member of this gene family with an assigned phenotype and verified gene structure. As with the NORK receptor kinase (*DMI2*) (*8*), *DMI1* is predicted to be a membrane-spanning protein and may participate in the formation of a receptor-complex for symbiotic signals. The fact that proline-rich and



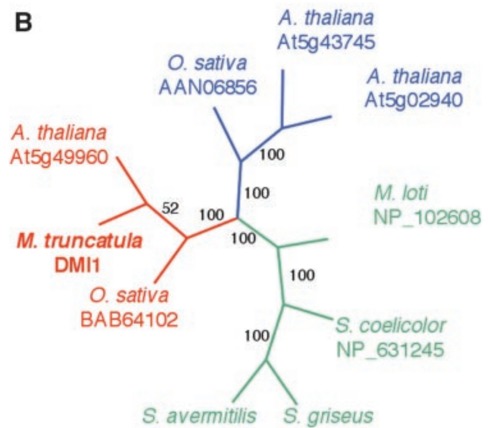


Fig. 4. Alignment and phylogenetic analysis of DMI1 protein homologs. (**A**) Partial alignment of DMI1 homologous proteins sequences by means of ClustalW with default parameters in the region sharing homologies with cation channels. This region contains the TrkA homologous region and also the third and fourth transmembrane domains of the DMI1 orthologs. *A. thaliana* At5 g49960 and rice BAB64102 DMI1 orthologs share about 80% similarity with the *M. truncatula* protein. *A. thaliana* At5 g02940 and rice AAN06856 proteins are 73% similar and share ~37% similarity with the orthologous grouping of DMI1-related proteins in *M. truncatula*, *Arabidopsis*, and rice. *Mesorhizobium loti* NP 102608 and the *Streptomyces* proteins represent a third clade of proteins, with 60% similarity between bacterial homologs and ~37% similarity to the DMI1 orthologs. Transmembrane domains (purple) were predicted using THMM, TMpred and TopPred 2 software programs (*26*). The presence of signal peptides (yellow) was assessed by using SignalP V2.0 software (*27*). The region homologous to cation channels was determined according to Anantharaman *et al*. (*21*) and annotated according to Jiang *et al*. (*22*). (**B**) Unrooted phylogenetic tree of DMI1 homologs. Full-length protein sequences were aligned using ClustalW with default parameters. The branch-and-bound parsimony method was used to infer phylogenetic relations and to identify two paralogous groups of plant genes and a single group of bacterial orthologs. Percentage bootstrap support is given to the side of each branch.

leucine zipper domains often participate in protein-protein interactions supports the idea of such a protein complex, but these motifs are present in the N-terminus of DMI1, a region of the protein that is poorly conserved among the various homologs. The DUF1012 protein family comprises the homologs of *Arabidopsis*, rice, *Mesorhizobium*, and *Streptomyces* proteins in the Pfam database (*20*), without functional assignment. The DMI1 DUF1012 domain overlaps substantially with a region of low, but global, similarity to the NAD-binding TrkA domain of certain bacterial potassium channels (*21*). In fact, the central portion of DMI1, from the third predicted transmembrane domain through the entire TrkA homology region, is predicted to share distant homology with multimeric, ligand-gated potassium channels of archaea (*22*) (Fig. 4A). DMI1 is conserved throughout both the pore and ring domains that are thought to constitute the functional cation channel (*22*) but is degenerate in the filter region, located between transmembrane domains 3 and 4, where structural modifications have been correlated with altered cation specificity (*23*). Whether DMI1 and its close phylogenetic relatives in plants and bacteria might also function as ligand-gated cation channels remains uncertain, but the possibility is particularly intriguing given the genetic requirement for DMI1 function in Nod factor–induced calcium oscillations within *Medicago* root hair cells.

Identifying DMI1-interacting proteins should expand our understanding of the role of the *DMI1* protein in symbiotic interactions. Candidate proteins to interact with DMI1 include LYK3 and 4 and NFR1 and 5 (*10*, *11*, *12*, *24*), NFP (*6*), DMI2 (*8*, *9*), and DMI3 (*5*).

About 80% of terrestrial plants establish mycorrhizal symbioses. These beneficial plant-fungal associations act to expand the effective root-soil interface; in addition to their importance in natural and agricultural ecosystems, they may have facilitated the colonization of land by plants by increasing access to vital soil nutrients in the primitive rootlike structures of nonvascular plants (*2*). The biological and molecular function of this group of proteins is likely to be conserved because of the high degree of conservation for *DMI1* orthologs among angiosperms (>80% similarity among rice, *Arabidopsis* and *Medicago* proteins) and because only a single gene with high similarity to *DMI1* is present in the fully sequenced genomes of rice and *Arabidopsis*. Other genes identified for Nod factor signaling (i.e., *LYK* homologs, *DMI2*) are less well conserved among angiosperms, and some may be members of large, fast evolving gene families. It is widely anticipated that nodulation in legumes arose from an ancient, conserved pathway for mycorrhizal associations, and we suggest that DMI1 is a pivotal component of this pathway.

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A Genome-Wide Screen Identifies Genes Required for Centromeric Cohesion

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During meiosis, two chromosome segregation phases follow a single round of DNA replication. We identified factors required to establish this specialized cell cycle by examining meiotic chromosome segregation in a collection of yeast strains lacking all nonessential genes. This analysis revealed Sgo1, Chl4, and Iml3 to be important for retaining centromeric cohesin until the onset of anaphase II. Consistent with this role, Sgo1 localizes to centromeric regions but dissociates at the onset of anaphase II. The screen described here provides a comprehensive analysis of the genes required for the meiotic cell cycle and identifies three factors important for the stepwise loss of sister chromatid cohesion.

In eukaryotes, the meiotic cell cycle allows for the formation of haploid gametes. This cycle is characterized by two rounds of chromosome segregation after a single DNA replication phase. During the first segregation phase, meiosis I, homologous chromosomes are separated. During meiosis II, sister chromatids separate. Three events must take place for meiosis to succeed [reviewed in (*1*)]: (i) One reciprocal recombination event (crossover) must occur between homologous chromosomes to physically link them, which is essential for accurate segregation during anaphase I. (ii) The kinetochores of sister chromatids must attach to microtubules emanating from the same spindle pole (co-orientation) during meiosis I to ensure sister chromatid cosegregation, but must attach to microtubules from opposite spindle poles (bi-orientation) for sister chromatids to separate during meiosis II (*2*). (iii) Cohesion between sister chromatids must be lost in a stepwise manner. Cohesion loss from chromosome arms is necessary for the resolution of chiasmata, the physical manifestation of crossover events, which is a prerequisite for homolog disjunction during meiosis I [reviewed in (*1*)]. Cohesion at centromeres, however, must be maintained during meiosis I for the faithful segregation of sister chromatids during meiosis II.

To identify genes required for meiotic cell cycle progression, we used a collection of *Saccharomyces cerevisiae* strains in which individual genes were deleted (*3*). Diploids were constructed that were homozygous for a particular deletion and carried green fluorescent protein arrays (GFP dots) on both copies of chromosome

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