Expression of the *Medicago truncatula* DMI2 Gene Suggests Roles of the Symbiotic Nodulation Receptor Kinase in Nodules and During Early Nodule Development

Anne Bersoult,¹ Sylvie Camut,¹ Ariana Perhald,² Attila Kereszt,² György B. Kiss,² and Julie V. Cullimore¹

¹Laboratoire des Interactions Plantes-Microorganismes, CNRS-INRA, BP52627, 31326 Castanet-Tolosan Cedex, France;

²Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, H-6701 Szeged, PO Box 521, Hungary

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The *Medicago truncatula* DMI2 gene encodes a receptorlike kinase required for establishing root endosymbioses. The DMI2 gene was shown to be expressed much more highly in roots and nodules than in leaves and stems. In roots, its expression was not altered by nitrogen starvation or treatment with lipochitooligosaccharidic Nod factors. Moreover, the DMI2 mRNA abundance in roots of the *nfp*, *dmi1*, *dmi3*, *nsp1*, *nsp2*, and *hcl* symbiotic mutants was similar to the wild type, whereas lower levels in some *dmi2* mutants could be explained by regulation by the nonsense-mediated decay, RNA surveillance mechanism. Using *pDMI2::GUS* fusions, the expression of DMI2 in roots appeared to be localized primarily in the cortical and epidermal cells of the younger, lateral roots and was not observed in the root apices. Following inoculation with *Sinorhizobium meliloti*, the DMI2 gene was induced in the nodule primordia, before penetration by the infection threads. No increased expression was seen in lateral-root primordia. In nodules, expression was observed primarily in a few cell layers of the pre-infection zone. These results are consistent with the DMI2 gene mediating Nod factor perception and transduction leading to rhizobial infection, not only in root epidermal cells but also during nodule development.

Additional keywords: legume–rhizobia (LR) symbiosis.

The legume–rhizobia (LR) symbiosis is characterized by the production of nodules on the roots of the plant in which the bacteria fix dinitrogen in exchange for nutrients derived essentially from the plants' ability to fix carbon dioxide. This mutualistic symbiosis is important not only because of its tremendous agricultural and ecological value but also because of its interest for studies of plant organogenesis, signaling, and plant–microbe interactions (Albrecht et al. 1999; Kistner and Parniske 2002; Limpens and Bisseling 2003).

The LR symbiosis generally is initiated in a region close to the root tips where root hairs are developing, called the susceptible zone (Caetano-Anollés and Gresshoff 1991). Effective nodulation requires two interlinked processes, nodule organogenesis and infection. Nodule organogenesis is initiated through cell divisions in the cortex and pericycle which lead to the

formation of the nodule primordium. This process is initiated earlier than infection, which generally occurs through curled root hairs followed by the production of infection threads which route the bacteria toward the developing primordium. Infection of the primordium leads to formation of a meristem from which the nodule develops. The nodule is a veritable organ with a defined ontogeny and function. Genera such as *Medicago*, *Trifolium*, and *Pisum* form indeterminate nodules in which the meristem is apical and persistent, leading to a gradation of cells of increasing age and developmental status from the nodule meristem to the root. Nodulation is associated with the expression of specific genes that are induced in nodules compared with roots (nodulin genes). Studies of early nodulin (*ENOD*) genes have shown that they can be used as markers of both early stages of nodulation and different developmental stages in the nodule (Albrecht et al. 1999).

Genetic analysis has revealed that the establishment of the LR symbiosis depends on the production of a specific rhizobial lipochitooligosaccharide signal (the Nod factor), whose synthesis is specified by the bacterial *nod* genes (Dénarié et al. 1992). The Nod factors are perceived and transduced by a specific plant signal transduction pathway (Limpens and Bisseling 2003; Oldroyd and Downie 2004). Some of the plant genes involved (synonymously called the “common SYM” or the “does not make infection” [*DMI*] genes) also are required for establishment of the symbiosis with arbuscular mycorrhizal fungi (the AM symbiosis). In *Medicago truncatula*, three *DMI* genes have been described: *DMI1*, *DMI2*, and *DMI3* (Catoira et al. 2000; Wais et al. 2000). The *DMI2* gene and its orthologs (*NORK* of *M. sativa* and *SYM10* of *Lotus japonicus*) have been shown to encode receptorlike kinases (RLKs) with large extracellular regions containing three leucine-rich repeats (Endre et al. 2002; Stracke et al. 2002). Because *dmi* mutants are able to respond to Nod factors in terms of a rapid calcium flux and root hair deformation (Catoira et al. 2000; Shaw and Long 2003), it appears that Nod factor perception occurs upstream of the *DMI2* receptor. Recently, genes required for these early responses have been identified (*NFP* of *M. truncatula*, *SYM10* of *Pisum sativum*, *NFR1*, and *NFR5* of *L. japonicus*) and shown to encode lysinelike motif (LysM)-RLKs, which are likely to be Nod factor-binding receptors (Ben Amor et al. 2003; Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003; Walker et al. 2000). Activation of the LysM-RLKs is postulated to activate the *DMI2* receptor and the common

SYM pathway. However, the *NFP* and *SYM10* genes are not required for establishment of the AM symbiosis; therefore, it appears that mycorrhizal fungi must use a different mechanism to activate DMI2.

From this genetic analysis, it is clear that the DMI2 receptor plays a key and pivotal role in establishing root endosymbiosis with two different symbionts—a bacterium and a fungus. In the rhizobial symbiosis, *DMI2* is required for Nod factor responses in the epidermal cells (calcium spiking and induction of *MtENOD11*) (Catoira et al. 2000; Vernoud et al. 2000; Wais et al. 2000). It also is required for elicitation of cell division in cortical cells and for Nod factor-induced *ENOD40* expression in the pericycle and cortex (Catoira et al. 2000). However, it is not known whether these responses in the internal tissues are cell autonomous events or depend on signals elicited by DMI2-dependent signal transduction in the epidermal cells. To date, the only work on the expression of *DMI2* or its potential orthologs is the report that the *SYMRK* gene is expressed constitutively in roots and is not induced by Nod factors or rhizobia (Stracke et al. 2002). In this article, we have studied the expression of the *M. truncatula* *DMI2* gene with the aim of analyzing the regulation of its expression and revealing potential roles during nodulation. This work has used fusions of the promoter region of *DMI2* with the β -glucuronidase (GUS) marker gene, a strategy which allows a sensitive mechanism for detecting the expression pattern of weakly expressed genes and which has faithfully reproduced the expression pattern of several nodulin genes in *M. truncatula* (de Billy et al. 1991; Fang and Hirsch 1998; Journet et al. 2001; Pichon et al. 1992; Vernoud et al. 1999).

Fig. 1. Regulation of expression of the *DMI2* gene. The abundance of *DMI2* mRNA was determined by Northern analysis in experiments on aeroponically grown plants. **A**, Expression during nodulation and in different organs of the plant. Whole root systems or isolated nodules were harvested at different times (h = hours, d = days) after inoculation with *Sinorhizobium meliloti* 2011; leaves and stems were isolated at 0 h. **B**, Expression in roots during nitrogen starvation. Plants grown on a nitrogen source of 5 mM ammonium nitrate (+N) were starved of nitrogen, and whole root systems were analyzed at different times (d-N). **C**, Expression in roots following addition of Nod factors. Roots of 5-day-old plants without a nitrogen source (0 h) were treated with 10^{-8} M Nod factors and whole root systems were analyzed at different times (h + NF). The abundance of mRNA for *MtENOD11* and *Histone* genes were used as controls. The RNA loading was verified by hybridization with an *rRNA* probe.

RESULTS

Analysis of DMI2 expression during nodulation and in different organs.

In order to determine whether the overall level of expression of the *DMI2* gene is regulated during nodulation, the level of *DMI2* mRNA was analyzed by Northern hybridization during a nodulation experiment. Plants were grown in aeroponic containers, starved of nitrogen for 4 days, and inoculated with *Sinorhizobium meliloti*. Whole root systems were harvested from 4 h to 14 days after inoculation and nodules were isolated at 5 and 14 days. The data on *DMI2* mRNA levels (Fig. 1A) were quantified and adjusted to equal gel loadings (using the *rRNA* signals) and compared with changes in expression of the early nodulin gene *MtENOD11* (an induced gene) and histone (a housekeeping gene). As reported in previous studies (Journet et al. 2001), *MtENOD11* was strongly induced during nodulation, with an increase in its mRNA being detectable by 51 h, and the level of its mRNA being much higher in young nodules than in roots (Fig. 1A). *DMI2* showed no such strong induction and the level of its mRNA varied by less than 30% throughout the experiment, except at 5 days, when an increase of approximately 70% was seen in both the root systems and isolated nodules. Histone showed similar changes to *DMI2* (less than 15% variation between the changes in expression of the two genes), except that it was much more highly expressed. In contrast to the underground organs, *DMI2* mRNA was much less abundant (approximately 8% of the level) in the stems and leaves of the plants. Examination of the *M. truncatula* expressed-sequence tag (EST) banks (Journet et al. 2002; Quackenbush et al. 2001) revealed 18 clones of the *DMI2* gene, 12 of them identified in root and nodule cDNA libraries and 5 in mixed tissue libraries; only 1 clearly came from a bank of aerial organs (clone GenBank BF640745 from the *M. truncatula* “local and systemic leaves following insect herbivory” library) (Quackenbush et al. 2001).

We also investigated whether the *DMI2* gene is induced by nitrogen starvation or addition of Nod factors. The level of mRNA of this gene in roots was similar (less than 30% variation) (Fig. 1B) whether the plants were grown on ammonium nitrate or starved of nitrogen for up to 5 days before inoculation. Moreover, a similar basal level of *DMI2* mRNA (less than 12% variation) was observed in roots following addition of Nod factors, whereas *MtENOD11* clearly was transiently induced by this treatment (Fig. 1C).

Cellular localization of DMI2 expression in roots and nodules.

To analyze the cell-specific expression of the *DMI2* gene in roots and nodules, constructs were made of the promoter and upstream regions of the gene fused to the GUS marker gene. Two fusions were made: *p1.2DMI2-GUS* and *p3.0DMI2-GUS*, corresponding to *DMI2* promoter and upstream regions of 1.2 and 3.0 kbp, respectively. These regions are functional because they direct complementation of *dmf2* mutants by the *DMI2* gene (Endre et al. 2002). These fusions were introduced into *Agrobacterium rhizogenes* and the strains were used to produce composite plants in which the roots, but not the shoots, are transgenic (Boisson-Dernier et al. 2001). Therefore, each plant results from a different transformation event and analysis of many plants eliminates the possibility that the observed GUS expression is due to the position of insertion of the transgene. In this article, at least 20 GUS-expressing plants were observed for each treatment or time point. The transgenic roots and nodules were analyzed histologically for GUS activity. Unless otherwise stated, results are described using the *p3.0DMI2-GUS* fusion and the *S. meliloti* 2011(pXLGD4) strain (Ardourel et al. 1994) which carries a constitutive *hemA-lacZ* fusion.

In noninoculated plants, GUS activity was observed over most of the root systems; however, noticeably, no activity was detected in the root apices (Fig. 2A and B). It was not possible to define where expression started in the roots, because the GUS activity was very low in the developing root hair zone (relative, for example, to expression from the *MtENOD11* or *12* promoters) (Journet et al. 2001; Pichon et al. 1992). However, GUS activity increased progressively in this zone and was highest in the region where the root hairs had just attained their maximal length (Fig. 2B). The older regions of the roots and, particularly, the primary roots showed lower GUS activity than the lateral roots (Fig. 2A). On root sections, most of the positive transgenic lines showed GUS staining in the cortex and also in the epidermis, including the root hairs (Fig. 2C).

In order to examine expression from the *pDMI2* promoter during nodulation, the transgenic composite plants were transferred to growth pouches. After 1 week, part of the root system was excised and analyzed histochemically for GUS activity. Sets of plants then either were inoculated with *S. meliloti* or 10^{-8} M Nod factors or mock inoculated, and GUS expression was analyzed in the remaining root system 2, 3, or 5 days later. At 2 days following rhizobial inoculation, 71% of the plants showed a clear increase in expression in the susceptible zone, which was significantly different than the number of plants reacting to the mock inoculation (Table 1). This rhizobial-induced expression generally was confined to the secondary roots and was not uniform but appeared as semi-discrete patches in the cortex (Fig. 2D). At 3 days, the increased cortical expression clearly was associated with the developing nodule primordia, whereas lateral root primordia showed basal or lower expression (Fig. 2E). At this time point, the number of plants responding to rhizobial inoculation was again significantly different than the mock inoculation, whereas addition of Nod factors did not lead to a significant change compared with this control (Table 1). At 5 days, 83% of the plants showed developing nodules emerging from the root, which showed stronger GUS expression than at the initial time point (Fig. 2F). At all three time points, the surrounding tissues of the root and the nonreactive roots showed activity similar to that of the roots harvested before inoculation (Table 1). Moreover, inoculation did not inhibit expression in the newly developing root tips and lateral roots that developed subsequent to rhizobial and Nod factor inoculation; these regions of the root expressed the *DMI2* gene at the basal level (Fig. 2F).

In order to pinpoint the localization of induced *DMI2* expression in relation to the developing infections, the bacteria in over 30 *pDMI2-GUS* plants were counterstained using Magenta-gal, due to their expression of the constitutive *hemA-lacZ* fusion. In addition, over 30 plants were assayed for GUS

expression using Magenta-gluc and the bacteria were stained with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside). Examination of these plants revealed that the youngest primordia that expressed the GUS fusion had not yet been infected (Fig. 2G), while slightly older primordia, with a limited ramification of the infection threads, showed similar, increased GUS expression in both infected and uninfected cells (Fig. 2H). Increased GUS activity was not observed specifically in cells in the epidermis or outer cortex through which the infection threads were passing.

Sectioning of some of the primordia and young, spherical nodules (assayed to measure the relative GUS activity in the different tissues) showed that the increased expression occurred throughout the internal tissues of the developing nodule, from the root stele towards the emerged apex, whereas the peripheral nodule tissues and the surrounding root tissues showed relatively little activity (Fig. 2I). At this stage, the internal tissues did not show differentiation into zones. Expression of *pDMI2-GUS* also was observed in sections of older nodules, 7 to 10 days following inoculation, in which the typical zonation of indeterminate nodules was apparent: a distal, apical, and persistent meristem (zone I) followed by zones of increasing cell age, comprising an infection zone (zone II), an interzone (II–III), and a nitrogen-fixation zone (zone III) (Vasse et al. 1990). Expression in all GUS-positive plants was localized in a small region at the distal part of the nodule (Fig. 2J). A similar localization of expression also was seen with the *p1.2DMI2-GUS* fusion, although GUS activity from the shorter promoter generally was lower (data not shown). This result suggests that the *cis*-acting elements required for the cell-specific expression must occur in the 1.2-kbp fragment. In addition the two widely used *S. meliloti* strains, 2011 or 1021 (Wais et al. 2002) induced similar patterns of expression (data not shown). Staining with 4',6-diamidino-2-phenylindole (DAPI) to reveal the nuclei (Fig. 2M) showed that the *pDMI2-GUS* expression in the apical zone of the nodule (Fig. 2K) occurred in cells proximal to the meristem. Using Nomarski microscopy to observe the infection threads (Fig. 2L), it appears that the strongest GUS activity occurred in cells distal to the main infection zone. Therefore, GUS activity, expressed from the *pDMI2* promoter, was located mainly in a region between the meristem and the main part of the infection zone. The transgenic plants showing highest GUS activity revealed expression in other regions of the nodule that were not detectable with low GUS-expressing lines. Many of these high-expressing lines showed GUS activity in the central, nitrogen-fixing tissue, and 2 of 22 plants expressed GUS activity also in or around the vasculature of both the nodule and the associated root irrigating the nodule (data not shown). In all of the GUS-positive plants, the nodule cortex showed either no or very low GUS expression. It should be

Table 1. Changes in expression of *DMI2* following treatment of roots with either *Sinorhizobium meliloti* or Nod factors^y

Treatment	Time point (days)	No. of plants tested	No. of plants responding ^z					
			Susceptible zones			Surrounding tissues		
			–	0	+	–	0	+
Mock	3	32	0	25	7	1	31	0
<i>S. meliloti</i>	2	21	0	6	15 a	0	21	0 c
	3	37	0	14	23 a	1	32	4 c
	5	31	0	5	26 a	3	28	0 c
	3	25	0	20	5 b	2	22	1 c
Nod factors	3	25	0	20	5 b	2	22	1 c

^y Part of the root system of transgenic plants expressing the *p3.0DMI2-GUS* construct was assayed histochemically for β -glucuronidase (GUS) activity and then the rest of the root system was treated either by a mock inoculation, with *S. meliloti*, or with 10^{-8} M Nod factors for 2, 3, or 5 days before re-assaying GUS expression. The GUS activity in the susceptible zones of the secondary roots in which nodules appear or in the surrounding tissues was compared with the initial time point for each plant and scored as either a decrease (–), no change (0), or increase (+) in expression.

^z a = Data significantly different than mock inoculation ($P < 0.002$), b = data not significantly different than mock inoculation ($P = 0.86$), and c = data not significantly different than mock inoculation ($P > 0.15$).

Fig. 2. Histochemical localization of *pDMI2-GUS* expression throughout various stages of nodulation in *Medicago truncatula*. β -Glucuronidase (GUS) activity was localized in transgenic plants expressing the *p3.0DMI2-GUS* fusion. **A** through **C**, Roots of nitrogen starved plants. **A**, Whole root segments of primary and secondary roots, showing GUS expression. **B**, Secondary roots showing lack of GUS expression in the root apices and increasing expression in the developing root hair zone. **C**, Section, 100 μ m thick, of a secondary root cut in the region behind the root apex, showing *pDMI2-GUS* expression in the root hairs and epidermal and cortical cells. **D** through **F**, Whole root segments of secondary roots showing expression of *pDMI2-GUS* in the developing nodule primordia (black arrows) at **D**, 2, **E**, 3, and **F**, 5 days after inoculation. The gray arrow shows a lateral-root primordium, with very low *pDMI2-GUS* expression. **G** through **H**, Whole root segments at **G**, 2 and **H**, 3 days after inoculation showing *pDMI2-GUS* expression in magenta and LacZ expression from the rhizobia in blue. Expression of *pDMI2-GUS* occurs before infection of the primordium cells and is not correlated with infection threads in the cells. **I**, Section of developing nodule at 5 days after inoculation showing *pDMI2-GUS* expression in the central, undifferentiated tissues of the developing nodule. **J**, Longitudinal section of 10-day-old nodule showing strong GUS expression in the pre-infection zone. The anatomical nodule zones are marked. **K** through **M**, Identical nodule section observed by **K**, light microscopy to show GUS activity, **L**, Nomarski microscopy to show infection threads, and **M**, dark field after diamidino-2-phenylindole (DAPI) staining to show the nuclei. The arrows are identically placed, in the meristematic region of these three images. Bars = 100 μ m, except in **A**, **B**, and **D** through **F**, where bars = 500 μ m.

noted that the intensity of the GUS staining in the preinfection zone of the nodule was always very much stronger than that associated with the nitrogen-fixing zone or the vasculature.

DMI2 mRNA levels in different mutant genotypes.

To investigate whether *DMI2* expression is regulated by known symbiotic genes, the *DMI2* mRNA levels were analyzed in roots of various symbiotic mutants (*nfp*, *dmi1*, *dmi3*, *nsp1*, *nsp2*, and *hcl*) and also in different allelic mutants of *dmi2* (TR25, TR26, P1, and R38). Genetic analysis has shown that *NFP* acts upstream; *DMI1* acts at the same level; and *DMI3*, *NSP1*, *NSP2*, and *HCL* all appear to act downstream of *DMI1/DMI2* in the Nod factor signal transduction pathway (Catoira et al. 2000, 2001; Ben Amor et al. 2003; Oldroyd and Long 2003). *DMI1*, *DMI2*, and *DMI3*, but not the other four genes, also are required for the establishment of the AM symbiosis. The level of *DMI2* mRNA measured in roots of the wild-type and most of the symbiotic mutants (Fig. 3) varied by less than 29%. However, two of the *dmi2* mutants, TR25 and TR26, contained much lower levels of *DMI2* mRNA (less than 18% of the wild type) compared with mutants P1 and R38. The size of the *DMI2* mRNA was similar in all plant lines tested.

DISCUSSION

The *DMI2* gene of *M. truncatula* was identified in screens for Nod⁻ mutants; thus, all alleles reported to date are either completely or severely restricted in nodulation. Further analysis has revealed that *dmi2* mutants are blocked in infection at the root epidermis and that these cells are defective in most Nod factor responses, including induction of some early nodulin genes and calcium spiking (Catoira et al. 2000; Wais et al. 2000). Thus, the gene plays an essential role in the early steps of Nod factor signal transduction leading to infection of the epidermal cells of the root. By studying the expression of the *DMI2* gene and fusions of the *DMI2* promoter with the *GUS* gene marker, data presented in this article suggest that *DMI2* plays also a role in the root cortex during early nodule development and in the preinfection zone of nodules.

Localization of *DMI2* expression.

Studies reported here using GUS fusions have shown that the *DMI2* gene is expressed in a very specific region of the nodules, located between the meristem and the main part of the infection zone (Fig. 2J through M). A similar, narrow zone of *DMI2* mRNA abundance recently has been shown by in situ hybridization (Mirabella 2004), thus validating the GUS fusion approach. This zone of expression has been termed the “prein-

fection zone” (Pichon et al. 1992) and is characterized by the induction of several nodulin genes of *M. truncatula*, including *MtENOD12* (Pichon et al. 1992), *MtENOD11* (Journet et al. 2001), *MtN1* (Gamas et al. 1998), *MtN6* (Mathis et al. 1999), and *MtAnn1* (de Carvalho-Niebel et al. 1998). By comparison of the depths of the zones in which the nodulin genes are expressed, *DMI2* and *MtENOD11* appear to have very similar, narrow expression patterns in this apical region of the nodule. Indeterminate nodules show a gradation of cells of increasing age from the meristem to the base (Vasse et al. 1990); therefore, this localization of expression suggests that the *DMI2* gene is expressed transiently during growth and differentiation of the nodule. However, it is noteworthy that *DMI2* also appears to be expressed, albeit at a lower level, in the nitrogen-fixing zone of the nodule.

In roots, studies with GUS fusions have shown that *DMI2* is expressed in the epidermis and cortex and throughout most of the root system but not in the root apices (Fig. 2A and B). Highest expression was observed in the younger part of lateral roots. During nodulation, the gene is strongly induced in the nodule primordia, forming particularly on the lateral roots, relative to the low expression in the surrounding tissues and newly developing roots. The low number of cells that showed induction did not lead to an obvious increase in the level of mRNA at early time points in the root systems as measured by Northern analysis (Fig. 1A), thus emphasizing the importance of studying spatial changes in gene expression in addition to overall mRNA levels. At 2 days following rhizobial inoculation, induction of *DMI2* could be seen clearly in patches of cells in the cortex and, at 3 to 5 days, this induction clearly was confined to the nodule primordia and the central, undifferentiated tissues of the young emerging nodules (Fig. 2D through F). At these stages of nodulation, the expression of *DMI2* resembles more closely the expression pattern of *MtENOD20* (Vernoud et al. 1999) and *MsENOD40-1* and *-2* (Fang and Hirsch 1998), rather than *MtENOD11* and *MtENOD12*, which are not induced in the primordia. However, the *ENOD* genes also are induced in the epidermal and cortical cells through which infection threads are initiated or passing (Journet et al. 2001; Pichon et al. 1992; Vernoud et al. 1999). We did not find a correlation between increased *pDMI2-GUS* expression and the presence of infection threads, as shown also in nodules. Despite certain similarities with the expression patterns of some nodulin genes, it is noteworthy that the expression of *DMI2* differs considerably from these genes by its basal level of expression in roots and lack of overall increase in mRNA levels in nodules (Fig. 1A).

Does the expression pattern of *DMI2* limit symbiotic responses?

Studies of nodulation in *M. sativa* have shown that the symbiosis with rhizobia is initiated in a susceptible zone behind the root apices primarily of lateral roots (Bhuvaneswari et al. 1981; Caetano-Anollés and Gresshoff 1991). Moreover, the expression of *MtENOD11* and *MtENOD12* by rhizobia also is initiated in a similar zone (Journet et al. 2001; Pichon et al. 1992). Although the low level of expression of the *pDMI2-GUS* fusion in roots has precluded a precise localization of the start of *DMI2* expression in roots, it is clear that the gene is poorly expressed in the apices and is more strongly expressed in the susceptible zone. Because both nodulation and expression of the two early nodulin genes depend on *DMI2* in roots, it appears that the pattern of expression of the *DMI2* gene may be a factor localizing the initiation of symbiotic activities to the start of the susceptible zone. However, the presence of similar levels of *DMI2* mRNA in roots of plants grown on high levels of an inorganic nitrogen source and following nitrogen starvation (Fig. 1B) suggests that the level of expression of

Fig. 3. Expression of the *DMI2* gene in roots of different symbiotic mutants. Aeropically grown plants of the indicated symbiotic mutants (*gene* and mutant) were starved of nitrogen for 4 days and RNA levels were analyzed by Northern analysis of whole root systems. A17 is the wild-type line.

DMI2 is not a factor, restricting establishment of the LR symbiosis to conditions of nitrogen deprivation.

In nodules, it is tempting to speculate that the expression of the *DMI2* receptor limits the pattern of induction of some early nodulin genes to the preinfection zone of the nodule. However, it should be noted that some of these nodulin genes have complex patterns of regulation (e.g., *MtENOD11* is induced by a *DMI*-independent pathway in the root cortex by a diffusible AM fungal factor) (Kosuta et al. 2003) and it has not been shown that their expression in nodules requires *DMI2*. Moreover, the lack of correlation of expression of *DMI2* with nodulin genes during early stages of infection suggests that other factors (e.g., receptor activation, other genes, and proteins) also may regulate or limit expression of the *ENOD* genes. Clearly, in this regard, the expression patterns of other genes required for Nod factor responses, such as the symbiotic LysM-RLKs, *DMI1*, and *DMI3*, also should be investigated in order to verify whether the regulation of *DMI2* expression may be a factor limiting symbiotic responses.

Regulation of *DMI2* expression.

As shown for *SYMRK* of *L. japonicus* (Stracke et al. 2002), we did not find an induction of the *DMI2* gene in roots by Nod factors (Table 1; Fig. 1C) or by nitrogen starvation (Fig. 1B). However, *DMI2* is strongly induced in nodule primordia before infection (Fig. 2) and the formation of these primordia requires a signal transduction pathway that is dependent on the symbiotic genes *NFP*, *DMI1*, *DMI2*, *DMI3*, *NSP1*, and *NSP2* (Ben Amor et al. 2003; Catoira et al. 2000; Oldroyd and Long 2003). Thus, by extrapolation, the expression of *DMI2* in the primordia should require its own and other genes defining this Nod factor signal transduction pathway, although this may not be a cell-autonomous event. We investigated whether these genes also are required for expression of *DMI2* in uninoculated roots. The same level of expression of the *DMI2* gene was observed in roots of all these mutants (except *dmi2*), suggesting that the basal level of *DMI2* expression in roots does not require the function of these other genes. In contrast, roots of some of the *dmi2* mutants had very low levels of mRNA from the mutated gene, whereas other alleles accumulated *dmi2* mRNA to wild-type levels (Fig. 3). Reduced levels of *dmi2* mRNA in TR25 (*dmi2-1*) recently have been reported by Mitra and associates (2004) and these authors have suggested that it may be due to an RNA surveillance mechanism called nonsense-mediated decay that detects and destroys mRNAs that contain a premature termination codon (Wagner and Lykke-Andersen 2002). The low levels of *DMI2* mRNA in TR25 and TR26 (which contain mutations leading to premature termination codons) and higher levels in the P1 and R38 alleles (which contain point mutations leading to a loss of exon6 and to a missense mutation, respectively) (Endre et al. 2002) are consistent with this hypothesis. However, these latter alleles are leaky for symbiotic phenotypes (Endre et al. 2002; Wais et al. 2000); therefore, we cannot eliminate the possibility that a functional *DMI2* receptor positively regulates its own expression in roots.

Role of *DMI2*.

Because *dmi2* mutants develop normally when grown with a combined nitrogen source, it appears that *DMI2* is essential only for establishing root endosymbioses. The much higher levels of mRNA in roots and nodules compared with leaves and stems (Fig. 1 and EST data) suggest a role in nodules in addition to roots and do not exclude functions in the aerial organs. Recently, roots of *dmi2* mutants have been found to be more sensitive to touch than those of wild-type plants (Esseling et al. 2004), suggesting that the gene may have a subtle, non-symbiotic role which should be investigated further.

Because *dmi2* mutants are blocked in infection of the epidermal cells by both rhizobia and AM fungi, a common symbiotic role of the gene has been suggested in the very early steps of infection. In the LR symbiosis, *DMI2* clearly plays a role in Nod factor-mediated signal transduction in roots prior to infection and, moreover, also is involved in establishing the nodule primordia (Catoira et al. 2000). The expression of *DMI2* in epidermal and cortical cells before inoculation is consistent with a role in perceiving the symbionts before allowing infection to proceed. In accord with an infection role in roots, the simplest explanation for the induction of the *DMI2* gene in the nodule primordium and in the preinfection zone of the nodule is that the *DMI2* receptor is required to prepare for infection during nodule organogenesis. Bacterial *nod* genes required for Nod factor synthesis seem to be induced not only in a molecular dialogue before root infection but also in the apical zone of nodules (Schlaman et al. 1998); therefore, it appears that *DMI2* is mediating Nod factor signal transduction in both roots and nodules. Moreover, the *DMI2* gene appears to be expressed, albeit at a lower level, in the fixation zone of the nodule, thus suggesting that *DMI2* may play a role in continual interactions between the plant and its functioning bacterial symbiont. From the expression pattern of the gene in proximity to infecting or nitrogen-fixing rhizobia, we cannot state whether the signal that binds to and activates the *DMI2* receptor is more likely to be derived from the bacterium or the plant (or, for the AM symbiosis, from the fungus); at present, the ligands for *DMI2* and related proteins remain unknown.

In conclusion, our studies on the localization of *DMI2* expression suggest that the gene plays a specific role in the root cortex and in nodules preceding or leading to rhizobial infection. In concurrence with its role in root epidermal cells, these results support the idea that Nod factor perception and transduction leading to rhizobial infection occur continuously during development of indeterminate nodules.

MATERIALS AND METHODS

Growth conditions.

M. truncatula Gaernt. cv. Jemalong lines A17 or J5 (Thoquet et al. 2002) and the mutant lines *nfp* C31 (Ben Amor et al. 2003); *dmi1* C71 and Y6 (Catoira et al. 2001); *dmi2* TR25, TR26, P1, and R38 (Catoira et al. 2000; Endre et al. 2002); *dmi3* TRV25; *nsp1* (Catoira et al. 2000); *nsp2* (Oldroyd and Long 2003); and *hcl* (Catoira et al. 2001) were grown in aeroponic chambers in a 16-h photoperiod at 23°C, usually for 2 to 3 weeks on a medium containing 5 mM NH_4NO_3 as described by Navarro-Gochicoa and associates (2003b). Plants then were starved of nitrogen for 4 days, unless otherwise stated, by replacing the culture medium with the same one lacking a nitrogen source. In some experiments, *S. meliloti* 2011 (pXLGD4) (Ardourel et al. 1990) then was added to the root medium. Whole root systems or organs were harvested at different times (as indicated in the figure legends), frozen in liquid nitrogen, and stored at -80°C before total RNA extraction. For the experiment in Figure 1C, seedlings were grown for 5 days in aeroponic chambers without a nitrogen source before treatment with 10^{-8} M Nod factors (biologically produced factors containing predominantly NodSmIV[Ac, S, C16:2], prepared essentially as described by Roche and associates [1991]).

Construction of *MtDMI2* promoter GUS fusions.

The *p3.0DMI2-GUS* construct was prepared by amplifying the 5' region of the *DMI2* gene from the BAC clone MtHI-67A11 (Endre et al. 2002) using the primers (TATAAGCTTAT

GATAAATAGTTTCTTAAA) and (ATAGGATCCAATCTGA AAGAGATTTTACCC) and cloning via the *Hind*III and *Bam*HI sites in the promoter probe vector, pPR97 (Szabados et al. 1995). For the *p1.2DMI2-GUS* construct, the 5' regions of *DMI2* were amplified by polymerase chain reaction (PCR) from plasmid BRC1667 (Endre et al. 2002) using the primers (M13 reverse) and (CATAATCTAGAAGAGATTTTAC), followed by cloning via the *Kpn*I-*Xba*I sites in front of GUS in the binary vector pGreenII 0029 (Hellens et al. 2000). Therefore, the constructs contained 3,077 and 1,243 bp of promoter region finishing 1 and 8 bp upstream of the initiating ATG in the *p3.0DMI2-GUS* and the *p1.2DMI2-GUS* constructs, respectively. The constructs were verified by DNA sequencing and the plasmids then were transformed into *A. rhizogenes* *ArQual* by electroporation.

***M. truncatula* transformation and treatment of transgenic plants.**

Composite plants expressing the *pDMI2-GUS* fusions in their roots were produced using the above *A. rhizogenes* strains as described by Boisson-Dernier and associates (2001). Plants producing roots on kanamycin at 20 µg/ml were transferred into growth pouches, two to three plants per pouch, and nontransformed roots were removed. The plants were grown for approximately 11 days with a medium containing 0.25 mM NH₄NO₃. Part of the root system containing several secondary roots then was excised and assayed histochemically for GUS activity. The next day, the rest of the root system was treated by pipetting aqueous solution at 1 ml/plant over the root systems containing either 10⁸ bacteria, 10⁻⁸ M NodSm factors, or just water (mock inoculation). Part of the root system was harvested at various times after inoculation (2, 3, or 5 days) and assayed for GUS activity as before. At least 20 plants were used for each time point. For the quantitative analysis in Table 1, roots were scored blind for a decrease, no change, or increase in GUS activity compared with the initial time point, either in the zone of the secondary roots, just behind the tips where nodules are predicted to develop (the susceptible zone), or in the surrounding regions. The data were analyzed by the χ^2 test of equality of multinomial distribution using the prop. test of the Splus software (version 3.4, release 1 for Sun SPARC; Mathsoft Inc., Cambridge MA).

Histochemical localization of GUS activity.

Histochemical fixation and measurement of GUS activity of whole roots and nodules were performed as described by Vernoud and associates (1999), generally using X-gluc as substrate although, in some experiments, Magenta-gluc was used. Stained whole roots were fixed with 2.5% paraformaldehyde and embedded into 3% agarose before making 150-µm-thick sections using a BioRad Micro Cut H1200 vibratome. Nodules (from 7 *p1.2DMI2-GUS* and 22 *p3.0DMI2-GUS* plants) were prefixed with 0.5% paraformaldehyde, embedded in 2% agarose, and sliced into 100-µm-thick sections before staining. Generally, both roots and nodules were stained for GUS activity for between 16 and 24 h, because the activity was low. Double detection of pDMI2-GUS expression and the LacZ activity of the bacteria was carried out as described by Pichon and associates (1994). Sections were observed by bright-field microscopy for roots and nodules. Nodule sections also were observed with Nomarski microscopy to view infection threads and with epifluorescent microscopy following DAPI (0.5 µg/ml) coloration to view nuclei. The roots were observed by phase contrast microscopy (Fig. 2H). Digital pictures were taken with a Leica camera using the Leica DC Viewer software and assembled using Adobe Photoshop (version 7.0).

RNA isolation and Northern analysis.

The methods to extract and quantify total RNA and prepare Northern blots are described by Navarro-Gochicoa and associates (2003a). DNA used to prepare the specific *DMI2* probes was a PCR product amplified from cDNA corresponding to the highly specific extracellular domain of unknown function. Control probes were produced from DNA fragments of *MtENOD11* (Journet et al. 2001), histone, and rRNA genes as described by Navarro-Gochicoa and associates (2003a). Hybridization was visualized using a Molecular Dynamics PhosphorImager and the signals were quantified using the ImageQuant 5.0 software. The signals of *DMI2*, *ENOD11*, and *histone* mRNA then were adjusted to identical loadings of rRNA.

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