api, A Novel *Medicago truncatula* Symbiotic Mutant Impaired in Nodule Primordium Invasion

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Genetic approaches have proved to be extremely useful in dissecting the complex nitrogen-fixing *Rhizobium*–legume endosymbiotic association. Here we describe a novel *Medicago truncatula* mutant called *api*, whose primary phenotype is the blockage of rhizobial infection just prior to nodule primordium invasion, leading to the formation of large infection pockets within the cortex of noninvaded root outgrowths. The mutant *api* originally was identified as a double symbiotic mutant associated with a new allele (*nip-3*) of the *NIP/LATD* gene, following the screening of an ethylmethane sulphonate–mutagenized population. Detailed characterization of the segregating single *api* mutant showed that rhizobial infection is also defective at the earlier stage of infection thread (IT) initiation in root hairs, as well as later during IT growth in the small percentage of nodules which overcome the primordium invasion block. Neither modulating ethylene biosynthesis (with L- α -(2-aminoethoxyvinylglycine or 1-aminocyclopropane-1-carboxylic acid) nor reducing ethylene sensitivity in a *skl* genetic background alters the basic *api* phenotype, suggesting that *API* function is not closely linked to ethylene metabolism or signaling. Genetic mapping places the *API* gene on the upper arm of the *M. truncatula* linkage group 4, and epistasis analyses show that *API* functions downstream of *BIT1/ERN1* and *LIN* and upstream of *NIP/LATD* and the *DNF* genes.

Additional keywords: *MtENOD*, nitrogen fixation, short root hair.

Legumes possess the remarkable capacity of establishing a symbiotic association with nitrogen-fixing soil bacteria known as rhizobia, thereby facilitating growth in nitrogen-limiting soils. This endosymbiotic interaction leads to the formation of specialized root organs known as nodules within which rhizobia reduce atmospheric dinitrogen for the benefit of the host plant (Mylona et al. 1995; Pawlowski and Bisseling 1996).

Rhizobial infection in temperate legumes such as *Medicago* or *Lotus* spp. is usually initiated in root hairs and is concomitant with nodule organogenesis in the root cortex (Gage 2004; Timmers et al. 1999). Rhizobial attachment to the tip of a growing root hair induces curling around the entrapped bacte-

ria, which subsequently enter the plant cell via a plasma membrane invagination and the formation of an intracellular tubular structure called the infection thread (IT). Bacteria divide within the IT, which progresses down the root hair and across the outer root cortex toward the newly formed nodule primordium comprising activated and dividing cells in both the inner and middle cortex (Timmers et al. 1999). Following penetration and ramification of the IT within the primordium, bacteria are released by endocytosis into host cells and subsequently differentiate into nitrogen-fixing bacteroids. In legumes such as *Medicago* spp. and pea, the continuous activity of the nodule apical meristem results in the formation of so-called indeterminate nodules characterized by consecutive histologically distinct regions, including the infection and nitrogen-fixing zones (Brewin 1991; Patriarca et al. 2004; Vasse et al. 1990).

Nodulation results from a series of tightly regulated molecular interactions between the two symbionts. Plants release flavonoids into the rhizosphere, which stimulate the production by rhizobia of specific lipochito-oligosaccharide signal molecules called Nod factors (NFs) that play a pivotal role in recognition and controlled host root infection (Dénarié et al. 1996; D'Haese and Holsters 2002). Purified NFs are sufficient to trigger many of the early plant symbiotic responses normally observed during preinfection stages of the interaction (Geurts et al. 2005; Oldroyd et al. 2005; Stacey et al. 2006). Intensive research based on the genetic and genomic dissection of the NF signaling pathway has led to increasingly integrated molecular and cellular models for the early steps of NF recognition and signal transduction leading to root epidermal responses, nodule organogenesis, and the initiation of infection (Andrianakaja et al. 2007; Charron et al. 2004; Geurts et al. 2005; Kistner et al. 2005; Marsh et al. 2007; Middleton et al. 2007; Oldroyd 2007; Oldroyd and Downie 2006; Radutoiu et al. 2007; Stacey et al. 2006). Recent findings also indicate that early NF signaling events leading to nodule organogenesis can be distinguished from the process of initial bacterial root hair entry (Gleason et al. 2006; Tirichine et al. 2006).

NF-dependent preinfection events in the host root are a prerequisite for the subsequent processes of bacterial entry and nodule morphogenesis, because all plant mutants defective in the early NF-signaling pathway also are defective either in the formation of colonized root hair curling (the Hac stage) or early in subsequent IT initiation or elongation. In addition, studies of infection mutants and the use of reverse genetic strategies for several legume species also have revealed a number of plant genes whose role is specific to the infection process itself. Mutants in two particular genes have phenotypes consistent with a defect in bacterial entry: *Medicago truncatula hcl* (Catoira et al. 2001) and *Mtin* (ortholog of *Ljnin* in

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Lotus japonicus and *sym35* in pea) (Borisov et al. 2003; Marsh et al. 2007; Schauser et al. 1999). The *nin* mutants and strong alleles of *hcl* are non-nodulating, and the absence of bacterial entry leads to exaggerated root hair curling in response to rhizobia. The *HCL* gene encodes the LysM receptor-like kinase LYK3 (Limpens et al. 2003; Smit et al. 2007) and *NIN* encodes a putative transmembrane transcriptional regulator with homology to *Drosophila* Notch (Marsh et al. 2007; Schauser et al. 1999). However, in contrast to *NIN*, *HCL* does not appear to be directly required for nodule organogenesis (Marsh et al. 2007).

Many legume mutants also have been reported which show defects in subsequent stages of rhizobial infection. In such mutants, ITs are generally blocked either in the root epidermis, in the underlying outer cortical layer, or at the later stage of bacterial release within the nascent nodule (*L. japonicus*: Lombardo et al. 2006; Murray et al. 2006; Sandal et al. 2006; Yano et al. 2006) (*M. truncatula*: Jones et al. 2007) (pea: Tsyganov et al. 2002). With the objective of identifying additional host symbiotic genes involved in rhizobial infection, we have screened an ethylmethane sulphonate (EMS)-mutagenized population of *M. truncatula*. In this article, we report the isolation and characterization of *api*, a new *M. truncatula* mutant that displays an original infection and nodulation phenotype. The *api* mutant fills an existing gap in the known sequence of infection mutant phenotypes because IT progression in *api* is primarily blocked just prior to nodule primordium invasion, with the consequent formation of large infection pockets within the cortex. We present physiological and genetic evidence suggesting that *API* function is not linked to ethylene metabolism or signaling and we demonstrate that *API* is a novel symbiotic locus positioned on *M. truncatula* linkage group 4.

RESULTS

Identification of a double symbiotic mutant carrying genetically distinct recessive mutations, *api* and *nip-3*.

A screen for symbiotic mutants with altered infection or nodulation phenotypes was performed on an EMS-generated M2 population derived from the wild-type nodulating *M. truncatula* L416 line (Journet et al. 2001) (Jemalong A17 genetic background). In this article, we report the genetic and detailed phenotypic analysis of one particular mutant line, T1-3, characterized by numerous small white outgrowths along the root system following inoculation with the symbiotic partner *Sinorhizobium meliloti* (Fig. 1A). Although the majority (>85%) of these outgrowths were not invaded by rhizobia, arrested infections were always observed in the outer cortex. T1-3 also showed reduced lateral root elongation (Supplemental Fig. 1B)

and shorter root hairs (not shown) compared with the wild type. All these phenotypes were stably inherited in the M4 and M5 generations.

To genetically characterize the T1-3 line, back-crosses (BC1) to the wild-type genotype A17 and parental genotype L416 were performed. All F1 plants displayed the wild-type nodulation phenotype, indicating that the nodulation defect of T1-3 is recessive. Surprisingly, nodulation analysis of the F2 progenies revealed two distinct symbiotic mutant phenotypes. The first was very similar to that of T1-3, typified by numerous noninvaded outgrowths (Fig. 1C, F and I) and hereafter named *api* (for “altered nodule primordium invasion”), whereas the second phenotype was blocked at a later stage and characterized by well-invaded nodule-like structures (Fig. 1E, H, and L), similar to the previously described *nip* phenotype (Veereshlingam et al. 2004). Phenotypic ratios in the F2 population were approximately 9:4:3 (wild-type/*api*/*nip*-like), consistent with segregation of two unlinked, monogenic, and recessive mutations (e.g., within F2 A17 × T1-3, 82:31:20; $\chi^2 = 1.8$, $P > 0.4$). In order to examine the *nip*-like mutation in T1-3, we performed reciprocal crosses between *nip* and T1-3. All F1 plants from both crosses presented the *nip* phenotype (Table 1), thus indicating that the T1-3 mutant indeed possesses a mutation in the *NIP* gene. Hereafter, we will refer to the first *nip* allele (Veereshlingam et al. 2004) as *nip-1*, the second *nip* allele (originally called *latd*) (Bright et al. 2005) as *nip-2*, and this new allele as *nip-3*.

In order to separate the *api* and *nip-3* mutations, 11 and 15 BC1 F2 plants displaying either the *api* or the *nip* phenotype, respectively, were selected to generate BC1 F3 progenies. Based on the segregation of nodulation and root phenotypes within these progenies, we were able to identify single-mutant lines carrying either the *api* (one line) or the *nip-3* (five lines) mutation. Complementation tests using these single-mutant lines confirmed that *api* is not allelic to *nip-3* and that *nip-3* is indeed allelic to *nip-1* (Table 1). The *api* single mutant was then back-crossed once more (BC2) into the A17 genetic background and we were able to verify that the frequency of the *api* phenotype in the BC2 F2 population is consistent with the 3:1 segregation of a single recessive allele (465:175; $\chi^2 = 3.5$, $P > 0.17$). These analyses also revealed that the short root hair phenotype of T1-3 is tightly linked to the *api* mutation, whereas the short lateral root phenotype is linked to the *nip-3* mutation (Supplemental Fig. 1B), consistent with the lateral root phenotype reported for *nip-1* and *nip-2* mutants (Bright et al. 2005; Veereshlingam et al. 2004).

To determine whether the *api* mutation belongs to a known *M. truncatula* complementation group, either T1-3 or the BC1 *api* line were crossed to a number of mutants defective in NF

Fig. 1. Root symbiotic phenotype of T1-3, *api*, *nip-3*, and control wild-type lines. **A** and **C** through **H**, Distribution of outgrowths or nodules in the primary nodulation zone; **C** through **E**, 5 days postinoculation (dpi); **A** and **F** through **H**, 12 dpi; **A**, T1-3 (initial *api nip-3* double mutant); **C** and **F**, *api*; **D** and **G**, a sibling BC2 wild-type line; **E** and **H**, *nip-3*. **B**, Presence of frequent outgrowths in lower regions of the *api* primary root at 12 dpi. **F**, Arrows point to the numerous arrested nodular outgrowths on the *api* primary nodulation zone and associated lateral roots; a few large, pinkish, nodule-like structures are also visible. **I** through **L**, Nodule and infection phenotypes 3 dpi for **I** and **J**, *api*; **K**, wild-type; and **L**, *nip-3*, after β -galactosidase histochemical staining of infecting rhizobia. White asterisk: nodule primordium; arrowhead: infection thread (IT) in outer cortex; double arrowheads: *api* infection pockets. Note that ITs appear normal in *api* root hairs whenever infection has proceeded into the cortex (**J**, arrow). **M** through **Q** and **S**, Tissue sections (4 μ m thick, stained with toluidine-blue) of *api* noninvaded, arrested outgrowths; **M** and **N**, 3 dpi; **O** and **P**, 12 dpi; **Q**, of rare *api* infected outgrowth at 5 dpi; and **S**, of *api* abnormal nodule-like structures at 12 dpi. Control wild-type nodules at **R**, 5 dpi and **T**, 12 dpi. Arrow in **M** shows a putative arrested IT in an abnormal periclinal orientation, just adjacent to the nodule primordium (white asterisk). **N**, Preinfection thread (PIT) structure (arrow), formed in medium root cortex and containing a swollen IT. **O** and **P**, Two noninvaded outgrowths (12 dpi) bearing infection structures of various shapes in the remnant of the outer cortex (arrowheads). In such outgrowths, an apical meristem-like group of cells (**O**, white asterisk) and initiating nodule vascular tissue (**O**, arrow) are frequently observed. Cortical cells containing these abnormal IT structures are often filled with magenta-staining fibrous material, probably rich in cellulose (**P**). **Q**, Complex IT network with large infection pockets (arrowhead) in outer cortex of a rare *api* outgrowth (5 dpi) undergoing infection; arrow shows an IT penetrating the primordium tissue. **R**, Nodule development and infected cell differentiation in the wild type is more advanced. **S**, *api* large nodule-like structure (12 dpi) with apical meristem (red asterisk), containing recurrent infection pockets in the central zone (double arrowheads) and less infected cells compared with the wild type (**T**). **T**, Arrows show typical wild-type ITs. Bars = 100 μ m for **I** and **K** through **L** and 50 μ m for **J** and **M** through **T**.

signaling, rhizobial infection or invasion, or later stages of nodule development (Table 1). Infection and nodulation phenotypes were assessed for F1 plants as well as for F2 progenies for a subset of mutants. T1-3/*api* complemented all mutants tested (Table 1), thus indicating that the *api* mutation corresponds to a novel symbiotic locus. Taken together, our results demonstrate that the original T1-3 line is a double symbiotic mutant carrying the two genetically unlinked, monogenic, and recessive mutations *nip-3* and *api*. The mutant *nip-3* is a new allele of the *NIP* gene, whereas *api* corresponds to a novel symbiotic locus. In addition, the fact that T1-3 expresses the *api* nodulation phenotype suggests that the infection process is blocked at an earlier stage in *api* compared with *nip-3*.

The novel symbiotic locus *API* maps to the upper arm of linkage group 4.

To establish the map position of the *api* mutation, the original *api nip-3* double mutant (line T1-3, A17 genetic background) was crossed to the two *M. truncatula* accessions DZA315.16 and A20. The F1 progeny from both crosses possessed a wild-type phenotype for nodulation, consistent with the recessive nature of both *api* and *nip-3* mutations. F2 mapping populations were generated from F1 plants and more than 120 individuals from each cross were phenotyped for infection and nodulation. The *api* and *nip-3* mutant phenotypes were similar to those observed for the A17 genetic background and, therefore, could be unambiguously distinguished. In all, 71 *api* mutant and 58 wild-type plants from the two F2 populations were selected and analyzed by using microsatellite markers regularly spaced on the

M. truncatula genetic map (T. Hugué and M. Cazaux, *unpublished data*). *API* was located in both populations on the upper arm of linkage group 4, between markers MTIC331 and McSSR5 in an interval spanning approximately 2.8 centimorgans (Fig. 2). The fact that no *M. truncatula* symbiotic mutation or gene with a symbiotic function has yet been located to this LG4 upper arm region (Table 1) is consistent with our conclusion from the complementation analyses that *API* is indeed a novel symbiotic locus.

Detailed characterization of rhizobial infection and nodulation in *api*.

With the exception of the reduced length of root hairs, no notable differences in shoot or root growth and overall development were observed for *api* mutant BC2 plants prior to inoculation (Supplemental Fig. 1A through C). For detailed characterization of the *api* infection and nodulation phenotype, plants were grown and inoculated in aeroponic chambers. After harvesting at various times after inoculation, whole roots were stained for the constitutive β -galactosidase marker to localize rhizobia within the root tissues. The basic *api* symbiotic phenotype described below was observed under all growth conditions tested and regardless of the rhizobial strain used for inoculation (RCR2011 or ABS7; Materials and Methods).

The *api* mutant phenotype, characterized by small and non-invaded nodular outgrowths, was clearly visible as early as 3 days postinoculation (dpi) (compare Fig. 1C with D; Fig. 3). Virtually all outgrowths contained swollen infection structures

Table 1. Allelism tests

Female parent	Male parent	F1 nodulation phenotype ^a	F2 phenotype segregation (WT:mutants) ^b	χ^2 P value ^c	Female mutant linkage group	Reference, source ^d
T1-3	<i>nip-1</i>	— ^e	nd	na	...	This work
<i>nip-1</i>	T1-3	— ^e	0:21	1.0	LG 1	Veereshlingam et al. 2004; Bright et al. 2005
<i>nip-3</i>	<i>api</i>	+	nd	na	LG 1	This work; R. Dickstein (<i>personal communication</i>)
<i>nip-1</i>	<i>nip-3</i>	— ^e	nd	na	LG 1	Veereshlingam et al. 2004; Bright et al. 2005
<i>nfp-1</i>	T1-3	+	nd	na	LG 5	Ben Amor et al. 2003
<i>dmi1-3</i>	T1-3	+	nd	na	LG 2	Catoira et al. 2000; Ane et al. 2002
<i>dmi2-1</i>	T1-3	+	nd	na	LG 5	Catoira et al. 2000; Ane et al. 2002
<i>dmi3-1</i>	T1-3	+	nd	na	LG 8	Catoira et al. 2000; Ane et al. 2002
<i>nsp1-1</i>	T1-3	+	nd	na	LG 8	Catoira et al. 2000; Smit et al. 2005
<i>nsp2-3</i>	T1-3	+	22:26	0.61	LG 3	C. Gough (<i>personal communication</i>); Kalo et al. 2005
<i>hcl-1</i>	T1-3	+	nd	na	LG 5	Catoira et al. 2001; Smit et al. 2007
<i>lin</i> (D8)	T1-3	+	nd	na	LG 1	C. Gough (<i>personal communication</i>); Kuppusamy et al. 2004
<i>rpg</i>	T1-3	+	nd	na	LG 1	J. F. Arrighi and C. Gough (<i>unpublished</i>)
<i>bit1-1</i>	T1-3	+	11:13	0.72	LG 7	Middleton et al. 2007
<i>rit1</i>	T1-3	+	31:27	0.08	LG 3	Mitra and Long 2004; G. E. D. Oldroyd (<i>personal communication</i>)
<i>Mtsym1</i>	T1-3	+	12:13	0.56	?	Benanben et al. 1995
<i>Mtsym14</i>	<i>api</i>	+	nd	na	?	Morandi et al. 2005
<i>Mtsym15</i>	<i>api</i>	+	nd	na	?	Morandi et al. 2005
<i>Mtsym16</i>	<i>api</i>	+	nd	na	?	Morandi et al. 2005
<i>Mtsym17</i>	<i>api</i>	+	9:11	0.31	?	Morandi et al. 2005
<i>Mtsym18</i>	<i>api</i>	+	22:14	0.68	?	Morandi et al. 2005
<i>Mtsym19</i>	<i>api</i>	+	nd	na	?	Morandi et al. 2005
<i>Mtsym20</i>	<i>api</i>	+	nd	na	?	Morandi et al. 2005
<i>Mtsym21</i>	<i>api</i>	+	nd	na	?	Morandi et al. 2005
<i>dnf1-1</i>	<i>api</i>	+	26:15	0.28	?	Starker et al. 2006
<i>dnf2</i>	<i>api</i>	+	21:21	0.76	?	Starker et al. 2006
<i>dnf3</i>	<i>api</i>	+	27:15	0.70	?	Starker et al. 2006
<i>dnf4</i>	<i>api</i>	+	nd	na	?	Starker et al. 2006
<i>dnf5</i>	<i>api</i>	+	nd	na	?	Starker et al. 2006
<i>dnf6</i>	<i>api</i>	+	26:18	0.74	?	Starker et al. 2006
<i>dnf7</i>	<i>api</i>	+	nd	na	?	Starker et al. 2006

^a For each cross, all the F1 tested (between 2 and 10) showed the same nodulation phenotype (+ = wild type and – = mutant).

^b For simplicity, detailed segregation of the various mutant phenotypes is not indicated here; WT = wild type, na = not applicable, and nd = not determined.

^c The χ^2 test was applied using F2 (wild type:mutant) expected segregations of 9/16:7/16 for crosses with the single mutant *api* and of 27/64:37/64 for crosses with the double mutant T1-3, respectively, except for the cross *nip-1* × T1-3, where the expected segregation is 0:1.

^d Reference or source for female mutant genotype and for corresponding linkage group number.

^e All F1 showed the *nip* phenotype.

filled with rhizobia which were located in the outer cortex adjacent to the primordium (compare Fig. 1I and J to K). Tissue sectioning revealed that only a limited number of nodule cell layers had formed within the inner cortex (Fig. 1M). Although abnormal, ITs followed the usual pathway defined by preinfection thread (PIT) alignments as far as the cortical cell layer adjacent to the nodule primordium. Despite the presence of both intra- and intercellular infection pockets within the cortex, ITs could not be detected in the underlying primordium tissues (Fig. 1N). At later time points, these noninvaded primordia showed very limited growth (Fig. 1F). In the larger outgrowths (e.g., 12 dpi) (Fig. 1O), it is possible to distinguish a zone with small meristematic-like cells at the apex, as well as the initiation of nodule vascular tissue development. However, rhizobia are still restricted to irregular ITs with balloon-like protrusions or to infection pockets located in the remnants of the outer cortex, and plant cells surrounding these arrested infection structures are filled with fibrous material (probably cellulose-rich) (Fig. 1O and P). The *api* phenotype is also characterized by the continuous formation of new outgrowths on younger parts of the primary and lateral roots (Figs. 1B and F and 3A). This is in contrast with the normal tight regulation of nodule number for the wild type (Fig. 3) and is probably a secondary consequence of the mutation (e.g., as discussed before by Kuppasamy and associates [2004]). The large majority of these new outgrowths remain noninvaded (Fig. 3).

Our observations also showed that ITs can occasionally penetrate nodule tissues of *api* outgrowths (approximately 3% of outgrowths at 3 dpi and 10% at 5 dpi). However, in these rare cases, abnormal sac-like infection structures are still observed

Fig. 2. *API* gene maps to the upper arm of *Medicago truncatula* linkage group 4 (LG4). Microsatellite genetic markers used for mapping on LG4 are shown with the genetic distances indicated to the left of the chromosome (Kosambi centimorgans [cM]). To the right, the number of recombinant haplotypes between each marker and the *API* locus is indicated in relation to the total number of F2 haplotypes tested. The *API* gene maps between MTIC331 and McSSR1 with an estimated interval of approximately 2.8 cM.

in the outer cortex. Furthermore, the often enlarged ITs only show limited progression within the nodule tissue and are frequently associated with the formation of infection pockets (compare Fig. 1Q with R). Such *api* outgrowths contain fewer infected plant cells and fewer bacteroids per infected cell than their wild-type counterparts (not shown). This small percentage of invaded outgrowths subsequently develop into large nodule-like structures (two- to threefold less abundant than for the wild type) (Figs. 1F and G and 3B) that appeared with a 2- to 3-day delay, mostly within the main nodulation zone of the primary root (Fig. 1F). These structures elongated at a normal rate and possessed a pink-colored central zone (from 10 to 12 dpi onward) (Fig. 1F). Sectioning (12 dpi) revealed the presence of enlarged ITs and a lower density of infected cells in the central zone (compare Fig. 1S with T). Moreover, large infection pockets were often present along the main axis of the nodule. Both of these abnormalities suggest the persistence of a defect in the infection process, even following nodule tissue invasion in the *api* mutant.

Taken together, these observations lead us to conclude that IT progression from the outer cortex into the developing nodule primordium is the major impairment responsible for the *api* nodulation phenotype, and that this block can subsequently be overcome at a low frequency. In addition, IT development in *api* is abnormal both prior to primordium infection following outer cortical penetration and also following rare nodule

Fig. 3. Striking differences in nodule infection between *api*, *nip-3*, and wild-type (WT) plants. Plants were grown in aeroponic chambers and inoculated with *Sinorhizobium meliloti* containing the *hemA::LacZ* fusion. **A**, Noninvaded nodule outgrowths and **B**, all types of infected nodule structures were counted in roots harvested 3, 5, 8, and 12 days postinoculation (dpi). The graphs indicate average numbers per plant with standard deviation values ($n = 10$). The statistical significance of pairwise comparisons between either mutant and the WT control for each time point is indicated by asterisks ($P < 0.05$ according to the Student *t* test).

colonization. By contrast, experiments have shown that the *api* mutant can be colonized successfully by the endomycorrhizal fungus *Glomus intraradices* (M. Chabaud and E.-P. Journet, unpublished data), suggesting that the *api* defect in symbiotic root invasion is specific to the rhizobial interaction.

Nodulated *api* plants are not self-sufficient for nitrogen fixation.

Despite the formation of large, pink, nodule-like structures, inoculated *api* plants consistently develop N-deprivation symptoms such as leaf chlorosis (not shown) and reduced root growth after 8 to 12 dpi (Supplemental Fig. 1D). To estimate N_2 fixation levels in the mutant, nitrogenase activity was assessed using the acetylene reduction activity assay (ARA). At 15 dpi, whole-plant ARA levels in *api* plants were only 40% compared with the wild type (Table 2). This is due to the combination of a lower overall nodular mass (approximately 60% of the wild type) coupled with a lower ARA activity in relation to nodule mass (approximately 65% of the wild type) (Table 2). This latter is consistent with the lower density of infected cells observed in the central tissues of *api* nodule-like structures (Fig. 1S).

api is also defective at early stages of root hair infection.

Because retarded IT development in the outer root cortex of the *api* mutant is already visible at 3 dpi, this suggested that earlier events of the infection process may also be defective. Further detailed microscopic analysis indeed showed that there is a significantly (sixfold) higher frequency of infections arrested at the hair curling stage (Hac) for *api* compared with wild-type inoculated plants (Supplemental Fig. 2A). In addition, the rhizobial microcolonies within these curls are larger than their wild-type counterparts (Supplemental Fig. 2B) and the total number of infection events is double in the mutant background (approximately 100 per 1 cm of root in the primary infection zone). Therefore, these observations suggest that the *api* mutation also impairs an early infection stage necessary for IT initiation.

Symbiotic marker gene expression confirms defects in infection steps in *api*.

To further characterize symbiotic defects in *api* at the molecular level, we used quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) to measure the expression levels throughout nodulation of several well-studied genes associated with various infection or nodule developmental stages. *MtENOD12* (Pichon et al. 1992) is a marker for both preinfection responses in the root epidermis and subsequent early infection in root tissues, and the nodule infection zone, *MtN6* (Mathis et al. 1999) is a preinfection marker for cells located

just ahead of growing ITs in the root cortex and nodule infection zone, and *MtENOD8* (Dickstein et al. 1993; Veereshlingham et al. 2004) is a marker for infected cell formation (i.e., bacterial release) in developing nodules. No significant differences in transcripts levels could be detected between *api* and wild-type roots at the preinfection stage (1 dpi) for any of the marker genes (Fig. 4). However, at 3 dpi, *MtENOD12* and *MtN6* mRNA

Fig. 4. Relative transcript levels for a range of symbiotic marker genes in *api* roots during early infection and nodule development. Transcript abundance of **A**, *MtENOD12*; **B**, *MtN6*; and **C**, *ENOD8* was determined by quantitative reverse-transcriptase polymerase chain reaction on total root RNA samples extracted from either wild-type (L416) or *api* roots harvested 1, 3, and 7 days postinoculation (dpi). Black bars: wild-type roots; hatched bars: *api* roots. Relative transcript abundance was calculated by normalization to EF-1 α expression. Each value represents the mean of four replicates (two technical and two biological) and error bars represent the standard deviation.

Table 2. N_2 fixation in *api* and *nip* mutants

Genotype	ARA/plant ^a		ARA/invaded nodule DM ^b	
	nmol/min/plant ^c	Ratio (%)	nmol/min/mg ^c	Ratio (%)
Wild type	4.82 $\pm$ 0.35	100	4.03 $\pm$ 0.62	100
<i>api</i>	1.96 $\pm$ 0.64	41	2.56 $\pm$ 0.46	63
<i>api</i> (invaded nodules removed) ^d	<0.025	<0.5	na	...
<i>nip-3</i>	0.32 $\pm$ 0.07	6.6	nd	...
<i>nip-1</i>	<0.025	<0.5	nd	...
<i>dmi2-1</i> ^e	<0.025	<0.5	na	...

^a The acetylene reduction activity (ARA) was assayed at 15 days postinoculation (dpi) on root systems of five plants of each genotype. Similar overall results were obtained at 21 dpi.

^b The dry mass (DM) of nodule-like structures in *api* was 62% of the nodule DM in the wild type.

^c In nmoles evolved ethylene per minute per plant or per milligram of nodule DM; average value $\pm$ standard deviation; na = not applicable; nd = not determined. All the ARA values above background are significantly different (*t* test, *P* < 0.01) in all pairwise comparisons.

^d Remaining ARA activity in *api* root systems after removal of all large (pink) nodule-like structures.

^e Negative control (no nodule organogenesis).

levels were significantly lower (10- and 11-fold, respectively) in *api* roots compared with the wild type. Four days later (7 dpi), mRNA levels for these two genes had dropped in wild-type roots and were no longer significantly different compared with levels in inoculated *api* roots. On the other hand, whereas transcriptional activation of *MtENOD8* is very pronounced in wild-type nodulated roots at 7 dpi, this gene is only weakly (20-fold less) activated in *api*. For all three genes, basal levels of expression in control uninoculated roots varied little over time and differences between the two genotypes were minimal (not shown). These gene expression data are clearly consistent with the defects in both infection and nodulation observed for the *api* mutant by microscopic analysis.

Could ethylene biosynthesis or signaling be involved in the *api* infection and root hair phenotypes?

Previous studies have shown that the plant hormone ethylene plays a central role in the regulation of successful rhizobial infection (Goormachtig et al. 2004; Guinel and Geil 2002; Mulder et al. 2005; Oldroyd et al. 2001; Penmetsa and Cook 1997), as well as during root hair development in a number of plant species (Bibikova and Gilroy 2003). We performed experiments to evaluate the possible involvement of altered either ethylene metabolism, signaling, or both in the *api* infection and root hair phenotypes. We first analyzed the infection phenotype of inoculated *api* seedlings grown in the presence of chemicals that either antagonize or promote ethylene biosynthesis. Addition of either the ethylene biosynthesis inhibitor L- α -(2-aminoethoxyvinylglycine) (AVG) or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) did not alleviate the basic *api* infection or nodulation phenotype (not shown). This suggests that the *api* phenotype is unlikely to be the result of altered ethylene metabolism or sensitivity. In addition, to test if the *api* infection phenotype is mediated by ethylene signaling, we introduced the ethylene-insensitivity and supernodulation mutation known as *sickle* (*skl*) (Penmetsa and Cook 1997) into the *api* background. Upon rhizobial inoculation, the *api skl* double mutant showed an additive nodulation phenotype with an approximately fivefold increase in *api* noninvaded nodule outgrowths per plant compared with the original *api* mutant (not shown). Importantly the frequency of rhizobial invasion of nodule primordia in the *api skl* double mutant was as low as for *api*. In contrast, the *skl* single mutant had an invasion frequency close to 100%, as is the case for wild-type plants (previously shown by Penmetsa and Cook 1997). The genetic inhibition of ethylene signaling thus fails to rescue the *api* infection and nodulation phenotype; therefore, we deduce that this phenotype does not result from a cascade of events involving ethylene signaling.

Mature root hairs on *api* roots are 40 to 50% shorter than those of wild-type roots (Supplemental Fig. 3A). Cosegregation of the root hair and nodulation defects was found for all 120 BC2 F2 *api* plants tested, indicating that the two phenotypes are either due to a single mutation or to tightly linked mutations. To evaluate whether the *api* short root hair phenotype is the result of altered ethylene endogenous levels or sensitivity, we measured the average length of mature root hairs of seedlings grown in the presence of AVG or ACC. These experiments show that both WT and *api* root hairs increase in length as the AVG concentration decreases or as the ACC concentration increases (Supplemental Fig. 3B). However, *api* root hairs always remain shorter (constant ratio) compared with wild-type root hairs grown under the same conditions. This suggests that the ethylene response of developing root hairs is normal in *api* and, thus, that the defect in root hair growth is unrelated to this particular plant hormone. Finally, *api* and wild-type seedlings treated with ACC or AVG showed

similar ethylene responsiveness with respect to root growth, root diameter, and the triple response of dark-grown seedlings (Guzman and Ecker 1990) (not shown).

api is epistatic to *nip-3* and to other Nod⁺ Fix⁻ mutations, whereas *bit1* and *lin* are epistatic to *api*.

Following the backcross of the initial T1-3 line and the isolation of the single *nip-3* mutation, we were able to observe several distinct characteristics of the *nip-3* phenotype compared with the *nip-1* allele. Although BC1 *nip-3* plants showed most of the root and nodulation mutant traits previously described for *nip-1* (Veereshlingam et al. 2004) (Figs. 1E, H, and L and 3), *nip-3* nodules appeared to be significantly more developed compared with *nip-1* (not shown). Indeed, *nip-3* nodules possess several layers of large cells filled with bacteroids (Supplemental Fig. 4), whereas *nip-1* is primarily blocked at the earlier stage of endocellular bacterial release (Veereshlingam et al. 2004). Consistent with this, *nip-3* nodules have a detectable N₂-fixation capacity, in contrast to *nip-1* (Table 2). Finally, primary root growth (but not lateral root elongation) in *nip-3* mutants is less inhibited than for *nip-1* (Supplemental Fig. 1B and C). Therefore, we conclude that *nip-3* is a weaker allele than *nip-1* and probably also weaker than *nip-2* (Bright et al. 2005).

The epistasis relationship of *api* to *nip*, inferred from the phenotype of the initial double mutant T1-3 (discussed above), was subsequently confirmed on a genetically verified BC1 *api nip-3* line. As expected, this line expresses the *api* phenotype, except that instead of the few large, pink, nodule-like structures typical of *api*, we observed a small number of invaded nodules with a typical *nip-3* phenotype which do not develop further. This shows that the rare infection events that overcome the main *api* defect are unable to progress beyond the *nip-3* blockage, and implies that *API* acts upstream of *NIP* in the sequence of events necessary for root infection and early nodule development.

The “leaky” character of the *api* nodulation phenotype made it possible to evaluate in a similar way the epistasis relationships between *api* and other mutations that impair later stages of nodule development. Phenotypic analyses of putative double mutants within F2 progenies derived from crosses between *api* and a number of Nod⁺ Fix⁻ mutants (Table 1, *Mtsym18*, *dnf1-2*, *dnf2*, *dnf3*, and *dnf6*; and not shown) lead to the conclusion that *API* acts upstream of all the five corresponding genes. On the other hand, in similar experiments using the early nodulation mutants *bit1-1* (Middleton et al. 2007) and *lin* (Kuppusamy et al. 2004), all the putative double mutants (identified from their phenotypes, Materials and Methods) displayed either the *bit1* or the *lin* nodulation phenotype, respectively, thus confirming that the corresponding genes *BIT1/ERN1* and *LIN* act upstream of *API* in the infection process.

DISCUSSION

The *API* gene product is required throughout rhizobial infection of host tissues.

Rhizobial penetration into host legume roots involves IT initiation and growth through the root hair, followed by IT elongation across several root cortical cell layers and then delivery to the nodule primordium. This involves complex and as yet poorly understood molecular and cellular processes which require tight coordination between the two symbiotic partners. Studies performed on *M. truncatula* and *L. japonicus* mutants so far have revealed essential functions for a number of plant genes during several stages of IT initiation and development. With the objective of identifying additional host symbiotic genes involved in rhizobial infection, we have isolated and characterized *api*, an

M. truncatula mutant that displays a novel, pleiotropic, and robust infection and nodulation phenotype.

The infection step which is most strongly impaired in *api* is IT progression from the outer cortex into the developing nodule primordium. Thus, the majority of ITs that penetrate into the cortex of the *api* mutant arrest growth in the innermost cortical cell layer adjacent to the nodule primordium. This leads to arrested root outgrowths containing abnormal cortical infection structures of variable shapes and sizes (one or more terminal spherical protrusions or larger infection pockets) filled with rhizobia. This *api* phenotype is slightly leaky, because this initial block can be occasionally overcome, leading to the development of invaded, nodule-like structures with abnormal ITs that are able to fix nitrogen, albeit at suboptimal levels. Our molecular data based on the expression of marker genes associated with various infection or nodule development stages are consistent with these observations. Another important characteristic of the *api* mutant is that defects can be identified throughout the entire infection process, whether at the preinfection step just before IT initiation, prior to primordium infection following outer cortical penetration, or following rare colonization of the nodule. Therefore, we deduce that the *API* gene is important for initiating and maintaining optimal infection in both root and nodule tissues. It should be emphasized that no signs of general defense responses, such as the accumulation of autofluorescent cell wall material, could be observed in *api* roots and nodule sections until at least 15 dpi. This is in marked contrast with *nip-3* roots and nodules (Supplemental Fig. 4) (Veereshlingam et al. 2004). The additional observation that the *api* infection phenotype is not alleviated in the ethylene-insensitive *skl* genetic background together leads us to conclude that *api* infection defects are probably not the consequence of strong defense reactions to rhizobial penetration.

The *api* mutant has a unique infection phenotype and maps to a novel chromosomal locus.

Our observations show that the symbiotic phenotype of *api* is novel when compared with other infection mutants previously reported in legumes, where ITs are generally blocked either in the root epidermis, in the underlying outer cortical layer, or at the later stage of bacterial release within the nascent nodule. A number of plant mutants falling into the first two categories have been reported in pea (Tsyganov et al. 2002), *L. japonicus* (*itd1* through *itd4*, Lombardo et al. 2006; *crinkle*, Tansengco et al. 2003, Yano et al. 2006; *sym6/Ljsym82* and *sym80*, Yano et al. 2006; *sym7*, *sym8*, and *sym10*, Schauser et al. 1998; *sym40* and *sym67*, Sandal et al. 2006; and nine suppressors of *Ljhar1-1*, Murray et al. 2006), and *M. truncatula* (*hcllyk3*, Catoira et al. 2001, Smit et al. 2007; *lin*, Kuppasamy et al. 2004; *bit1*, Middleton et al. 2007; *rit1*, Mitra and Long 2004). The *lin* mutant shows a reduced number of infections and arrested ITs in the root epidermis; the *bit1* and *rit1* mutants form ITs that abort in the epidermis or outer cortex of the root. Plant mutants affected in bacterial delivery to nodule cells have been described in pea (Morzhina et al. 2000; Tsyganov et al. 1998), *L. japonicus* (*alb1/Ljsym74*; Imaizumi-Anraku et al. 1997; Kawaguchi et al. 2002; Yano et al. 2006), and *M. truncatula* (*Mtsym1*, Benaben et al. 1995; *Mtsli*, Haynes et al. 2004; Pislariu and Dickstein 2007; *Mtnip/latd*, Bright et al. 2005; Veereshlingam et al. 2004). In *sli* and *nip*, almost all nodules contain rhizobia trapped within ITs; whereas, in *Mtsym1*, release of rhizobia from the nodule IT network is hampered, and engulfed bacteria undergo limited replication but fail to elongate into bacteroids. Equally, none of the rhizobial symbiotic mutants so far described elicit an infection phenotype similar to that of *api* (Frayssé et al. 2003; Jamet et al. 2007; Jones et al. 2007). Nor does the *api* abnor-

mal infection phenotype resemble those observed in RNAi knock-down or overexpression experiments designed to examine the role of various host genes (e.g., *NFP*, Arrighi et al. 2006; *DMI2*, Limpens et al. 2005; *DMI3*, Godfroy et al. 2006; *LYK3*, Limpens et al. 2003; *SrSymRK*, Capoen et al. 2005; *MtHAP2-1*, Combier et al. 2006; *MtMPL1*, Combier et al. 2007; *MtCRE1*, Gonzales-Rizzo et al. 2006; *MtCDPK1*, Ivashuta et al. 2005). Thus, the *api* mutant fills an existing gap in the known sequence of infection mutant phenotypes. Following the phenotypic code proposed by Tsyganov and associates (1998) for IT development in pea, the major *api* mutant phenotype can be defined as a block between the Itr stage ("IT differentiation inside root cortical cells") and the Itn stage ("IT differentiation inside the nodule tissues"). Therefore, we propose the addition of the "Api" stage ("altered nodule primordium invasion") to integrate the *api* infection phenotype.

Our genetic data shows that *api* is indeed a novel mutant locus, positioned on the upper arm of *M. truncatula* linkage group 4. Based on the developmental phenotypes of individual mutants and of double mutants analyzed in this study, we propose the following position of *API* within the sequence of genes required for the infection or nodule maturation process: [*BIT1/ERN1*, *LIN*] → *API* → [*NIP/LATD*, *MtSYM18*, *DNF1*, *DNF2*, *DNF3*, *DNF6*]. In addition, it has been proposed by Veereshlingam and associates (2004) that *NIP/LATD* acts upstream of the *DNF* genes and by Mitra and Long (2004) that *DNF1* acts upstream of *DNF2*, *DNF3*, and *DNF6*.

What role for *API* during rhizobial infection?

Abnormalities in IT initiation, morphology, and growth kinetics suggest that the *api* mutation affects a component that is important throughout the entire infection process. Furthermore, the strong genetic link between the infection and short root hair phenotypes suggests that *API* also is involved in root hair development. Although both infection and root hair development are known to be regulated by the phytohormone ethylene (Bibikova and Gilroy 2003; Mulder et al. 2005), we have shown that ethylene signaling is not involved in the expression of either phenotype and that the *api* mutant is not altered in its overall responsiveness to ethylene. Thus, it is unlikely that *API* function has a close link with ethylene metabolism or signaling. It is also noteworthy that inoculation with an *S. meliloti* strain that constitutively overproduces NFs (*nodD3-syrM* plasmid) does not rescue the *api* phenotype, indicating that the infection defect is unlikely to be a consequence of a reduced sensitivity of *api* root tissues to NFs (J. Garcia and E.-P. Journet, unpublished data) (Den Herder et al. 2007).

Rhizobium and host plant mutations affecting various stages of the infection process often lead to the formation within either cortical or nodule tissues of aberrant IT structures (Combier et al. 2007). However, there are relatively few reports of large infection pocket formation in legume mutants. In the *L. japonicus alb1-1* mutant, which is defective in endocellular release of rhizobia (Imaizumi-Anraku et al. 1997; Kawaguchi et al. 2002; Yano et al. 2006), strikingly large intercellular infection pockets form within central nodule tissues. In the case of the *Lotus crinkle* mutant, where most ITs are blocked in the epidermis or outer cortex (Tansengco et al. 2003; Yano et al. 2006), smaller infection pockets with bacterial aggregates were infrequently observed within nodules. Thus, the systematic formation of spherical protrusions or larger infection pockets relatively early after inoculation (3 to 5 dpi) within the outer and medium root cortex of noninvaded outgrowths is so far unique to the *api* mutant.

Whatever the molecular, cellular, or physiological defects caused by the mutation, the *api* infection phenotype suggests that there is a critical checkpoint controlling IT entry into the

outer cell layer of the nodule primordium. What makes this transition so special? One obvious possibility is that the *API* gene product plays an essential role at this stage of the infection process. Another nonexclusive explanation is that there is a limited time window for IT entry into the primordium, such that the infection block in *api* is an indirect consequence of a delay in IT growth through the root hair and outer cortex. Indeed, Timmers and associates (1999) have proposed that, for successful infection in wild-type *Medicago* spp., ITs must reach the primordium before the meristem forms. Although no typical meristem can be identified in *api* outgrowths at 3 to 5 dpi, it is possible that an alternative temporal restriction may be operating. Finally, it remains to be determined whether the "leaky" character of the *api* nodulation phenotype is due to this particular mutant allele or to functional redundancy between *API* and other plant genes involved in infection. Therefore, we anticipate that the future molecular characterization of *API* will contribute to our understanding of rhizobial infection of both root and nodule tissues and may also provide insights into links between IT progression and root hair elongation.

MATERIALS AND METHODS

Plant material, bacterial strains, and growth conditions.

The registered wild-type plant lines used in this study are the *M. truncatula* mapping parental genotypes cv. Jemalong A17, DZA315-16 (Thoquet et al. 2002), and A20 (Penmetsa and Cook 2000), as well as the wild-type nodulating, transgenic line L416 carrying the *pMtENOD11-GUS* reporter gene fusion (Charron et al. 2004; Journet et al. 2001). All *M. truncatula* mutants used are referenced in Table 1, except *skl-1* (*skl*) (Penmetsa and Cook 1997). The isolation of T1-3, *api*, and *nip-3* mutants and sibling back-crossed wild-type lines is described in the following subsection. The *S. meliloti* bacterial strains used are the wild-type strain RCR2011 harboring the pXLGD4 plasmid carrying the constitutively expressed *hemA::lacZ* fusion (Leong et al. 1985) (strain GMI6526) (Ardourel et al. 1994), the Nod⁺Fix⁺ strain ABS7(pXLGD4) (Benaben et al. 1995), and the strain RCR2011(pMH682) carrying a *nodD3-syrM* construct (GMI6390) (Roche et al. 1991). Rhizobia were grown at 30°C in tryptone-yeast medium supplemented with 6 mM CaCl₂ and tetracycline at 10 µg ml⁻¹ for pXLGD4 selection.

M. truncatula plants were germinated, transferred to an aeroponic chamber, and grown on inorganic medium as described by Garcia and associates (2006) and Barker and associates (2006). For nodulation and transcript analysis experiments, seedlings were grown on nitrogen-free medium and inoculated 0 to 3 days after planting with a late log-phase culture of *S. meliloti*. To assess the robustness of *api* and *nip-3* nodulation phenotypes, plants were also grown and inoculated in pouches (up to 15 dpi), Fahræus-agar plates, and tubes (up to 4 weeks postinoculation [wpi]) and soil (6 wpi) (Barker et al. 2006). For root hair growth and ethylene responsiveness assays, seedlings were grown for 2 to 3 days on Fahræus-agar plates supplemented with appropriate concentrations of AVG (Sigma, St. Louis) or ACC (Sigma). For the triple-response assay, germinating seed were incubated in the dark on a range of ACC concentrations (0 to 100 µM) as described by Penmetsa and Cook (1997).

Mutant isolation and genetic analysis.

The initial nodulation mutant screen was performed on an EMS-generated M2 population derived from the wild-type-nodulating *M. truncatula* L416 line, kindly provided by Maria Harrison. M2 plants were grown in aeroponic chambers and root systems were examined 7 to 12 dpi with *S. meliloti* strain

GMI6526. This nodulation assay was used for all subsequent screens of back-cross and mapping populations. A single individual of the M3 self-progeny from the M2 candidate mutant T1-3 was back-crossed to both A17 and L416 lines (manual crossing procedure of Chabaud and associates [2006]). BC1 F2 population screening and further F3 or F4 progeny analysis resulted in the isolation of one BC1 *api* and five BC1 *nip-3* single-mutant lines, and one BC1 *api nip-3* double-mutant line. Following a second back-cross of BC1 *api* to A17, several BC2 *api* and sibling wild-type F3 lines were isolated and used for most of the detailed phenotypic analyses.

For mapping studies, the line T1-3 (A17 genetic background) was crossed to the two *M. truncatula* accessions, DZA315.16 and A20. In all, 71 *api* mutant and 58 wild-type individuals from the two resulting F2 populations were selected and leaf genomic DNA was extracted from each plant for PCR amplification. The map position of the *api* locus was determined by using microsatellite markers (SSR) regularly spaced on the *M. truncatula* genetic map (Julier et al. 2003; T. Huguet, unpublished data) and the additional McSSR1 and McSSR5 markers defined on the *M. truncatula* upper LG4 arm by M. Cazaux and C. Jacquet (unpublished data). The primers used were 5'-CTCACGAGAACAGCATGAGG-3' and 5'-AATTGCATGTGCTGCATACTTA-3' for McSSR1 and 5'-GACTTCCAACACTGACGACAATA-3' and 5'-CTGGTTTCTTGGCACTCGT-3' for McSSR5. PCR conditions were 94°C for 4 min; followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; followed by 6 min at 72°C. Marker bands were separated by gel electrophoresis (3.5% agarose) and scored as codominant markers. Genetic distances between the *api* locus and molecular markers were estimated by combining recombinant frequencies observed in the two F2 mapping populations.

To generate an *api skl* double mutant, *skl* was crossed to the BC1 *api* line and an F2 plant showing a presumptive double-mutant phenotype was selected to produce F3 progeny. The genetic nature of the double mutant was confirmed by test crosses to both *skl* and *api* single mutants. For genetic epistasis analyses between *api* and *bit1* or *lin* mutations, 200 individuals in each F2 population derived from a cross between *bit1-1* or *lin* and T1-3 were first screened on Fahræus-agar plates to select *api* individuals based on their short root hair phenotype (approximately 25% of each population). The selected plants were then grown in aeroponic chambers and the nodulation phenotypes determined at 12 dpi. The *bit1* or *lin* nodulation phenotypes were present in these F2 subsets and segregated with *api* in a 1:3 ratio; therefore, it was inferred that the plants expressing the *bit1* or *lin* phenotype were actually *api bit1* or *api lin* double mutants, respectively, thus leading to the conclusion that, in such plants, the *api* nodulation phenotype was masked by the earlier *bit1* or *lin* nodulation block.

Cytological root and nodule analysis.

To characterize infection and nodulation phenotypes, plants were harvested at various time points after inoculation with *S. meliloti* strain GMI6526, fixed, and stained for rhizobial β -galactosidase activity using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Biosynth AG, Staad, Switzerland) as described by Boivin and associates (1990). To facilitate examination of the rhizobial colonization of root or nodule structures, stained whole-root samples were cleared with sodium hypochlorite as described by Truchet and associates (1989). Samples were observed under a Wild/Leitz stereomicroscope (Leica Microsystems, Wetzlar, Germany) or a Zeiss Axiophot light microscope (Carl Zeiss, Oberkochen, Germany). For fine histological examination, unstained nodulated root samples were fixed in 2.5% (vol/vol) glutaraldehyde buffered in 0.1 M sodium phosphate (pH 7.2), dehydrated in an alcohol series, and

embedded in Technovit 7100 resin (Hereaus Kulzer, Wehrheim, Germany). Semithin (4 µm) nodule sections were observed by brightfield microscopy after counterstaining in a 0.2% aqueous toluidine blue solution and by epifluorescence microscopy prior to counterstaining.

Acetylene reduction assay.

Nitrogenase activity of individual plants was determined by measuring ARA on whole-root systems harvested from an aeroponic culture at 15 dpi. Excised tissue was incubated at 25°C in 60-ml sealed vials in the presence of 10% (vol/vol) acetylene. Time series of three samples (200 µl) per vial were taken over a 30- to 120-min period and injected into an Intersmat IGC 120 FL gas chromatograph. Ethylene was quantified by comparison to a 100-ppm ethylene standard, and the values were normalized for incubation times.

Real-time PCR analysis.

Root tissues (two independent biological replicates) were collected from both inoculated and control plants at 1-, 3-, and 7-dpi time points. For 1- and 3-dpi samples, the root tips were excised from the rest of the root before freezing samples for RNA extraction. Total RNA was extracted using the Macherey-Nagel total RNA isolation kit (Macherey-Nagel, Hoerd, France) according to the manufacturer's instructions. First-strand cDNA synthesis with 0.5 µg of total RNA was performed with an anchored oligo(dT) (17T+V) and Superscript II reverse transcriptase (Invitrogen, Renfrewshire, U.K.) following the manufacturer's protocol. An in vitro transcribed nebulin RNA (30 ng) was included in each first-strand cDNA reaction in order to control the efficiency of the reverse transcriptase. Gene-specific primers were designed based on the sequence information available at National Center for Biotechnology Information databases. To allow detection of amplification from contaminant genomic DNA, primers were chosen within exons and selected to span one or more introns when present. The specific primers were 5'-ATAGGCATCCTCCAGCAGAA-3' and 5'-AACTTGGCCTTGCCCATAC-3' for *MtENOD12* (X68032), 5'-GGCTTCTCTGTACTCCCAGGTT-3' and 5'-AAACAACCTTCGCGAGCATTC-3' for *MtN6* (AJ133118), 5'-ACATTGGTGTGTTGTTGTGGA-3' and 5'-GGAGGATCATTGAAACTCCAG-3' for *MtENOD8* (AF064775), and 5'-CTTTGCTTGGTGCTGTTTATAGATGG-3' and 5'-ATTCCAAAGGCGGCTGCATA-3' for the elongation factor housekeeping gene *EF1-α* used as an internal standard to check RNA quality and for normalization of transcript levels. PCR fragments corresponding to each gene were cloned into pGEM-T (Promega, Charbonnières, France) and verified by sequencing, and serial dilutions of these plasmids starting with an initial concentration of 1 ng/µl were used to generate a calibration standard curve. qRT-PCR reactions were performed with the Light Cycler Fast Start Reaction Mix Master^{PLUS} SYBR Green I (Roche Applied Sciences, Mannheim, Germany) on a Roche Light Cycler real-time PCR apparatus according to the manufacturer's instructions. Each reaction was carried out with 2 µl of a 1:25 (vol/vol) dilution of the first cDNA strand and 0.5 µM each primer in a total reaction volume of 10 µl. The cycling conditions were 95°C for 9 min followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 10 s, and extension at 72°C for 10 to 20 s. At the end of the PCR cycles, PCR amplification specificity was verified by the analysis of a dissociation curve generated by heating the samples from 60 to 95°C. Background subtraction and determination of the maximum second derivative for each amplification curve was performed using the LightCycler 3.5 analysis software (Roche Applied Sciences). The cycle numbers defining the second maximum derivative of the standards were plotted against their

logarithmic concentrations to derive a standard curve for each of the selected genes. Transcript abundance corresponding to specific genes in the unknown samples was interpolated from this standard curve.

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