New Nodulation Mutants Responsible for Infection Thread Development in *Lotus japonicus*

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Legume plants develop specialized root organs, the nodules, through a symbiotic interaction with rhizobia. The developmental process of nodulation is triggered by the bacterial microsymbiont but regulated systemically by the host legume plants. Using ethylmethane sulfonate mutagenesis as a tool to identify plant genes involved in symbiotic nodule development, we have isolated and analyzed five nodulation mutants, *Ljsym74-3*, *Ljsym79-2*, *Ljsym79-3*, *Ljsym80*, and *Ljsym82*, from the model legume *Lotus japonicus*. These mutants are defective in developing functional nodules and exhibit nitrogen starvation symptoms after inoculation with *Mesorhizobium loti*. Detailed observation revealed that infection thread development was aborted in these mutants and the nodules formed were devoid of infected cells. Mapping and complementation tests showed that *Ljsym74-3*, and *Ljsym79-2* and *Ljsym79-3*, were allelic with reported mutants of *L. japonicus*, *alb1* and *crinkle*, respectively. The *Ljsym82* mutant is unique among the mutants because the infection thread was aborted early in its development. *Ljsym74-3* and *Ljsym80* were characterized as mutants with thick infection threads in short root hairs. Map-based cloning and molecular characterization of these genes will help us understand the genetic mechanism of infection thread development in *L. japonicus*.

Legume plants establish a symbiotic interaction with rhizobia, resulting in the formation of root nodules. In the central nodule tissue, rhizobia fix atmospheric nitrogen to ammonium to be used by the host plants. This legume–rhizobia symbiosis starts with chemical signaling between the host plants and the rhizobia. Rhizobia synthesize Nod factors when they perceive flavonoid compounds released from host plants (Oldroyd and Downie 2004). This results in the curling of growing root hairs which entrap the rhizobia. Infection threads then are developed from the point of curling, allowing the rhizobia to penetrate into the cell. Inside the roots, cortical cells divide to develop nodule primordia. Infection threads elongate toward the nodule

primordia by forming a network structure and, finally, rhizobia are released into the host cells (Gage 2004).

Recently, many symbiotic mutants have been isolated from the model legumes *Lotus japonicus* and *Medicago truncatula*. Several symbiotic genes have been identified in which mutation causes symbiotic phenotypes, designated non-nodulation (Nod[−]), nonfixation (Fix[−]), or hypernodulation (Nod⁺⁺). Nod[−] mutants include *nin* (Schauser et al. 1999), *symrk/nork* (Endre et al. 2002; Stracke et al. 2002), *nfr1* and *nfr5* (Madsen et al. 2003; Radutoiu et al. 2003), *castor* (Imaizumi-Anraku et al. 2005), *pollux/dmi1* (Ané et al. 2004; Imaizumi-Anraku et al. 2005), *dmi3* (Lévy et al. 2004; Mitra et al. 2004), *nsp1* (Smit et al. 2005), and *nsp2* (Kaló et al. 2005). These mutants fail to undergo infection thread initiation and cortical cell division. Except for *nin*, these mutants also show no root hair curling. The gene has been identified from a Fix[−] mutant, *sst1* (Krusell et al. 2005). Nod⁺⁺ mutants represent *har1* (Krusell et al. 2002; Nishimura et al. 2002a) and *astray* (Nishimura et al. 2002b). These mutants develop extra nodules compared with the wild-type plants.

To understand the interaction between host plants and rhizobia, it is necessary to reveal not only the early responses to Nod factors but also the succeeding processes of nodule development, including infection thread formation, cortical cell division, and nodule differentiation. Infection threads are very important structures that allow the delivery of rhizobia from the rhizosphere to the host cells. Ardourel and associates (1994) demonstrated that a *nodF/nodL* double mutant of *Sinorhizobium meliloti*, which synthesizes Nod factors that lack an O-acetyl group and have a modified N-acyl group, does not induce infection thread formation, although it colonizes curled root hairs of *M. truncatula*. Limpens and associates (2003) showed that LYK3 and LYK4, LysM domain-containing receptor-like kinases of *M. truncatula*, are important for infection thread development in response to specific Nod factor structures. When they suppressed expression of these genes by RNA interference (RNAi), infection thread elongation was affected by inoculation with a *nodFE* mutant of *S. meliloti*, which synthesizes Nod factors that have a modified N-acyl group, but not by inoculation with wild-type *S. meliloti*. *Pssym35*, which has a mutation in the pea *Nin* gene, induces root hair curling accompanied with colonization of rhizobia, but fails to initiate infection thread formation (Borisov et al. 2003; Tsyganov et al. 1999, 2002). *Nin* encodes a putative transcriptional regulator (Borisov et al. 2003; Schauser et al. 1999). It is possible that this regulator affects the expression of a set of genes necessary for infection thread initiation. With limited information on how infection threads are initiated at the molecular level, the process of infection thread development is far from clear. To date, several mu-

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tants with aberrant infection thread development have been isolated in *L. japonicus* and *M. truncatula* (Bénaben et al. 1995; Imaizumi-Anraku et al. 1997; Kuppusamy et al. 2004; Mitra and Long 2004; Schauser et al. 1998; Tansengco et al. 2003; Veereshlingam et al. 2004). In pea, at least nine loci are responsible for infection thread development (Tsyganov et al. 2002), suggesting that we still can identify novel mutants that show defects in infection thread development. Here, we present the isolation and characterization of five mutant lines, *Ljsym74-3*, *Ljsym79-2*, *Ljsym79-3*, *Ljsym80*, and *Ljsym82*, from the model legume *L. japonicus*. After detailed analysis of infected roots, we have determined that these mutants are affected at different developmental stages in infection thread development in root hairs. Mapping analysis and complementation tests reveal that three mutants are allelic with known loci. Recent progress on the *Lotus* genome project will help us to identify genes responsible for infection thread development.

RESULTS

Isolation of novel symbiotic mutants with incomplete nodule development.

M2 seed of *Lotus japonicus* B-129 'Gifu' that had been treated with ethylmethane sulfonate (EMS) by Kawaguchi and associates (2002) were screened for nodulation mutants. Preliminary tests on nodulation showed that five lines were candidate mutants with incomplete nodule development. The mutant

lines and the wild-type B-129 parent were inoculated with *Mesorhizobium loti* MAFF303099 and grown under B&D medium with a low concentration of potassium nitrate (Broughton and Dilworth 1971). After 1 month, all mutant lines were nitrogen starved and growth retarded compared with the wild type (Fig. 1). Under high concentrations of potassium nitrate, mutants appeared to be wild type for growth (data not shown).

Complementation tests.

Several infection thread mutants have been identified in *L. japonicus*. *Ljsym74* (*alb1*) has short, thick infection threads arrested in the epidermis (M. Hayashi, *unpublished results*). The *Ljsym74* locus is mapped at the northernmost part of the linkage group 1 (Kawaguchi et al. 2005). *Ljsym79* (*crinkle*) shows distortion of segregation, has aborted infection threads in the epidermis, and has crinkled trichomes (Kawaguchi et al. 2002; Tansengco et al. 2003). When trichome development was observed, trichomes of the wild type were straight, whereas those of *Ljsym79-3* were crinkled (Fig. 2). The same trichome phenotype was noted in *Ljsym79-2* (data not shown). This suggested that both *Ljsym79-2* and *Ljsym79-3* were allelic with *Ljsym79*. From the results of phenotype analysis and mapping (also discussed below), we hypothesized that *Ljsym74-3* was an allele of *Ljsym74*, and *Ljsym79-2* and *Ljsym79-3* were alleles of *Ljsym79*. When *Ljsym74-3* was crossed with *Ljsym74-1*, two pods were harvested after successful crossing. F₁ progenies (four and two plants from each pod) were used

Fig. 1. Growth phenotype of wild type and mutants. Plants were grown for 1 month after inoculation with *Mesorhizobium loti* MAFF303099. **A**, Wild type; **B**, *Ljsym74-3*; **C**, *Ljsym79-2*; **D**, *Ljsym79-3*; **E**, *Ljsym80*; **F**, *Ljsym82*. Bar: 1.0 cm.

for nodulation assays, and none developed functional nodules. *Ljsym79-2* was crossed with *Ljsym79* and resulted in five successful crossings. Twenty-five F₁ plants (five plants from each pod) were inoculated with rhizobia, and all these plants formed small, white bumps. In addition, the trichome phenotype of the F₁ plants was identical with that of *Ljsym79*. F₁ plants (two and three plants from two pods) from the crossing between *Ljsym79-3* and *Ljsym79* showed the same phenotype. Because of the results on *Ljsym79* allelism tests, *Ljsym79* is referred to as *Ljsym79-1* hereafter.

Segregation and mapping of incomplete nodulation mutants in F₂ progenies.

Segregation of *Ljsym74-3*, *Ljsym79-2*, *Ljsym79-3*, *Ljsym80*, and *Ljsym82* in the F₂ progenies from the crosses between each mutant and *L. japonicus* MG-20 'Miyakojima' was examined. Except for *Ljsym79-2* and *Ljsym79-3*, all mutants showed

segregation of wild-type to mutant phenotype as a 3:1 ratio in the F₂ progeny (Table 1). Cosegregation of the nodule and trichome phenotypes was observed in *Ljsym79-2* and *Ljsym79-3*, suggesting that these two phenotypes were caused by a single mutation (data not shown). Distortion of segregation observed in *Ljsym79-2* and *Ljsym79-3* was described already in *Ljsym79-1*, possibly due to the male gametophytic deficiency (Tansengco et al. 2004). The distortion of segregation in this region was not markedly observed in a B-129 × MG-20 cross (Hayashi et al. 2001). F₂ populations were then utilized for low-resolution mapping using simple-sequence-length polymorphism (SSLP) markers. *Ljsym74-3* was mapped at the northernmost locus of the linkage group 1, where a suppression of recombination was observed (Fig. 3) (Hayashi et al. 2001). *Ljsym80* was mapped between TM0122 and TM0105 on linkage group 1. *Ljsym82* was mapped between TM0076 and TM0021 on linkage group 2. Finally, *Ljsym79-2* and *Ljsym79-3* loci were mapped between TM0151 and TM0024 on linkage group 5.

Nodule structure

of *Ljsym74-3*, *Ljsym79-3*, *Ljsym80*, and *Ljsym82*.

Because all mutant lines examined above showed typical nitrogen-starvation symptoms after inoculation with *M. loti*, detailed analysis of nodule structure was done. *Ljsym79-2* was omitted from the examination because it was allelic with *Ljsym79-3*. One month after inoculation with *M. loti* MAFF303099, all mutants formed many bumps, but failed to develop mature nodules (Fig. 4). To examine whether rhizobia were released in the cells of the central nodule tissue, semithin sections of nodules were prepared. In the wild type, many infected cells were observed in the central nodule tissue (Fig. 5A). In all four mutants, however, infected cells were never observed (Fig. 5B through E). After longer incubation in all mutants except in *Ljsym82*, there were sporadic release events in some parts of the nodules, especially in *Ljsym80* (Fig. 5F). This phenotype has been described previously as type II nodules (Imaizumi-Anraku et al. 1997; Tansengco et al. 2003). The above results led us to investigate whether infection thread development was affected in *Ljsym74-3*, *Ljsym79-3*, *Ljsym80*, and *Ljsym82*.

Abortion of infection thread development

in *Ljsym74-3*, *Ljsym79-3*, *Ljsym80*, and *Ljsym82*.

To visualize infection thread development, *Ljsym74-3*, *Ljsym79-3*, *Ljsym80*, and *Ljsym82* were inoculated with *M. loti* ML001 carrying a *lacZ* reporter gene. Two weeks after inoculation, β-galactosidase activity was assayed. The wild-type B-129 showed typical infection thread development and successful invasion of rhizobia in the inner nodule tissue based on the presence of blue staining (Fig. 6A). In contrast, mutant nodules failed to exhibit visible staining, suggesting the absence of invasion process due to aborted infection threads in the epidermis (Fig. 6B through D). In *Ljsym82*, two types of infection thread abortion were observed. One abortion was at the initiation, where rhizobia failed to enter into the root hair cells, although rhizobia colonized inside the curled tip of root hairs (Fig. 6E). The second point of arrest was during elongation,

Fig. 2. Trichomes on sepals of wild-type and *Ljsym79-3* plants. **A**, Wild type; **B**, *Ljsym79-3*. Bar: 1.0 mm.

Table 1. Segregation of mutant phenotypes in the F₂ progeny of crosses between mutants and MG-20

Line	Locus	F ₂ progeny	Wild-type phenotype	Mutant phenotype	χ^2 ^a
S64	<i>Ljsym74-3</i>	182	143	39	1.24
S66	<i>Ljsym79-2</i>	112	104	8	19.0
1-5A	<i>Ljsym79-3</i>	100	91	9	13.7
N2	<i>Ljsym80</i>	156	116	40	0.03
N4	<i>Ljsym82</i>	158	111	47	1.90

^a $P > 0.05$ when $\chi^2 < 3.84$.

Fig. 3. Mapping of infection thread mutations. Mutant loci were mapped using simple-sequence-length polymorphism markers. The map is based on that of *Lotus japonicus* MG-20 'Miyakojima'.

Fig. 4. Nodulation phenotype of wild-type and mutant plants. Plants were grown for 1 month after inoculation with *Mesorhizobium loti* MAFF303099. **A**, Wild type; **B**, *Ljsym74-3*; **C**, *Ljsym79-3*; **D**, *Ljsym80*; **E**, *Ljsym82*. Arrow indicates nodule. Arrowheads indicate bumps. Bars: 1.0 mm.

when infection threads stopped at the middle of the root hair cell (Fig. 6F). The morphological phenotype of infected root hairs of *Ljsym74-3*, *Ljsym79-3*, and *Ljsym80* was compared with the wild type. Mutant *Ljsym79-3* had infected root hairs similar to that of the wild type (Fig. 6C); however, *Ljsym74-3* and *Ljsym80* typically showed short infected root hairs with thick infection threads inside (Fig. 6B and D).

Numbers of nodules and infection threads in *Ljsym74-3*, *Ljsym79-3*, and *Ljsym80*.

At 2 to 3 weeks after inoculation with *M. loti* ML001, the total number of nodules and bumps and the number of infec-

tion threads were examined (Table 2). To avoid contamination, plants were grown in glass bottles. We were unable to grow *Ljsym82* plants under these conditions, possibly because of the presence of high humidity in the bottles. Two weeks after inoculation with *M. loti*, no significant difference in the total number of nodules and bumps was noted between the wild-type and the mutants. However, 3 weeks after inoculation, the total number of nodules and bumps in *Ljsym74-3* and *Ljsym79-3* were 2 and 1.6 times greater than in the wild-type plants, respectively. For the number of infection threads 3 weeks after inoculation, *Ljsym79-3* and *Ljsym80* were 60 and 40%, respectively, less than in the wild-type plants.

Fig. 5. Histological examination of wild-type and mutant nodules. Plants were grown for 1 month after inoculation with *Mesorhizobium loti* MAFF303099, except F, which was grown for 2 months after inoculation. Nodules were fixed and embedded in Histo-Technovit 7100. Sections were stained with 4',6-diamidino-2-phenylindole (for nuclei) and fluorescent brightener 28 (for cell wall). A, Developing nodule of the wild type; B, *Ljsym74-3*; C, *Ljsym79-3*; D, *Ljsym80*; E, *Ljsym82*; F, a type II nodule of *Ljsym80*. Arrowheads indicate infected cells. Bar: 100 μ m.

DISCUSSION

We have isolated novel symbiotic mutants from the model legume *L. japonicus*. These mutants formed small, white bumps that failed to develop into mature nodules (Fig. 4). When semithin sections of nodules were examined, no infected cells were observed in these mutants (Fig. 5). Detailed analysis showed that development of incomplete nodules lacking infected cells was correlated closely with abnormal development of

infection threads (Fig. 6). From the results of mapping and complementation tests, we have identified *Ljsym74-3*, *Ljsym79-2*, *Ljsym79-3*, *Ljsym80*, and *Ljsym82* (Fig. 3; Table 1). Three of these mutants are allelic with previously reported mutants of *L. japonicus* (Imaizumi-Anraku et al. 1997; Tansengco et al. 2003), but two mutants, *Ljsym80* and *Ljsym82*, have never been characterized in detail. Previously, several other mutants of *L. japonicus* have been isolated that formed small, white bumps (Schauser et al. 1998; Szczyglowski et al. 1998). It is

Fig. 6. Development of infection threads in wild-type and mutant plants. Plants were grown for 2 weeks after inoculation with *Mesorhizobium loti* ML001. **A**, Wild type; **B**, *Ljsym74-3*; **C**, *Ljsym79-3*; **D**, *Ljsym80*; **E**, arrest of infection thread initiation in *Ljsym82*; **F**, arrest of infection thread elongation in a root hair cell of *Ljsym82*. Arrows indicate infection threads. Arrowheads indicate colonized rhizobia. Bar: 100 μ m.

Table 2. Number of nodules, bumps, and infection threads in wild-type and mutant plants 2 and 3 weeks after inoculation with *Mesorhizobium loti* ML001^a

Plants	Plants tested		Nodules		Bumps		Total		Infection threads	
	2	3	2	3	2	3	2	3	2	3
Wild-type	8	7	2.0 $\pm$ 0.76	3.9 $\pm$ 0.78	1.3 $\pm$ 1.28	1.1 $\pm$ 0.90	3.3 $\pm$ 1.04	5.0 $\pm$ 0.82	35.1 $\pm$ 6.29	52.1 $\pm$ 4.45
<i>Ljsym74-3</i>	6	7	0	0	4.5 $\pm$ 1.52	10.7 $\pm$ 2.29	4.5 $\pm$ 1.52	10.7 $\pm$ 2.29	26.3 $\pm$ 4.72	46.9 $\pm$ 5.61
<i>Ljsym79-3</i>	7	6	0	0	3.1 $\pm$ 1.21	8.0 $\pm$ 1.41	3.1 $\pm$ 1.21	8.0 $\pm$ 1.41	11.3 $\pm$ 2.50	19.5 $\pm$ 4.32
<i>Ljsym80</i>	6	5	0	0	2.5 $\pm$ 0.84	6.0 $\pm$ 2.23	2.5 $\pm$ 0.84	6.0 $\pm$ 2.23	14.7 $\pm$ 2.25	31.0 $\pm$ 7.48

^a Numbers shown at 2 and 3 weeks after inoculation.

possible that incomplete nodule development in these mutants is influenced by the abortion of infection thread formation, as shown in the mutants we have identified here. Schauser and associates (1998) reported one example of such phenotype in *Ljsym8* mutant.

Initiation of infection thread development.

In a new mutant, *Ljsym82*, cortical cell division was initiated, although infection thread development was arrested at the early stage (Fig. 6E and F). Nodule primordia of *Ljsym82* also failed to develop into mature nodules (Figs. 4E and 5E). A similar mutant of *Medicago truncatula*, *lin*, also initiates cortical cell division with arrested infection thread initiation at the epidermis (Kuppusamy et al. 2004). Recently, the macrosynteny between *L. japonicus* and *M. truncatula* was investigated (Choi et al. 2004). *Ljsym82* locus is mapped between TM0076 and TM0021 on linkage group 2 (Fig. 6). This region is conserved with linkage group 5 of *M. truncatula*. On the other hand, the *LIN* locus is located on linkage group 1 of *M. truncatula* (Kuppusamy et al. 2004). This fact suggests that the gene responsible for *Ljsym82* is different from the *LIN* gene. Furthermore, *Ljsym82* fails to establish a symbiosis with arbuscular mycorrhizal fungi; arbuscules develop poorly in the cortical cells (K. Senoo, *personal communication*). Recently, it has been reported that *Ljsym82* is allelic with *Ljsym6* (Sandal et al. 2006). The mutant *Ljsym6* shows a symbiotic phenotype with incomplete nodule development and almost no arbuscle formation, which is similar to that of *Ljsym82* (Kistner et al. 2005). There are two candidates for the *Ljsym82* ortholog in pea. One is *Pssym14*, which shows abortion in the infection thread initiation and weak colonization of arbuscular mycorrhizal fungi (Tsyganov et al. 1999). The other is *Pssym36*, which shows aberrant development of infection threads before reaching the bottom of epidermal cells (corresponding to DK24 reported by Sagan and associates [1994]), and loss of arbuscule development (corresponding to RisNod24 in Gianinazzi-Pearson 1996). Because *Pssym14* does not show any cortical cell division, whereas *Pssym36* develops nodule primordia (Tsyganov et al. 2002), *Pssym36* is a more likely candidate for *Ljsym82* ortholog. Still, there is a possibility that this phenotype is determined by the plant species (i.e., pea or *L. japonicus*).

Pleiotropic phenotype of *Ljsym79*.

Ljsym79-2 and *Ljsym79-3* exhibited not only arrested infection threads but also crinkled trichomes and distorted segregation in the F₂ progenies (Figs. 6C and 2B; Table 1). Distorted segregation in nodulation mutants has been reported in a pea mutant, *Pssym40*, which shows hypertrophied infection threads and abnormal endocytosis of bacteria (Tsyganov et al. 1998). Because *Ljsym79*, as well as *Ljsym74*, shows hypertrophied infection threads with incomplete nodules (Imaizumi-Anraku et al. 1997; Tansengco et al. 2003), *Pssym40* is a potential candidate for the *Ljsym79* ortholog, although its trichome phenotype has not been reported. In addition, the primary nodulation phenotype of *Ljsym79*, which is the abortion of infection threads in the epidermis, is not observed in *Pssym40*. Arrest of infection thread development in the epidermis without accompanying cortical cell division is a typical phenomenon in the wild-type *L. japonicus* (M. Hayashi, *unpublished results*). However, in *Ljsym74*, *Ljsym79*, and *Ljsym80*, aborted infection threads are observed with nodule primordia. It is possible that aborted infection threads sometimes are incorporated in nodule tissue, which resulted in the formation of hypertrophied infection threads in a later stage of nodule development. This is in accordance with the observation of two types of nodules in these mutants (Fig. 5F) (Imaizumi-Anraku et al. 1997; Tansengco et al. 2003). Abortion of infection thread development in such mutants is

supposed to be leaky, occasionally giving rise to a limited number of infected cells. Two types of nodules are also identified in the mutants of pea (Novák et al. 1995; Tsyganov et al. 1998) and *M. truncatula* (Bénaben et al. 1995).

Suppression of recombination in *Ljsym74* locus.

Ljsym74 was mapped at the northernmost locus of the linkage group 1 (Fig. 3). This is the region with a severe degree of recombination suppression, probably caused by a translocation of a chromosome segment between *L. japonicus* B-129 'Gifu' and MG-20 'Miyakojima' (Hayashi et al. 2001). Therefore, we need crossing partners other than MG-20 to perform positional cloning of *Ljsym74*. Recently, *L. burtii* and *L. filicaulis* were reported as alternative crossing partners to MG-20 (Kawaguchi et al. 2005; Sandal et al. 2002, 2006). The severe suppression of recombination in this region was not observed when they were crossed with *L. japonicus* B-129 'Gifu' (Kawaguchi et al. 2005; Sandal et al. 2002). Preliminary data indicate that crossing *Ljsym74-3* and *L. burtii* resulted in a good recombination in this region.

Development of infection threads.

We have shown that infection thread development in *Ljsym74-3*, *Ljsym79-3*, and *Ljsym80* were arrested in the epidermal cells (Fig. 6B through D). This result suggests that the invasion of infection threads from the epidermal cell to the outermost cortical cell is tightly regulated. The morphology of infection threads in *Ljsym79-3* seems not to be affected by the mutation because infected root hairs of *Ljsym79-3* appeared to be wild type (Fig. 6C). *Ljsym74-3* and *Ljsym80* have short infected root hairs with thick infection threads compared with the wild type (Fig. 6B and D). Infection threads of *L. japonicus* often develop in elongated root hairs (Szczygłowski et al. 1998). This phenotype also is observed in white clover (Callaham and Torrey 1981) but not in soybean, wherein infection threads develop in short root hairs (Turgeon and Bauer 1982). We hypothesize that genes responsible for developing infection threads or curling of root-hair tips in developing root hairs are affected in *Ljsym74* and *Ljsym80*. It is interesting to note that infected root hairs in these mutants often are accompanied by thick infection threads which occupy almost the whole diameter of the root hair cell. A similar phenomenon of infection thread development has been observed in *Pssym43* (Sagan et al. 1994).

Autoregulation of nodule and infection thread development.

Three weeks after inoculation with *Mesorhizobium loti* ML001, the number of bumps in *Ljsym80* was similar to the total number of nodules and bumps in the wild type (Table 2). However, the number of bumps in *Ljsym74-3* and *Ljsym79-3* was significantly higher than that of nodules and bumps in the wild type (Table 2). This is possibly caused by autoregulation of nodulation, which is affected to a certain degree by Nod factors (van Brussel et al. 2002). Developed nodules are already present 3 weeks after inoculation, and also may trigger autoregulation of nodulation (Caetano-Anollés et al. 1991; Nutman 1952). It is possible that autoregulation triggered by Nod factors is effective in *Ljsym74-3* and *Ljsym79-3* but not that triggered by mature nodules. Thus, these two mutants developed more nodules than wild-type but less than *har1* mutants (Kawaguchi et al. 2002; Wopereis et al. 2000). Autoregulation is also applicable to infection thread development (Vasse et al. 1993). Three weeks after inoculation with *M. loti* ML001, the number of infection threads in all mutants, except in *Ljsym79-3*, was comparable with the wild type (Table 2). This result indicates that autoregulation of infection thread development is not affected in such mutants. Fewer infection threads in *Ljsym79-3*

possibly are caused by the aberrant development of root hairs, as shown in *crinkle* mutant (Tansengco et al. 2003)

Position of infection thread mutants vis-à-vis symbiotic signal transduction pathway.

In summary, we suggest that genes responsible for *Ljsym74*, *Ljsym79*, *Ljsym80*, and *Ljsym82* function downstream of *Nin* because all these mutants show a certain degree of nodule development. From the cytological observation among the mutants, the gene mutated in *Ljsym82* acts earliest in infection thread development because arrest of infection thread initiation was observed only in this mutant. The genes that are involved in fixation, *LjSst1* and *LjLb* (symbiotic leghemoglobin genes), are responsible for later stages, because infected cells are observed in plants generated by either mutation or knockdown (Krusell et al. 2005; Ott et al. 2005). Map-based cloning and molecular characterization of these genes will help to clarify the genetic mechanism of infection thread development in *L. japonicus*.

MATERIALS AND METHODS

Plant materials and bacterial strains.

Mutant lines S64 (*Ljsym74-3*), S66 (*Ljsym79-2*), 1-5A (*Ljsym79-3*), N2 (*Ljsym80*), and N4 (*Ljsym82*) were isolated from M2 seed of *L. japonicus* B-129 'Gifu' treated with EMS by Kawaguchi and associates (2002). Plant lines in the M6 generation were used for further phenotypic characterization. All mutant and wild-type seed were scarified with sandpaper, sterilized with 10% NaClO for 10 min, washed three times with sterile water, and kept in sterile water for 6 to 8 h. Imbibed seeds were germinated on 1.0% agar plates and incubated with shading under a cycle of 16 h at 26°C and 8 h at 23°C in a growth cabinet (LPH-350S; Nippon Medical and Chemical Instruments, Osaka, Japan). Three-day-old seedlings were used for each experiment.

Mesorhizobium loti MAFF303099 was obtained from the Ministry of Agriculture, Forestry and Fisheries, National Institute of Agrobiological Sciences, Japan. *M. loti* MAFF303099 derivative ML001 carrying pGD499 constitutively expressed a *lacZ* reporter gene (Nukui et al. 2004). Rhizobia were cultured at 28°C in tryptone-yeast extract medium with phosphomycin at 100 µg/ml for *M. loti* MAFF303099 and tetracycline at 15 µg/ml for *M. loti* ML001.

Investigation of nodule structure.

Seedlings of mutants and the wild type were transferred to pots containing vermiculite supplied with nitrogen-free B&D medium (Broughton and Dilworth 1971) and 10 µM potassium nitrate. Seedlings were inoculated with *M. loti* MAFF303099 and grown under a cycle of 16 h of light at 26°C and 8 h of dark at 23°C in the growth cabinet. After 1 month, nodules were observed under a SZX12 microscope (Olympus, Tokyo). Nodules were subsequently excised and embedded in Histo-Technovit 7100 (Kulzer, Wehrheim, Germany). Fixation, dehydration, and embedding were carried out as previously described by Tansengco and associates (2003). Semithin 2-µm sections were prepared at using a glass knife with an Ultracut S microtome (Leica, Deerfield, IL, U.S.A.). Sections were stained with a solution consisting of 4',6-diamidino-2-phenylindole at 10 µg/ml and fluorescent brightener 28 at 5 µg/ml (Sigma-Aldrich, St. Louis). Thin sections were observed under a BX50 microscope (Olympus).

Examination of β-galactosidase activity in infection threads.

Seedlings of mutants and the wild type were transferred to pots or 500-ml glass bottles containing vermiculite:perlite mix

(5:1, wt/vol) supplied with B&D medium and 10 µM potassium nitrate. *M. loti* ML001 at a cell density of 10⁸ cells/ml was inoculated. The plants were grown under 16 h of light at 26°C and 8 h of dark at 23°C in a growth cabinet. After 2 or 3 weeks, roots were vacuum infiltrated for 20 min with fixative solution (2% [vol/vol] glutaraldehyde in 1× phosphate-buffered saline [PBS], pH 7.5) and incubated for 1 h at 37°C. Fixed samples were washed three times with PBS and stained for β-galactosidase activity using a solution containing 0.2× PBS (pH 7.5), X-gal at 0.8 mg/ml, 2.5 mM K₃[Fe(CN)₆], 2.5 mM K₄[Fe(CN)₆], and 5-bromo-4-chloro-3-indolyl-β-D-galactoside at 0.8 mg/ml (Nacalai Tesque, Kyoto, Japan). After 20 min of vacuum infiltration, samples were incubated overnight at room temperature in the staining solution. Infection threads were observed under an SZX12 microscope.

Mapping.

Low-resolution mapping was performed using the F₂ progeny resulting from crosses between each mutant and *L. japonicus* MG-20 'Miyakojima' (Kawaguchi et al. 2001). F₂ progenies were inoculated with *M. loti* MAFF303099 and grown in the modified pillow system (Tansengco et al. 2003) supplied with B&D medium and 10 µM potassium nitrate. After approximately 2 months, each plant was scored as either wild type or mutant based on the nodulation phenotype. Low-resolution mapping was carried out with SSLP markers as described previously (Nakamura et al. 2002; Sato et al. 2001). Marker fragments were amplified at 94°C for 5 min; followed by 45 cycles of 94°C for 50 s, 55°C for 30 s, and 72°C for 1 min; and, finally, 72°C for 10 min with GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, U.S.A.). Polymerase chain reaction (PCR) products were subjected to gel electrophoresis in 13% native polyacrylamide, 3% NuSieve 3:1 agarose (Cambrex, Rockland, ME, U.S.A.), or 3% MetaPhor agarose (Cambrex).

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