Root, Root Hair, and Symbiotic Mutants of the Model Legume *Lotus japonicus*

Masayoshi Kawaguchi,^{1,6} Haruko Imaizumi-Anraku,² Hiroyuki Koiwa,³ Sinobu Niwa,⁴ Akira Ikuta,⁴ Kunihiro Syono,⁵ and Shoichiro Akao²

¹Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 153-8902 Japan; ²National Institute of Agrobiological Resources, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602 Japan; ³Iwate Biotechnology Research Center, Narita 22-174-4, Kitakami, Iwate 024-0003 Japan; ⁴Science University of Tokyo, 2641 Yamazaki, Noda-shi, Chiba 278-8510 Japan; ⁵Nihon Women's University, Mejirodai 2-8-1, Bunkyo-ku, Tokyo 112-8681 Japan; ⁶PRESTO, Japan Science and Technology Corporation

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To gain an overview of plant factors controlling nodule number and organogenesis, an extensive screening using model legume *Lotus japonicus* was carried out. This screening involved 40,000 M2 seeds, and 32 stable mutant lines were isolated. From these, 16 mutant lines maintaining the phenotypic variation were selected and genetically analyzed. With respect to nodule number, four loci were identified, *Ljsym77*, *Ljsym78*, slippery root (*slp*), and radial organization1 (*rdo1*). The former two mutants have an increased number of nodules, while the latter two have a decreased number. *Ljsym78-1* and *Ljsym78-2* are hypernodulating mutants with a branched root system and were found to be allelic to *Ljsym16*. The phenotype of the *Ljsym77* mutant was highly pleiotropic, being deficient in light and gravity responses. The *slp* mutant was isolated as a low-nodulating mutant lacking root hairs. Concerning nodule organogenesis, nine symbiotic loci were identified, including the two loci *alb1* and *fen1*. Mutants affecting the developmental process of nodule organogenesis were placed in three phenotypic categories: Nod⁻ (*Ljsym70* to *Ljsym73*), Hist⁻ (*alb1-1*, *alb1-2*, and *Ljsym79*), and Fix⁻ (*fen1*, *Ljsym75*, and *Ljsym81*).

Additional keywords: root development.

The formation of a nitrogen-fixing root nodule is a typical example of postembryonic development regulated by environmental stimuli. For example, Nod factors (lipo-chitoooligosaccharides [LCO]) are produced by symbiotic rhizobia and serve as positive regulators of nodule formation and infection (Dénarié et al. 1996; Schultze and Kondorosi 1998; Spaink 1992; Spaink and Lugtenberg 1994). In contrast, ethylene (Heidstra et al. 1997; Penmetsa and Cook 1997) and nitrogen in the form of NO₃⁻ and NH₄⁺ are negative regulators.

In the last 20 years, extensive studies on the rhizobial genes have resulted in the successful identification and characterization of various kinds of symbiotic genes (Fischer 1994;

Gonzalez et al. 1996; Hirsch 1992; Long 1996). To dissect the genetic network underlying the process of nodule organization from the plant side, symbiotic mutants have been isolated from pea (Borisov et al. 1992; Duc and Messenger 1989; Jacobsen and Feenstra 1984; Kneen and LaRue 1988; Kneen et al. 1994), soybean (Devine and Kuykendall 1996), common bean (Park and Buttery 1992; 1994), fava bean (Duc 1995; Haser et al. 1992), alfalfa (Dudley and Long 1989), and sweet clover (Caetano-Anolles and Gresshoff 1991; Kneen and LaRue 1988; Stougaard 2001; Sugauma 1999). Characterization of these mutants has revealed the existence of genes responsible for nodule initiation, bacterial invasion, nitrogen fixation, and autoregulation. However, because the tools for molecular genetic analysis of leguminous plants are still in the process of being developed, there has been only one report of the molecular cloning of a symbiotic gene.

In order to establish a more tractable approach to the study of plant genes involved in nodulation, recent studies have used several diploid autogamous legumes as genetic systems. Besides the pea, soybean, and sweet clover models, studies have been initiated on *Lotus japonicus* as a determinate-nodulation host (Handberg and Stougaard 1992) and *Medicago truncatula* as an indeterminate nodulation host (Barker et al. 1990; Cook 1999). In *L. japonicus*, transformation procedures via either *Agrobacterium tumefaciens* or *A. rhizogenes*, or both, have been published (Martirani et al. 1999; Handberg et al. 1994; Oger et al. 1996; Stiller et al. 1997). In *Medicago truncatula*, symbiotic mutants and natural variants have been reported and characterized (Bénaben et al. 1995; Catoira et al. 2000; Penmetsa and Cook 1997; Sagan et al. 1995; Tirichine et al. 2000). The infrastructure of a genome analysis has been developed by several researchers (Jiang and Gresshoff 1997; Kawaguchi et al. 2001; Men et al. 2001; Sandal and Stougaard 1998). In parallel with these studies, symbiotic mutants deficient in rhizobial and mycorrhizal associations have been isolated and characterized (Hayashi et al. 2000; Stougaard 2001) for nodulation (Imaizumi-Anraku et al. 1997, 2000; Schauser et al. 1998; Szczyglowski et al. 1998; Wopereis et al. 2000) and for mycorrhization (Bonfante et al. 2000; Senoo et al. 2000; Solaiman et al. 2000; Wegel et al. 1998). Noteworthy is the *Ac* transposon tagging that was developed for *L. japonicus* (Thyckjær et al. 1995). From this, the molecular cloning of the symbiotic gene *Nin*, which is responsible for nodule inception (Schauser et al. 1999), was carried out.

To obtain an overview of factors that are controlled by the plant genome, an extensive screening of mutants affecting nod-

Corresponding author: M. Kawaguchi; Department of Environmental Sciences, Faculty of Science, Niigata University, Ikarashi, Ninomachi 8050, Niigata City, Niigata, 950-2181, Japan; Telephone and Fax: +81-25-262-6172; E-mail: masayosi@sc.niigata-u.ac.jp

Current address of S. Niwa: Amersham-Pharmacia Japan, 3-25-1 Hyakunincho, Shinjuku, Tokyo 169-0073 Japan.

ule number and organogenesis was conducted using the model legume *L. japonicus*.

RESULTS

Isolation of mutants affecting nodule number and organogenesis.

L. japonicus 'Gifu' B-129 seeds (8,000) treated with 0.4% ethylmethane sulphonate (EMS) were sown and germinated in a greenhouse setting. M2 seeds were collected from approximately 3,000 M1 plants. Screening of 40,000 M2 seeds (10 seeds per seed family) enabled the isolation of 32 mutant lines with phenotypes that were stably inherited up to the M4 and M6 generations. The mutants were divided into two classes, root and root hair mutants affecting the number of effective nodules (5 mutant lines) and symbiotic mutants affecting nodule organogenesis (27 mutant lines). In the root and root hair mutants class, we identified two loci that conferred an increased number of nodules when mutated (*Ljsym77* and *Ljsym78*) and two loci that produced fewer nodules (slippery root [*slp*] and radial organization1 [*rdo1*]). Allelism tests were also carried out using 11 representative mutants out of the total 27 symbiotic mutants. Nine loci affecting nodule organogenesis (*Ljsym70* through *Ljsym76*, *Ljsym79*, and *Ljsym81*) were subsequently identified including *alb1* and *fen1*, which had been previously reported by Imaizumi-Anraku and associates (1997). Phenotypic traits of 16 mutants, except *Ljsym79*, were segregated as monogenic recessives (Table 1).

Root and root hair mutants affecting nodule number.

Enhanced nodulation mutant, Ljsym77. The *Ljsym77* mutant was isolated by screening on agar plates. The number of nodules observed on the roots of the *Ljsym77* mutant grown on

agar plates was two to three times greater than that on wild-type plants (Fig. 1B and C). The zone of nodule differentiation in the *Ljsym77* mutant was wider than that of wild type (Fig. 1B and C). In contrast to typical hypernodulating mutants, the *Ljsym77* mutant phenotype is highly pleiotropic. When grown on agar plates in the absence of bacteria, the hypocotyls and shoots of the *Ljsym77* mutant were longer than those of wild type. In addition, the lateral roots grew in a nearly horizontal direction (Fig. 1A). Furthermore, the root and hypocotyl of the mutant were thinner than those of wild type (data not shown). When illuminated with white light, the surfaces of wild-type nodules and roots turned green (Fig. 1B), whereas those of the *Ljsym77* mutant did not (Fig. 1C), indicating that the root is also defective in light response. Although the *Ljsym77* plant was not as strong as the hypernodulating mutants, it was self-supporting when the seedlings were placed on the agar plate.

Hypernodulating mutants with a branched root system, Ljsym78. Two mutants, *Ljsym78-1* and *Ljsym78-2*, were isolated as hypernodulating mutants with a highly branched root system. When the mutant seedlings were grown in vermiculite immersed with bacteria-free B & D medium (Broughton and Dilworth, 1971) containing 0.5 mM potassium nitrate, the numbers of lateral roots in the *Ljsym78-1* and *Ljsym78-2* mutants were 3.2 and 1.7 times greater than those in wild-type seedlings, respectively (Fig. 2A and B). The primary roots of both mutant seedlings were shorter than wild-type roots (Fig. 2A). When *Mesorhizobium loti* JRL501 bacteria was inoculated onto the roots, the growth of the *Ljsym78* seedlings was severely inhibited compared with wild-type growth (Fig. 2C), due to excessive nodule formation. The *Ljsym78-1* mutant seedlings developed approximately two times more nodules than *Ljsym78-2* under various concentrations of potassium nitrate (Fig. 2D). From the dose-response analysis of nitrate on

Table 1. Sixteen representative mutants of *Lotus japonicus* affecting nodule number and organogenesis^a

Genetic loci	Renaming of loci	Phenotypic classes	F2 segregation ($P > 0.05$)	Mycorrhizal colonization ^b	Pleiotropy	Other comments
Nodule organogenesis						
<i>Ljsym70</i>		Nod ⁻	121:38 ($\chi^2 = 0.102$)	Ami ⁺		
<i>Ljsym71-1</i>		Nod ⁻	131:48 ($\chi^2 = 0.314$)	Co ⁻		Mycorrhizal colonization blocked at exodermis
<i>Ljsym71-2</i>		Nod ⁻	ND	Co ⁻		Mycorrhizal colonization blocked at exodermis
<i>Ljsym72</i>		Nod ⁻	89:24 ($\chi^2 = 0.852$)	Ami ^{-c}		Mycorrhizal colonization blocked at epidermis
<i>Ljsym73</i>		Nod ⁻	53:17 ($\chi^2 = 0.0190$)	Ami ⁺		Nodulation at very low frequency
<i>Ljsym74-1</i>	<i>alb1-1</i>	Hist ⁻	324:108 ($\chi^2 = 0.240$) ^d	Ami ⁺		Deficient in bacterial infection and nodule vascular bundle formation
<i>Ljsym74-2</i>	<i>alb1-2</i>	Hist ⁻	103:29 ($\chi^2 = 0.646$)	Ami ⁺		Strong allele of <i>alb1</i>
<i>Ljsym75</i>		Fix ⁻	95:28 ($\chi^2 = 0.327$)	Ami ⁺		Many small vacuoles
<i>Ljsym76</i>	<i>fen1</i>	Fix ⁻	126:39 ($\chi^2 = 0.163$) ^d	Ami ⁺		
<i>Ljsym79</i>		Hist ⁻	114:14 ($\chi^2 = 13.5$) ^e	Ami ⁺	Pleiotropic	Small pod
<i>Ljsym81</i>		Fix ⁻	85:29 ($\chi^2 = 0.0116$)	Ami ⁺		Many small vacuoles
Nodule number (increase)						
<i>Ljsym77</i>		Nod ⁺⁽⁺⁾ ^f	50:15 ($\chi^2 = 0.128$)	ND	Pleiotropic	Reduced sensitivity to light and gravity
<i>Ljsym78-1</i>	<i>har1-4</i>	Nod ⁺⁺	59:19 ($\chi^2 = 0.0171$)	Arb ^{+++g}	Pleiotropic	Branched root system, allelic to <i>Ljsym16</i>
<i>Ljsym78-2</i>	<i>har1-5</i>	Nod ⁺⁺	119:31 ($\chi^2 = 1.50$)	Arb ^{+++g}	Pleiotropic	Branched root system, allelic to <i>Ljsym16</i>
Nodule number (decrease)						
slippery root	<i>slp</i>	Low nod	63:16 ($\chi^2 = 0.949$)	Ami ⁺	Pleiotropic	Lack of root hairs
radial organization1	<i>rdo1</i>	Low nod	103:32 ($\chi^2 = 0.120$)	ND	Pleiotropic	Thick and short root, counterclockwise-directed leaflets

^a This table comprises half of the 32 mutants generated by Kawaguchi's laboratory. The remaining half comprises seven Nod⁻, four Hist⁻, and five Fix⁻ mutants. To illustrate complementation groups of all mutants, we are mapping them on the *L. japonicus* linkage map.

^b According to genetic nomenclature guidelines (Stougaard et al. 1999) and recent data (Senoo et al. 2000, Solaiman et al. 2000).

^c Completely no formation of arbuscules and vesicles.

^d Previously reported by Imaizumi-Anraku and associates (1997, 2000).

^e Distorted segregation in F2 progeny.

^f Two to three times more nodules on agar plate.

^g Increased arbuscule formation (Solaiman et al. 2000).

nodule development, *Ljsym78-1* and *Ljsym78-2* mutants appear to be tolerant to 3 mM potassium nitrate (Fig. 2D). However, higher concentrations of nitrate significantly inhibited the development of *Ljsym78-1* and *Ljsym78-2* nodules but not the formation of nodule primordia (data not shown). Unexpectedly, the tendency seemed to be similar in both wild and mutant types (Fig. 2D). In contrast to nodulation, the number of lateral roots in the wild type and the *Ljsym78* mutants was apparently not suppressed by higher concentrations of potassium nitrate (data not shown). The phenotype of the *Ljsym78* mutants is very similar to that of the *har1-1* and its allele *Ljsym16* mutants reported previously (Schauser et al. 1998; Szczygłowski et al. 1998; Wopereis et al. 2000). Allelism tests revealed that *Ljsym78-1* and *Ljsym78-2* are allelic with *Ljsym16*, indicating that they are *har1-4* and *har1-5* respectively (data not shown).

Root hair mutant, *slp*. The *slp* mutant, a low-nodulating mutant, was isolated by careful screening on agar plates. Microscopic observation of the *slp* mutant revealed that the root almost completely lacks root hairs (Fig. 3A and B). The *slp* mutant could grow as well as wild type in spite of the lack of root hairs; a trait that differs from the root-hairless mutants of *Arabidopsis*, which are all seedling lethal (Schneider et al. 1997). When the *slp* seedlings were pulled out of the agar, the roots came out easily without breaking due to the lack of root hairs, which, if present, would keep the roots entrenched within the agar. Upon inoculation with *M. loti*, bacteria could be seen colonizing the *slp* even though very few root hairs were present. Moreover, once bacteria entered the root cells, nodules developed normally. (Fig. 3C and D). This is the first example found in legumes of a low-nodulating mutant deficient in the development of root hairs.

Mutant affecting radial organization, *rdo1*. The *rdo1* mutant was isolated as a low-nodulating mutant with aberrant radial organization. The *L. japonicus rdo1* mutant exhibited the thick- and short-root phenotype (Fig. 4A). Shoots of the *rdo1* mutant were also thicker and shorter than those of wild type. These phenotypes are similar to those shown by *sym17*, a pleiotropic mutant of pea (Kneen et al. 1994). Transverse sections of the

primary root 1 cm distal to the root tip revealed that the cell number and the volume of the primary root of the mutants were greater than those of the wild-type root, especially in the root cortex (Fig. 4B and C). Interestingly, leaflets from the *rdo1* mutant originated from the shoot tip in a counterclockwise direction when the seedling was observed from above (Fig. 4D and E).

Developmental and infectious pathways to an effective nodule.

Mutants affecting the developmental process of nodule organogenesis were placed in three phenotypic categories: Nod⁻ (*Ljsym70* to *Ljsym73*), Hist⁻ (*alb1-1*, *alb1-2*, and *Ljsym79*), and Fix⁻ (*fen1*, *Ljsym75*, and *Ljsym81*). Hist⁻ is a mutant category used to represent defects in 'cooperative histogenesis' elicited by symbiotic interactions, and it is defined as the stage between Nod⁻ and Fix⁻. Fix⁻ is defined as a mutant category with ineffective nodules irrespective of the presence of many infected cells.

Nod⁻ mutants.

Four genes were identified from allelism tests using five Nod⁻ mutant lines. Genes *Ljsym70*, *Ljsym71-1*, *Ljsym71-2*, and *Ljsym72* were nonnodulating (Nod⁻), but very small bumps on the *Ljsym70* root were induced after prolonged incubation with *M. loti* TONO and JRL501 strains. In contrast, the *Ljsym73* gene elicited effective, large nodules at a very low frequency. Among these mutants, no clear strain specificity could be observed. Nod⁻ phenotypes can be due either to a mutation in Nod-factor signaling or the lack of *nod*-gene inducers secreted from the root. To distinguish between these two possibilities, we utilized *M. loti* JRL501 and TONO strains carrying the plasmid pMP2112, which produces Nod factor in response to the *nod*-gene inducer naringenin. Production of Nod factors was confirmed by high-pressure liquid chromatography analysis with an octyldecyl silane column after the application of naringenin at a concentration of 10⁻⁷ M (Niwa et al. 2001). Inoculation of roots with *M. loti* JRL501 pMP2112 in the pres-

Fig. 1. Phenotypes of the *Ljsym77* mutant grown on an agar plate. **A**, Wild-type (left) and *Ljsym77* mutant seedlings (third from the right) in the absence of *Mesorhizobium loti*. **B**, Wild-type and *Ljsym77* mutant (right) seedlings 31 days after inoculation with *M. loti* JRL501. **C**, Seedlings of the *Ljsym77* mutant 31 days after inoculation with *M. loti* JRL501. Note that the zone of nodule development is wider than in the *Ljsym77* mutant seedling. Bars = 5 mm.

Fig. 2. Phenotypes of the hypernodulating mutants. **A**, Seedlings of wild type (left) and *Ljsym78-1* mutant (right) with branched root system. The seedlings were grown in vermiculite immersed with bacteria-free B & D medium (Broughton and Dilworth 1971) containing 0.5 mM potassium nitrate for 3 weeks. **B**, Number of lateral roots in the bacteria-free seedlings. Lateral roots were counted 3 weeks after germination. **C**, Appearance of seedlings on agar plate containing B & D medium 17 days after inoculation with *Mesorhizobium loti* JRL501. Wild type is on the left, and the *Ljsym78-2* mutant is on the right. Bar = 5 mm. **D**, Effects of potassium nitrate on nodule number in wild-type and *Ljsym78* seedlings. Small developing and mature nodules were counted 3 weeks after inoculation with *M. loti* MAFF 303099. Mean values with standard deviations were obtained from at least seven seedlings.

ence of naringenin did not overcome the Nod^- phenotype, indicating that the Nod^- phenotype of *Ljsym70* to *Ljsym73* mutants is not due to the absence of *nod*-gene inducers. The four loci were further differentiated by the phenotypic characterization of mycorrhizal colonization (Table 1) (Senoo et al. 2000). The *Ljsym71-1*, *Ljsym71-2*, and *Ljsym72* genes are mycorrhizal mutants, while *Ljsym70* and *Ljsym73* are not, indicating that the former three genes are part of a common signaling pathway between rhizobia and mycorrhiza, whereas the latter two genes are required specifically for rhizobial interaction.

Hist⁻ mutants.

The *alb1-1* (*Ljsym74-1*) mutant was previously reported as an ineffective mutant deficient in successful bacterial entry (Imaizumi-Anraku et al. 1997) and development of nodule vascular bundles, concomitant with strong suppression of *ENOD40* gene expression (Imaizumi-Anraku et al. 2000). In the *alb1-1* nodule, bacteria often failed to enter the host cells and, consequently, resided in intercellular spaces in the nodule (Fig. 5A). Possible necrotic patches were also observed on the *alb1-1* nodule surface (Fig. 5C).

From genetic analysis of the Hist⁻ mutants, we found another allele of the *alb1* mutant, *alb1-2*. At an early stage, the phenotype of the *alb1-2* mutant was indistinguishable from that of a Nod^- mutant. Three weeks after inoculation, the *alb1-2* mutant elicited nodulelike structures on the root (Fig. 5B and D). Histological characterization revealed that the nodulelike structure was made of a nodule primordium but lacked nodule vascular bundles, a differentiated nodule endodermis, and a bacterial infection zone (Fig. 5B). When inoculated with *M. loti* JRL507 containing a *gusA* gene, few infection events were observed in the *alb1-2* roots 22 days after inoculation (Fig. 5D), while bacteria colonized in the intracellular space of *alb1-1* nodules (Fig. 5C). The phenotype of the *alb1* mutants resembles that of the *Ljsym8* mutant reported by Schauser and associates (1998). Allelism tests found *alb1* to be distinct from *Ljsym8*.

The *Ljsym79* mutant induced nodulelike structures on roots at a low frequency. Infection of *M. loti* JRL501 was severe (data not shown). In addition, the pods of the *Ljsym79* plant were smaller than those of wild-type plants, even when the mutant was grown in nutrient-rich soil. The *Ljsym79* mutant was backcrossed twice, but the inheritance of the mutant phenotype was distorted in the F₂ population and did not fit typical Mendelian ratios (Table 1). The *Ljsym79* mutant appeared to be a pleiotropic mutant that affects both nodule organogenesis and pod development.

Fix⁻ mutants.

The nodules of wild type and three ineffective mutants were compared 22 days after inoculation. Brightfield microscopy of longitudinal sections of the wild-type nodule revealed enlarged infected cells packed with bacterial endosymbionts in the central zone of the nodule (Fig. 6A). In contrast, many small vacuoles were clearly visualized in the infected cells of the *Ljsym75* nodules prior to maturation (Fig. 6B). Many vacuoles were also observed in the infection cells of the *Ljsym81* nodules (data not shown). However, in the *fen1* mutant, which had been reported as an ineffective mutant enlarging infected cells (Imaizumi-Anraku et al. 1997), no prominent accumulation of small vacuoles was found in infected cells (Fig. 6C). In place of vacuoles, granulelike structures were observed in several infected cells of the *fen1* nodule (Fig. 6C). In order to monitor the colonization of symbionts, we used *M. loti* JRL507, which is marked with *gusA*. Upon inoculation of wild-type roots with *M. loti* JRL507, a blue color was found in nodules throughout development (Fig. 6D). Interestingly, in the *Ljsym75* and *fen1*

mutants, a blue color was observed in developing nodules but not in fully formed nodules (Fig. 6E and F).

Finally, a summary of the symbiotic mutants affecting nodule organogenesis is found in Figure 7.

DISCUSSION

Defects in root and root hair development affect nodule number.

The results of this study indicate that the mutant *Ljsym77* is highly pleiotropic with enhanced nodulation. The hypocotyls and shoots of the *Ljsym77* mutant were longer than those of the wild type. The lateral roots of the *Ljsym77* mutant grew in a nearly horizontal direction (Fig. 1A). When illuminated with white light, the surfaces of wild-type roots turned green (Fig. 1B), whereas those of the *Ljsym77* mutant did not (Fig. 1C). These features exhibited by the *L. japonicus Ljsym77* mutant are reminiscent of those shown by the *Arabidopsis hy5* mutant that is deficient in a variety of responses, including light-dependent hypocotyl elongation and gravitropism (Oyama et al. 1997). Interestingly, the *hy5* mutant elicits enhanced lateral root formation. The *HY5* gene has been isolated by T-DNA tagging and shown to encode a transcriptional factor with a bZIP motif (Oyama et al. 1997). The unphosphorylated isoform of the *HY5* gene product in *Arabidopsis* strongly prevents the generation of lateral roots, suggesting that the phosphorylation of the *HY5* protein regulates lateral root formation (Hardtke et al. 2000). This information leads us to speculate that the *Ljsym77* mutant may encode an *HY5* homolog and act as a regulator of nodule initiation in place of lateral root formation. A detailed characterization of the *Ljsym77* mutant and the molecular cloning of the gene responsible for the pleiotropic mutant phenotype will be published later.

The *Ljsym78-1* and *Ljsym78-2* mutants were isolated as hypernodulating mutants with a branched root system. It was found that the branched system was formed by the restriction of primary root growth and the promotion of lateral root formation (Fig. 2A and B). This root system was also prominent in the absence of *M. loti*. Since these phenotypes resemble *L. japonicus har1* hypernodulating mutants (Wopereis et al. 2000), the *Ljsym78-1* and *Ljsym78-2* mutants were reciprocally crossed with *Ljsym16*, an allele of the *har1* locus, *har1-3*. As a result, our *Ljsym78-1* and *Ljsym78-2* mutants were found to be allelic to *Ljsym16*, named *har1-4* and *har1-5* respectively. Analysis of the effects of nitrate on nodule number showed that both mutants seemed to be tolerant of up to 3 mM of potassium nitrate. Contrary to our expectation, nodule formation on the *Ljsym78-1* and *Ljsym78-2* roots was apparently suppressed by the application of 5 or 10 mM of potassium nitrate, although the formation of nodule primordia was not inhibited by high concentrations (Fig. 2D). The tendency seemed to be similar in both wild type and mutants. Therefore, detailed physiological analysis (for example, the analysis of the effects of nitrogen compounds on nitrogen-fixation activity as well as nodule formation) will be required to reveal the relationship between the hypernodulation phenotype of *har1* mutants and nitrate tolerance. Wopereis and associates (2000) have reported that nodule morphogenesis on *har1-1* mutant plants was insensitive to 5 to 15 mM of combined nitrogen. Although we did not examine the effects of nitrogen compounds under the same conditions, the different responses to nitrate among alleles suggest that *har1-1* may be the strongest allele of this locus. Recently, a mutation in the *Ljsym78* locus has been shown to enhance mycorrhiza formation and arbuscule differentiation (Solaiman et al. 2000), suggesting that the *LjSym78* gene product negatively regulates mycorrhiza formation as well as lateral organ development.

Radial organization of the root is important for nodule formation, because the location of the cell divisions for the development the nodule primordium is highly restricted. For example, in a determinate-nodule host, cell divisions leading to nodule primordia formation are initiated in the outer cortex, whereas in an indeterminate-nodule host, they take place in the inner cortex (Hirsch 1992). The *rdol* mutant with its decreased number of nodules is not a symbiotic mutant per se, but rather, a root mutant. The increased number and volume of cortical cells in the roots of the *rdol* mutant is correlated with a decrease in the efficiency of nodule formation. In contrast, the root of the *har1* mutant, which shows a dramatic increase in nodule number, is thinner and shorter than the wild-type root. A short and thin root was shown to be due to a reduced number or volume of cells in the radial orientation (Wopereis et al. 2000). The root of the *Ljsym77* mutant is both thinner and longer than wild-type roots. An increase in nodule number may correlate with the formation of thin roots.

Fig. 3. Phenotypes of the *slp* mutant. **A and B**, Roots of seedlings two weeks after inoculation with *Mesorhizobium loti* JRL501. **A**, Wild type. **B**, *slp* mutant. **C and D**, Colonization of *M. loti* JRL507 carrying the *gusA* gene. **C**, Wild type; **D**, *slp* mutant. Arrowheads indicate colonization of *M. loti* JRL507 on nodule primordia. Bars = 1 mm.

Fig. 4. Phenotypes of the *rdo1* mutant. **A**, Seedlings of wild type (left) and *rdo1* mutants (two plants on the right) 22 days after inoculation. **B and C**, Transverse sections of primary root 1 cm above root tips. **B**, Wild type; **C**, *rdo1* mutant. Bars = 50 μ m. **D and E**, Seedlings observed from above. **D**, Wild type; **E**, *rdo1* mutant. Note that leaflets of *rdo1* mutant are generated in the counterclockwise direction.

Fig. 5. Phenotypes of the *alb1* mutants. **A and B**, Longitudinal sections of nodulelike structures 22 days after inoculation. **A**, *alb1-1*; **B**, *alb1-2*. Asterisk indicates bacteria remaining in intercellular space. Bars = 50 μ m. **C and D**, Colonization of *Mesorhizobium loti* JRL507 carrying the *gusA* gene. **C**, *alb1-1* nodule. Arrowheads indicate possible necrotic patches. **D**, *alb1-2* nodule primordium.

The significance of the root hair for nodulation was demonstrated by the isolation of the *slp* mutant, which was almost hairless. Nevertheless, bacterial colonization of the root, and even nodule formation, was not completely inhibited in this mutant (Fig. 3D). Careful observation of the early stages of infection should provide new insights into bacterial entry and nodule formation.

Cooperative histogenesis leading to a nitrogen-fixing nodule.

Until now, symbiotic mutants incapable of forming effective nodules have been divided into two categories, *Nod*[−] and *Fix*[−]. In this case, *Fix*[−] phenotypes include small bumps, bumps, nodulelike structures, and outgrowths, as well as ineffective nodules containing infected cells. When arranged according to the phenotypic variation of the symbiotic mutants, it was not always easy to distinguish between *Nod*[−] and *Fix*[−]. For example, a wild-type seedling elicits effective nodules 3 weeks after inoculation, while the *alb1-2* mutant induces only small nodulelike structures (Fig. 5B and D). The early stages of the *alb1-2* and *Ljsym79* mutants were indistinguishable from those of *Nod*[−] mutants. In contrast, we believe that it would be feasible to divide the *Fix*[−] category into an infectious process accompanied by cooperative histogenesis and a functional process exhibiting symbiotic nitrogen fixation. The former produces a nodulelike structure lacking a developed infection zone consisting of infected and uninfected cells, whereas the latter causes the development of an infection zone. In this report, the

former mutants are placed into a category named *Hist*[−]. Judging from the definition, the *alb1-1* mutant, which is defective in tissue differentiation such as nodule vascular bundles and bacterial infection (Imaizumi-Anraku et al. 1997, 2000), is a typical *Hist*[−] mutant, and the *alb1-2* and *Ljsym79* mutants can also be placed in this category. In contrast, the *Fix*[−] category is defined as having symbiotic mutants with ineffective nodules irrespective of the presence of many infected cells, and therefore, *fen1*, *Ljsym75*, and *Ljsym81* can be placed into the *Fix*[−] category.

Possible necrotic patches were conspicuous on the *alb1-1* nodule surface (Fig. 5C). The cell is reminiscent of brown cells elicited by abortive infection during the *Sinorhizobium meliloti*–alfalfa symbiotic interaction (Vasse et al. 1993). Although it is unclear whether the brown patches are related to abortive infection of *M. loti*, it is important to characterize the *alb1-1* mutant according to the plant defense response.

Nod[−] mutants and *Fix*[−] mutants.

The phenotype of the *Ljsym70* to *Ljsym73* mutants was not overcome by inoculation of their roots with *M. loti* JRL501 pMP2112 in the presence of naringenin. This result indicates that the *Nod*[−] phenotype appears to be unrelated to the absence of *nod*-gene inducers. It seems possible that the nonnodulating phenotype of the *Nod*[−] mutants is caused by a mutation in *Nod* factor recognition and subsequent signaling. Recently, LCO were purified from the culture of *M. loti* strains and major

Fig. 6. Histological characteristics of two *Fix*[−] mutants. **A to C**, Longitudinal sections of nodules 22 days after inoculation with *Mesorhizobium loti* JRL501. **A**, Wild type. **B**, *Ljsym75*. Arrows indicate infected cells containing many vacuoles. **C**, *fen1*. Arrowheads indicate infected cells having granulike structures. Bars = 50 μ m. **D to F**, Colonization of *M. loti* JRL507 carrying the *gusA* gene 3 weeks after inoculation. **D**, Wild type; **E**, *Ljsym75*; **F**, *fen1*.

components were identified (López-Lara et al. 1995; Niwa et al. 2001). Nodule primordia, together with the expression of *LjENOD40* and *LjENOD2*, could be efficiently induced by the application of LCO (Niwa et al. 2001). The purified LCO could be used to assess the sensitivity of *Nod*⁻ mutants. Equally, it would be worthwhile to examine the effect of LCO on *Hist*⁻ and *Nod*⁺ mutants.

In the *Ljsym75* and *fen1* mutants, a blue color indicating colonization of *M. loti* JRL507 was observed in developing nodules but not in fully formed nodules (Fig. 6E and F). Histological analysis revealed that infected cells were highly vacuolated in the *Ljsym75* nodule (Fig. 6B), whereas granulelike structures were observed in the infected cells of the *fen1* nodule (Fig. 6C). As reported previously, highly vacuolated, infected cells are the most typical phenotype of premature senescence in ineffective nodules. For example, Morzhina and associates (2000) described how infected cells of pea *sym32* mutants were heavily vacuolated and rapidly destroyed. Without vacuolation, however, disintegration of the cytoplasm was advanced in the infected cells of *sym26*, *sym27*, and *RisFixN* mutant lines. To address the question of which *Lotus* mutants phenotypically correspond to these pea ineffective mutants, more detailed characterization of the *Ljsym75*, *Ljsym81*, and *fen1* mutants will be required.

Determining the order of the mutant loci in *L. japonicus*.

The aim of this study was to obtain an overview of the mutational spectrum of *L. japonicus* relative to nodule number and organogenesis through an extensive screening of mutants. From this study, we discovered several novel mutants. However, due to the presence of numerous symbiotic mutants (Schauser et al. 1998; Szczygłowski et al. 1998), we did not

carry out complete allelism tests among all mutants. As long as the symbiotic loci are unmapped, it will take a long time to identify the new locus responsible for nodule number and organization. Recently, an early flowering *L. japonicus* 'Miyakojima' MG-20 has been shown to be a suitable crossing partner of a standard 'Gifu' B-129 (Kawaguchi 2000; Kawaguchi et al. 2001). A high-density linkage map is currently being constructed using the F2 population derived from a cross between 'Gifu' and 'Miyakojima'. Similar linkage maps have also been constructed using *L. filicaulis* (Sandal and Stougaard 1998) or *L. japonicus* 'Funakura' (Jiang and Gresshoff 1997) as a crossing partner of 'Gifu' B-129. These mapping strategies will allow us to order the many mutant loci in *L. japonicus*.

MATERIALS AND METHODS

Plant materials and bacterial strains.

Seeds of *L. japonicus* 'Gifu' B-129 were used as the parental line. For the symbiont, we used the *M. loti* JRL501 strain, a nalidixic acid-resistant variant of *M. loti* MAFF 303099 (National Institute of Agrobiological Resources [NIAR], Tsukuba, Japan) (Imaizumi-Anraku et al. 1997), the TONO strain (a wild symbiont isolated from a Japanese accession of *L. japonicus*, MG-10 'Tono'), and the NZP2235 strain. These strains, which have good nodule-forming ability, were cultured in tryptone-yeast extract medium at 28°C and maintained on yeast extract-mannitol agar plates at 4°C.

The plasmid pMP2112, which contains the *nodD* gene of *Rhizobium leguminosarum* bv. *trifoli* (López-Lara et al. 1995), was introduced into *M. loti* JRL501 from *Escherichia coli* KMBL1164 by a triparental mating. *M. loti* JRL507 containing

Fig. 7. Summary of symbiotic mutants affecting nodule organogenesis.

a constitutive *gusA* expression construct was obtained by introducing pmTn5SS*gusA*20 from *E. coli* S17-1 (Wilson et al. 1995) into *M. loti* JRL501. *M. loti* JRL501 (pMP2112) and JRL507 were maintained on YM agar plates with 100 μ M spectinomycin.

Screening of mutants.

Inbred seeds of *L. japonicus* 'Gifu' B-129 were mutagenized with 0.4% EMS for 16 h. After rinsing with distilled water, 8,000 M1 seeds were sown and germinated in Power Soil (Kanto Hiryo Kogyo Co. Ltd., Tokyo) at a density of one plant per 20 cm². M1 plants were grown in an air-conditioned greenhouse at NIAR, in order to later collect the M2 seeds. Twenty-nine thousand M2 seeds (10 seeds per seed family) were then sown in vermiculite, watered with nitrogen-free ¹/₂ nutrient solution (Imaizumi-Anraku et al. 1997), and grown in a greenhouse under a 16-h day/8-h night cycle at 25°C.

In order to screen for root and root hair mutants, 11,000 M2 seeds (10 seeds per seed family) were aseptically germinated on 0.8% agar plates containing nitrogen-free B & D nutrient solution (Broughton and Dilworth 1971) in square plastic plates (Iwaki Co. Ltd., Tokyo). The bottoms of the plates were sealed with black tape, and the sides and tops were wrapped in gas-permeable surgical tape (3M Health Care, St. Paul, MN, U.S.A.). The seedlings were grown under a 16-h day/8-h night cycle at a light intensity of 55 μ Es⁻¹ m⁻² at 22°C in a Biotron LH-100 (Nihon-ika Co. Ltd., Tokyo). The plates were oriented vertically to facilitate straight root growth. Three days later, the roots were inoculated with *M. loti* JRL501 or TONO strains at a cell density of 10⁷ cells per ml. Putative mutants were screened by visual assay 1 month after inoculation. The inheritance of the mutant phenotype was then followed to the M6 generation.

GUS staining of root nodules.

Seedling roots (5-mm long) were excised and immersed in 200 μ l of GUS assay solution (0.02% sodium dodecyl sulfate; 20% ethanol; 50 mM phosphate buffer, pH 7.0; 0.96 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide [X-Gluc; Wako Pure Chemical Industries Ltd., Osaka, Japan]) in a microfuge tube. After a 10-min vacuum treatment, the samples were incubated overnight at 37°C. The reaction was stopped by replacing the X-Gluc solution with 70% ethanol. Nodules and roots infected by *M. loti* JRL507 were viewed under a microscope (SZH10; Olympus Kougaku Co., Tokyo).

Light microscopy.

Nodules and root segments were fixed overnight in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C and postfixed with 2% OsO₄ for 3 h. The fixed segments were dehydrated through an acetone series to 100% acetone at room temperature, embedded in Kushida's low-viscosity epoxy resin containing Quetol 651/MNA/DMP-30 (22:28:1) (TABB Laboratories, Berkshire, England), and polymerized at 60°C (Imaizumi-Ankaru et al. 1997). Semithin or ultrathin sections were obtained using an ultramicrotome (Reichert Ultracut S; Leica USA, Deerfield, IL, U.S.A.) Serial semithin sections were stained with 1% toluidine blue in 0.5% sodium tetraborate (pH 9.0) and viewed through an AXIOPLAN light microscope (Carl Zeiss, Stuttgart, Germany).

Allelism tests.

Reciprocal crossing was carried out according to Jiang and Gresshoff (1997), using Nod⁻ and Hist⁻ mutant categories or Hist⁻ and Fix⁻ categories. *Ljsym8* and *Ljsym16* were used to carry out allelism tests with *alb1-1* (*Ljsym74-1*) and the hyper-nodulating mutants (*Ljsym78-1* and *Ljsym78-2*), respectively,

using mutant lines provided by Jens Stougaard (Schauser et al. 1998).

Effects of nitrate on number of nodules and lateral roots.

Seeds of the *Ljsym78-1* and *Ljsym78-2* mutants were germinated on filter paper. Three days later, the seedlings were placed on vermiculite immersed with different concentrations of potassium nitrate and simultaneously inoculated with *M. loti* MAFF 303099 at a cell density of 10⁷ cells per ml. The seedlings were grown under a 16-h day/8-h night cycle at a light intensity of 55 μ E s⁻¹ m⁻² at 22°C in a Biotron LH-100. The number of developing and mature nodules was counted 3 weeks after inoculation. In order to evaluate the number of lateral roots, *Ljsym78-1* and *Ljsym78-2* mutant seedlings were grown in vermiculite immersed with bacteria-free B & D medium containing 0.5 mM potassium nitrate for 3 weeks.

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