# Characterization of the *Lotus japonicus* Symbiotic Mutant *lot1* That Shows a Reduced Nodule Number and Distorted Trichomes[1]

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We isolated a recessive symbiotic mutant of *Lotus japonicus* that defines a genetic locus, *LOT1* (for low nodulation and trichome distortion). The nodule number per plant of the mutant was about one-fifth of that of the wild type. The *lot1* mutant showed a moderate dwarf phenotype and distorted trichomes, but its root hairs showed no apparent differences to those of the wild type. Infection thread formation after inoculation of *Mesorhizobium loti* was repressed in *lot1* compared to that in the wild type. The nodule primordia of *lot1* did not result in any aborted nodule-like structure, all nodules becoming mature and exhibiting high nitrogen fixation activity. The mutant was normally colonized by mycorrhizal fungi. *lot1* also showed higher sensitivity to nitrate than the wild type. The grown-up seedlings of *lot1* were insensitive to any ethylene treatments with regard to nodulation, although the mutant showed normal triple response on germination. It is conceivable that a nodulation-specific ethylene signaling pathway is constitutively activated in the mutant. Grafting experiments with *lot1* and wild-type seedlings suggested that the root genotype mainly determines the low nodulation phenotype of the mutant, while the trichome distortion is regulated by the shoot genotype. Grafting of *har1-4* shoots to *lot1* roots resulted in an intermediate nodule number, i.e. more than that of *lot1* and less than that of *har1-4*. Putative double mutants of *lot1* and *har1* also showed intermediate nodulation. Thus, it was indicated that *LOT1* is involved in a distinct signal transduction pathway independent of *HAR1*.

Leguminous plants form nitrogen-fixing root nodules postembryonically with symbiotic bacteria called rhizobia. This cross-kingdom symbiosis is initiated by reciprocal signal exchange between the two organisms (for review, see Geurts and Bisseling, 2002; Oldroyd and Downie, 2004).

Nodulation in legumes is tightly controlled. The best characterized control mechanism is termed autoregulation of nodulation, in which the nodule formation on one part of a rhizobium-infected root systematically inhibits subsequent nodulation of nearby regions (Nutman, 1952; Kosslak and Bohlool, 1984; Caetano-Anollés and Gresshoff, 1991; van Brussel et al., 2002). A defect of autoregulation results in supernodulation or hypernodulation of soybean (*Glycine max*) mutants (Carroll et al., 1985a, 1985b; Gremaud and Harper, 1989; Akao and Kouchi, 1992), which are regulated by the shoot genotype (Delves et al., 1986; Sheng and Harper, 1997). Similar hypernodulation mutants have been isolated from *Lotus japonicus* (Schauser et al., 1998; Szczyglowski et al., 1998; Kawaguchi et al., 2002), a model legume (Handberg and Stougaard, 1992; Jiang and Gresshoff, 1997), and termed *har1* for hypernodulation aberrant root formation (Wopereis et al., 2000).

The mobile signal molecules involved in autoregulation have not yet been identified. Besides autoregulation, it is generally known that leguminous plants do not form root nodules when they are exposed to high concentrations of a nitrogen source such as nitrate (Streeter, 1988; Carroll and Mathews, 1990). Ethylene is another negative factor; insensitivity to ethylene causes hypernodulation of *Medicago truncatula*, another model legume (Penmetsa and Cook, 1997). In addition to the above well-known signaling mechanisms, there are other mechanisms for control of the nodule number. For example, the *astray* mutant of *L. japonicus* starts nodule development early and forms approximately twice the number of nodules on a wider area of roots compared to the wild type, and shows normal sensitivity to ethylene and nitrate (Nishimura et al., 2002b, 2002c). It remains to be clarified if the recently reported *sunn* mutant of *M. truncatula* (Penmetsa et al., 2003) is orthologous to *har1* of *L. japonicus*.

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Ethyl methanesulfonate-mutagenized *L. japonicus* symbiotic mutants fall into four basic categories: (1) nonnodulation (Nod$^-$); (2) hypernodulation (Nod$^{2+}$); (3) defect in cooperative histogenesis (Hist$^-$); and (4) ineffective nodulation that often accompanies early senescence (Fix$^-$; Kawaguchi et al., 2002). Along with developing fundamentals of *L. japonicus* and *M. truncatula* genomics, an increasing number of symbiotic genes have been cloned. These include *NIN* (Schauser et al., 1999; Borisov et al., 2003), *SYMRK/NORK* (Endre et al., 2002; Stracke et al., 2002), *NFR1* and *NFR5* (Madsen et al., 2003; Radutoiu et al., 2003), *LYK3/4* (Limpens et al., 2003), *DMI1* (Ané et al., 2004), and *DMI3* (Levy et al., 2004; Mitra et al., 2004) for the Nod$^-$ phenotype. On the other hand, for the Nod$^{2+}$ phenotype, the *HAR1/NARK* (Krusell et al., 2002; Nishimura et al., 2002a; Searle et al., 2003) and *ASTRAY* (Nishimura et al., 2002b) genes have also been cloned.

In this article, we report the isolation and initial characterization of a *L. japonicus* mutant, named *lot1*, which shows unprecedented low nodulation, distorted trichomes, and moderate dwarfism.

## RESULTS

### Plant Phenotype and Growth Kinetics of the *lot1* Mutant

When the *lot1* mutant was inoculated with *Mesorhizobium loti* Tono, it formed apparently healthy nodules, but the number was around 20% that of the wild type (Figs. 1A and 2A).The *lot1* mutant also showed a moderate dwarf phenotype (Fig. 1A). Both the shoots and roots of *lot1* were shorter than those in the wild type when the plants were grown not only on a nitrogen-free medium with *M. loti* (Fig. 2, C and D) but also on a nitrogen-rich medium without *M. loti* (Fig. 2, E and F). At 8 weeks after *M. loti* inoculation, nodules were formed much more sparsely than in the wild type (Fig. 1, B and C). In addition, the *lot1* mutant formed wavy trichomes in calyx regions (Fig. 1D) and on the abaxial side of leaflets (Fig. 1F). According to the proposed guidelines for *L. japonicus* genetic nomenclature (Stougaard et al., 1999), we named the mutant *lot1* for low nodulation and trichome distortion. In contrast to the unique nodulation phenotype, the *lot1* mutant was colonized by mycorrhizal fungi as effectively as the wild type (Fig. 2B).

### Monogenic and Recessive Inheritance of the *lot1* Phenotype

When the *lot1* mutant was backcrossed with the parental wild type *L. japonicus* Gifu B-129, all $F_1$ progenies formed as many nodules as the wild type. The $F_1$ plants were naturally self-crossed, and the resulting $F_2$ progenies segregated at the ratio of 189:53 (3:1, $\chi^2$ = 0.228), indicating recessive and monogenic Mendelian inheritance of the low nodulation phenotype. This finding was confirmed by crossing the *lot1* mutant with another wild-type line, *L. japonicus* Miyakojima MG-20 (Kawaguchi et al., 2001). The $F_2$ progenies segregated at the ratio of 129:42 (3:1, $\chi^2$ = 0.002). As far as we examined the $F_2$ population, no genetic segregation was found among low nodulation, moderate dwarfism, and crinkly trichomes.

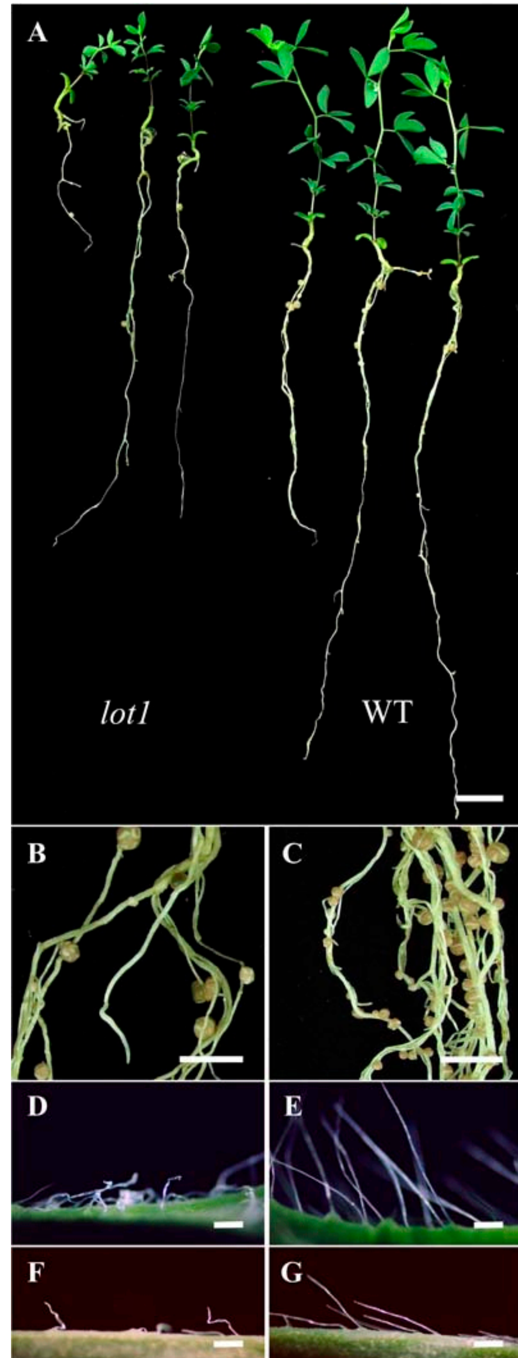


**Figure 1.** The appearance of the *lot1* mutant and wild type. A, Plants grown for 4 weeks after inoculation of *M. loti* Tono. Left, *lot1*; right, wild type. B and C, Mature nodules of *lot1* and the wild type, respectively, at 8 weeks postinoculation (wpi). D and E, Trichomes in calyx regions of *lot1* and the wild type, respectively. F and G, Trichomes on the abaxial side of leaflets of *lot1* and the wild type, respectively. Bars in A to C, 1 cm; bars in D to G, 300 $\mu$m.

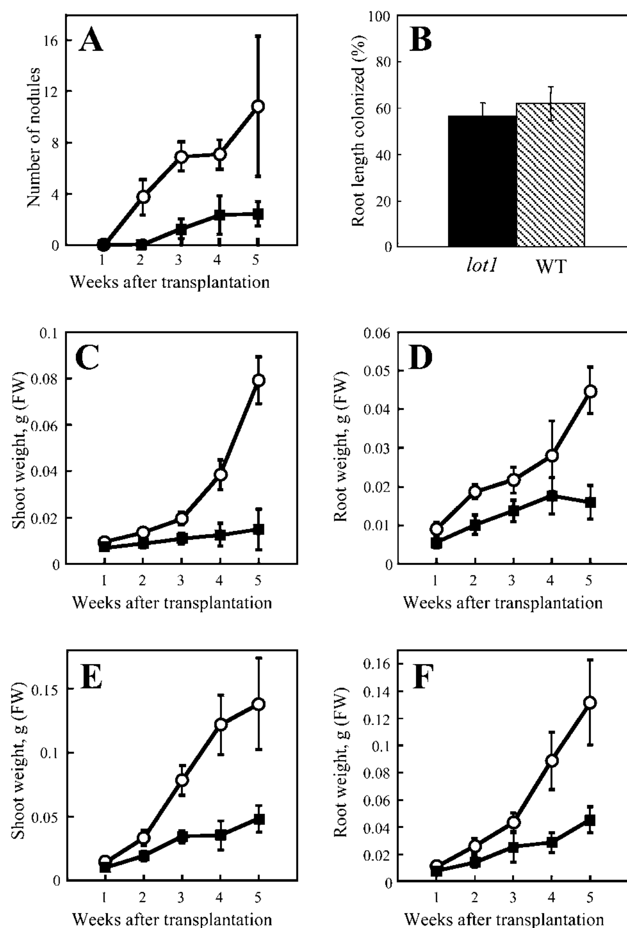


**Figure 2.** Nodulation, mycorrhizal colonization, and growth kinetics of *lot1* and the wild type. A, Numbers of nodule primordia and mature nodules as a function of time. One week after transplantation, inoculation of *M. loti* Tono was carried out as described in "Materials and Methods." The means and SDs are presented ($n = 10$). B, Comparison of mycorrhizal colonization between *lot1* and the wild type. The means and SDs ($n = 3$) are not significantly different at $P < 0.01$ according to the *t* test. C to F, Growth kinetics of shoots and roots. The means and SDs are presented ($n = 10$). C and D, Shoot weight and root weight, respectively. Seedlings at 7 d after germination were transplanted onto nitrogen-free B & D medium on vermiculite, and, 1 week later, they were inoculated with *M. loti* Tono. E and F, The seedlings were transplanted onto nitrogen-rich 0.5× Gamborg B5 medium on vermiculite and then grown without bacteria. White circles and black squares in all sections except B indicate the wild type and *lot1* mutant, respectively.

### Repressed Formation of Nodule Primordia on *lot1* Roots

A series of experiments was conducted to determine why the *lot1* mutant forms a smaller number of nodules than the wild type. Root hair deformation of the *lot1* mutant induced by *M. loti* was indistinguishable from that of the wild type (data not shown). In our experimental system, the infection threads and nodule primordia were first observed at 3 and 7 d postinfection (dpi), respectively. As shown in Table I, infection thread formation with green fluorescent protein (GFP)-labeled *M. loti* BNO2 was significantly blocked compared to that of the wild type, although the shape of the infection threads was apparently normal in the mutant (data not shown). Similar results were obtained with *lacZ*-labeled *M. loti* ML001 (data not shown). Abortion at some step after initiation might also have occurred (Table I). It is noteworthy that once nodule primordia were formed on *lot1* roots, they did not result in any aborted nodule-like structure, all nodules becoming mature. The inside structure of mature *lot1* nodules was normal, bacteroid-infected cells and uninfected cells being indistinguishable from those of the wild type (data not shown). The nitrogenase activity of *lot1* nodules determined as acetylene reduction was as high as that of the wild type (Table II).

### Sensitivity of the *lot1* Mutant to Exogenous Nitrate

It is known that exogenous nitrate inhibits nodule formation by legumes (Streeter, 1988; Carroll and Mathews, 1990). Accordingly, we examined the effects of varying concentrations of nitrate in the medium on nodule formation by *lot1* mutant and wild-type plants. As shown in Figure 3, the nodule formation by the *lot1* mutant was reduced with 1 mM nitrate and completely blocked with more than 3 mM nitrate. On the other hand, wild-type plants grown with 1 mM nitrate formed a similar number of nodules to a control without nitrate. The wild type kept forming nodules even with 10 mM nitrate, although the number was reduced to some extent (Fig. 3). The latter results are similar to those of Hussain et al. (1999) and Nishimura et al. (2002c). Thus, it was suggested that *lot1* shows higher sensitivity to exogenous nitrate than the wild type.

### Nodule Formation by the *lot1* Mutant with Various Rhizobia

It is generally known that Rhizobium mutants lacking nitrogen fixation form more nodules than wild-type bacteria (Nutman, 1949; Hirsch and Smith, 1987). The low nodule number of the *lot1* mutant might be caused by higher sensitivity to fixed nitrogen of bacteroids. Therefore, we checked nodule formation by a *nifH*-deficient mutant of *M. loti*. As shown in Table III, the *nifH*-deficient mutant formed more nodules not only on wild-type roots but also *lot1* roots. Thus, *lot1* is capable of perceiving fixed nitrogen. However, the inoculation of ineffective *M. loti* onto *lot1* roots resulted in a smaller increase in nodule number than in the case of the wild type (Table III). Unlike the *nifH*-deficient mutant, *Rhizobium etli* CE3, a heterologous symbiont that forms partly effective nodules on the wild type (Banba et al., 2001), scarcely formed nodules on the *lot1* mutant (Table III).

### Ethylene Sensitivity of the *lot1* Mutant

We examined the sensitivity of the *lot1* mutant to ethylene, which inhibits nodule formation. As shown

**Table I.** *The nodulation process in lot1 mutant and the wild type*

Infection threads were observed for the first time and counted microscopically at 3 dpi for *M. loti* ML001, which expresses GFP constitutively. At 7 dpi, nodules and primordia were observed for the first time and counted again. The successful nodulation ratios were then calculated by dividing the numbers of nodules and primordia by those of infection threads at 3 dpi. The means and SDs are presented ($n = 8$). Each value for *lot1* was significantly lower than that for the wild type at $P < 0.01$ according to the *t* test.

| Parameter | dpi | Wild Type | *lot1* |
|---|---|---|---|
| Number of infection threads | 3 | 17.7 ± 6.1 | 2.8 ± 2.3 |
| | 5 | 68.4 ± 20.0 | 29.2 ± 16.1 |
| | 7 | 153 ± 39.2 | 51.9 ± 21.5 |
| Number of nodules and primordia | 7 | 3.8 ± 1.4 | 0.1 ± 0.3 |
| Successful nodulation (%) | | 21 | 4 |

in Figure 4A, application of 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor (Adams and Yang, 1979), on germination in the dark caused a typical triple response, i.e. inhibition of root and hypocotyl elongation, radial swelling of hypocotyls, and hypocotyl hook formation, of the *lot1* mutant as well as the wild type. The effects of ACC on nodule formation by grown-up seedlings (Fearn and LaRue, 1991; Penmetsa and Cook, 1997; Wopereis et al., 2000; Penmetsa et al., 2003) were investigated next. As expected, 10 $\mu$M ACC reduced the nodule number of the wild type (Fig. 4B). On the other hand, ACC showed no effect or caused a slight increase in the nodule number of the *lot1* mutant. In parallel, we applied silver thiosulfate (STS), an inhibitor of the ethylene action (Veen, 1983; Nukui et al., 2004), to the seedlings. Five micromolar STS clearly increased the nodule number of the wild type (Fig. 4B). The effect of STS on *lot1* nodule formation was not significant. Application of 1 $\mu$M aminoethoxyvinylglycine (AVG), a potent inhibitor of ethylene synthesis (Yu et al., 1979), to the seedlings caused an increase in the nodule number of the wild type, but the nodule number of *lot1* did not change either (Fig. 4C). Overall, *lot1* showed insensitivity to ethylene with regard to nodulation, although its triple response was normal.

### Grafting with *lot1* and Wild-Type Plants

To examine what portion determines the low nodulation phenotype of *lot1*, we conducted grafting experiments with the *lot1* mutant and wild type. As shown in Figure 5, grafting of wild-type shoots onto *lot1* roots resulted in a slightly but significantly larger nodule number than that for one control, *lot1*/*lot1*. In addition, grafting of *lot1* shoots to wild-type roots gave a slightly smaller nodule number than that for another control, wild type/wild type. However, the wild-type shoots were larger than *lot1* shoots, and so *lot1* roots with wild-type shoots are longer than those with *lot1* shoots (Fig. 5A). These secondary differences in nodule number (Fig. 5D) would be attributable to different photosynthetic activity of the grafted shoots. Sparse nodules are one of the hallmarks of the *lot1* mutant (Fig. 1B). Notably, *lot1* roots grafted with wild-type shoots formed nodules much more sparsely than wild-type roots with *lot1* shoots (Fig. 5, B and C). These results suggest that the root genotype determines the low nodulation phenotype of the *lot1* mutant, although we cannot exclude a small effect of shoot genotype completely. This is in contrast to *har1/nark* mutants, in which the shoot genotype determines the hypernodulation phenotype in a clear manner (Delves et al., 1986; Sheng and Harper, 1997; Jiang and Gresshoff, 2002; Krusell et al., 2002; Men et al., 2002; Nishimura et al., 2002a; Searle et al., 2003). Unlike the nodule formation, trichomes on *lot1* shoots grafted to wild-type roots remained wavy. In addition, trichomes on wild-type shoots grafted to *lot1* roots were normal (data not shown). Therefore, trichome morphology is determined by the shoot genotype.

### Independence of *LOT1* and *HAR1* Functions

The above-described high nitrate sensitivity and low nodulation are just opposite to the case of the nitrate-tolerance and hypernodulation phenotype of *har1* mutants of soybean (Carroll et al., 1985a, 1985b) and *L. japonicus* (Wopereis et al., 2000), although some *har1* mutants of *L. japonicus* show similar nitrate sensitivity to that of the wild type (Kawaguchi et al., 2002). As a first step to determine whether or not the *LOT1* gene product is involved in the *HAR1* regulation pathway, we estimated the relative expression levels of the *HAR1* transcript in the *lot1* mutant and wild type by real-time reverse transcription (RT)-PCR. *HAR1* mRNA levels were similar in both the mutant and wild-type plants, decreasing in shoots, roots, and root nodules in that order (data not shown). This expression pattern is consistent with that reported by Nishimura et al. (2002a). As a control, the constitutive $\beta$-actin gene (Matamoros et al., 2003) was also amplified, showing a similar expression level in all tissues (data not shown). Thus, it was shown that the *lot1* mutant expresses the *HAR1* gene as highly as the wild type.

Next, we carried out grafting experiments with *lot1* and *har1* mutants. Since the *har1* mutant shows a dwarf phenotype too, especially when inoculated with *M. loti*

**Table II.** *Acetylene reduction activity of nodules formed by lot1 and the wild type*

Acetylene reduction activity was determined with dissected root portions at 8 wpi. The means and SDs are presented ($n = 6$). Different letter suffixes within a column indicate significant differences at $P < 0.01$ according to the *t* test.

| | $C_2H_2$ Reduction Activity | |
|---|---|---|
| | $\mu mol\ h^{-1}\ plant^{-1}$ | $\mu mol\ h^{-1}\ g^{-1}$ (fresh nodule weight) |
| Wild type | 1.12 ± 0.49 a | 17.4 ± 2.1 a |
| *lot1* | 0.20 ± 0.09 b | 16.2 ± 4.6 a |

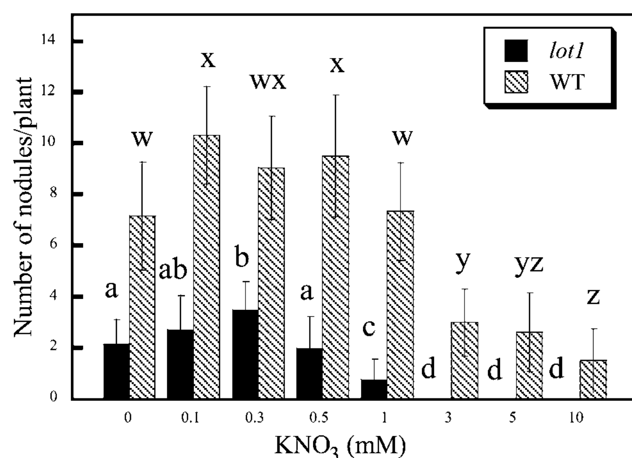


**Figure 3.** Effects of varying concentrations of nitrate on nodule formation by *lot1* and the wild type. Seedlings of 7 d after germination were transplanted into B & D medium on vermiculite containing the indicated concentrations of $KNO_3$, and, after 1 week, they were inoculated with *M. loti* Tono. The seedlings were grown for 3 weeks, and then the number of mature nodules was determined. The means and SDS ($n = 6$) are presented. Different letters above the bars indicate significant differences at $P < 0.01$ according to the *t* test.

(Wopereis et al., 2000), we do not have to take into account the secondary effect caused by the difference in shoot growth in the experiments in Figure 6. The short root phenotype of the *har1* mutant seems to be determined by the shoot genotype (Krusell et al., 2002) as well as super/hypernodulation (Delves et al., 1986; Sheng and Harper, 1997; Nishimura et al., 2002a; Searle et al., 2003). Interestingly, grafting of *har1-4* shoots onto *lot1* roots resulted in an intermediate number of nodules. The grafted plants formed more nodules than one control, *lot1/lot1*, and much less nodules than another control, *har1-4/har1-4* (Fig. 6C). Grafting of *lot1* shoots onto *har1-4* roots gave similar results.

Finally, we prepared putative double mutant lines of *lot1* and *har1-4*, named F3-45, F3-55, and F3-94. As shown in Figure 7, all putative double mutants showed intermediate nodule number between those of the single mutants. Unexpectedly, however, the putative double mutants showed straight trichomes (data not shown) and short root phenotype like *har1* (Fig. 7) when inoculated with *M. loti*. The homozygous *har1-4* allele seems to suppress the *lot1* phenotypes to some extent. Taken together, these results indicate that the *LOT1* gene product acts independently from the *HAR1* gene product in the regulation of nodule number.

### Chromosomal Mapping

Using the $F_2$ plants obtained by crossing *lot1* and Miyakojima MG-20, linkage analysis with a total of 24 simple sequence repeat markers was carried out. The results suggested that *LOT1* is near an intraspecific translocation site between Gifu B-129 and Miyakojima MG-20 involving chromosomes 1 and 2 (data not shown). Combined with the phenotypical difference of the *lot1* mutant from thus far reported *L. japonicus* mutants (Schauser et al., 1998; Szczyglowski et al., 1998; Kawaguchi et al., 2002; Tansengco et al., 2003) and pea (*Pisum sativum*) mutants (Provorov et al., 2002), this finding strongly suggests that *LOT1* is a novel locus on the *L. japonicus* genome.

## DISCUSSION

We isolated a novel *L. japonicus* symbiotic mutant, *lot1*. Although the *lot1* mutant is monogenic and recessive, it shows some distinct phenotypes such as low nodulation, trichome distortion, and moderate dwarfism (Figs. 1 and 2). These findings indicate that *LOT1* is involved not only in control of nodule formation but also in trichome formation and growth control. In Arabidopsis, a gene that controls both trichome and root hair formation has been reported (Rerie et al., 1994; Ohashi et al., 2003). The *crinkle* mutant of *L. japonicus* also shows alteration of both trichome and root hair formation (Tansengco et al., 2003). In the case of the *lot1* mutant, however, the root hairs were normal. Other pleiotropic nodulation mutants have been described (Caetano-Anollés and Gresshoff, 1991; Penmetsa and Cook, 1997; Wopereis et al., 2000; Nishimura et al., 2002a, 2002c; Tansengco et al., 2003, 2004). It is thought that most nodule-enhanced genes, or nodulin genes, are recruited for nodule-specific functions from among preexisting common genes (Hata et al., 1998; Gualtieri and Bisseling, 2000; Nakagawa et al., 2003; Szczyglowski and Amyot, 2003), although there is an exceptional galegoid legume-specific gene family (Mergaert et al., 2003). At least some key genes involved in nodule formation seem to have also been recruited from among preexisting genes involved in coordinated plant development. These include *LOT1* (this study),

**Table III.** *Nodulation of lot1, har1-4, and the wild type on inoculation of various rhizobia*

A *nifH*-defective mutant of *M. loti* (Δ*nifH*; J. Maruya and K. Saeki, unpublished data) and its parent strain, MAFF 303099, were inoculated to determine the numbers of nodules formed on *lot1*, *har1-4*, and the wild-type *L. japonicus* Gifu B-129. Another wild type, *M. loti* Tono, and *R. etli* CE3 (Banba et al., 2001) were also examined. The means and SDS at 6 wpi are presented ($n = 12$–$18$). Different letter suffixes within a column indicate significant differences at $P < 0.05$ according to the *t* test.

| Rhizobia | Number of Nodules | | |
|---|---|---|---|
| | *lot1* | *har1-4* | Gifu B-129 |
| *M. loti* MAFF 303099 | 3.3 ± 1.5 a | ND[a] | 8.1 ± 1.8 a |
| Δ*nifH* | 5.8 ± 2.9 b | ND | 26.3 ± 4.2 b |
| *M. loti* Tono | 2.2 ± 0.8 a | 23.4 ± 7.3 a | 6.8 ± 1.7 a |
| *R. etli* CE3 | 0.2[b] ± 0.4 c | 13.1 ± 4.3 b | 7.3 ± 2.2 a |

[a]ND, Not determined. [b]About 17% of the *lot1* mutant inoculated with *R. etli* CE3 formed only a single nodule, the others not forming any nodules.

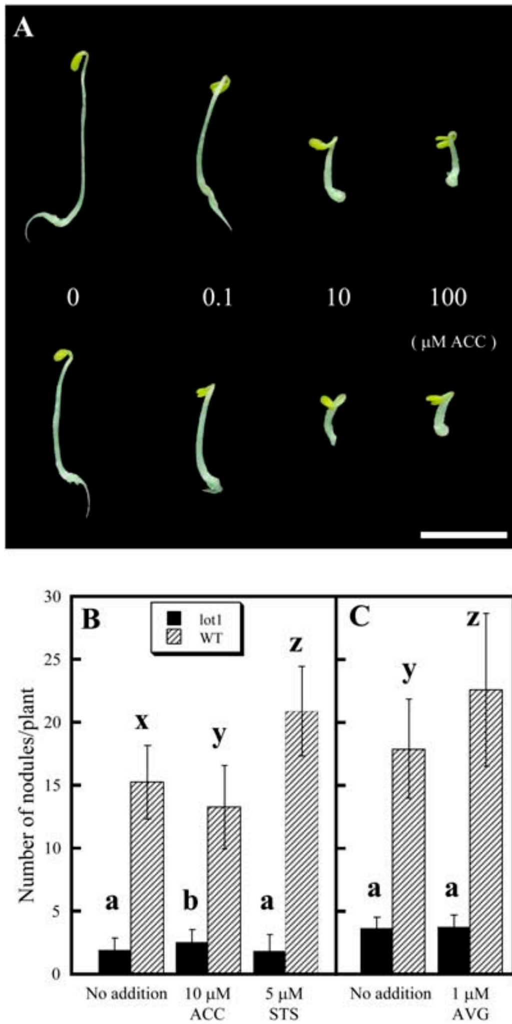


**Figure 4.** Production and sensitivity to ethylene of *lot1* mutant. A, Triple response of the wild type (top) and the *lot1* mutant (bottom). Seeds were germinated in the dark in the presence of varying concentrations of ACC. Bar, 1 cm. B, Effects of ACC and STS on the nodule number of *lot1* and wild-type plants. Seedlings were inoculated with *M. loti* Tono and grown for 5 weeks in the absence or presence of 10 $\mu$M ACC or 5 $\mu$M STS, and then the mature nodules were counted. C, Effect of 1 $\mu$M AVG on the nodule number in an experiment independent of B. The means and SDs are presented ($n$ = 16–30 for *lot1*, $n$ = 24–30 for the wild type). Different letters within a section indicate significant differences at $P < 0.05$ according to the $t$ test.

*HAR1/NARK* (Wopereis et al., 2000; Krusell et al., 2002; Nishimura et al., 2002a; Searle et al., 2003), *ASTRAY* (Nishimura et al., 2002b, 2002c), and *CRINKLE* (Tansengco et al., 2003, 2004). This view is in accord with the prediction of Szczyglowski and Amyot (2003).

Although the shape of infection threads was normal, their formation was significantly blocked (Table I). Subsequent nodule primordia formation may also be repressed compared to the wild type (Table I). Since it has long been known that a high proportion of infection results in abortion (Nutman, 1962), careful examination like that of Vasse et al. (1993) would be necessary to determine the blocked step(s). In any case, once nodule primordia were formed on *lot1* roots, all nodules developed fully (Fig. 1; Table II). This is in contrast to *crinkle* (Tansengco et al., 2003) and

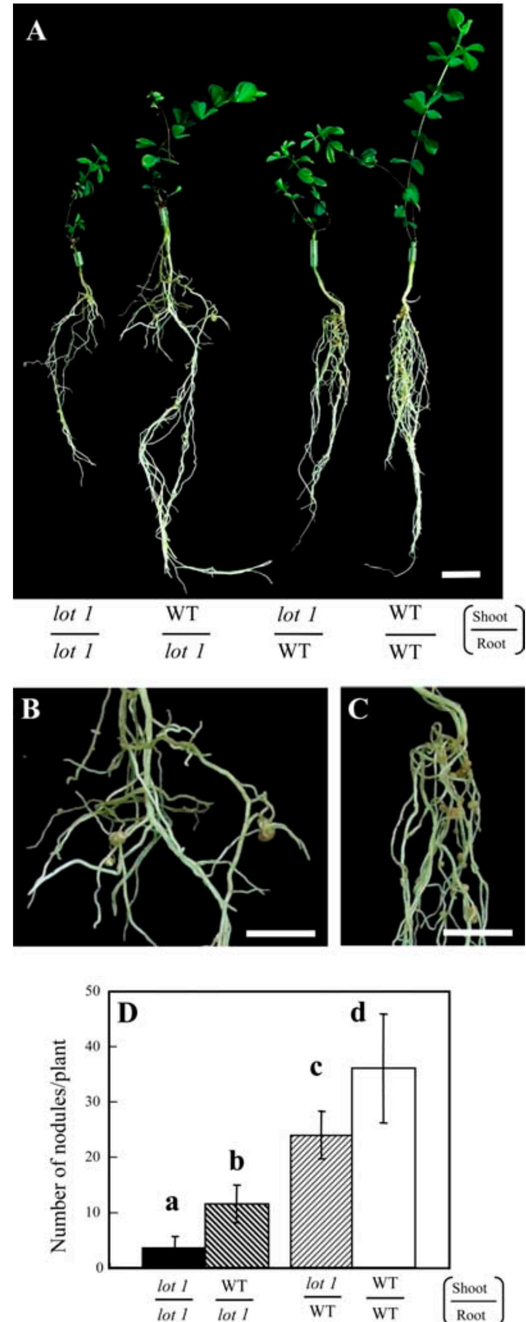


**Figure 5.** Grafting experiments with *lot1* and the wild type (WT). A, Plants grown for 6 weeks after grafting and 5 wpi of *M. loti* Tono. Bar, 2 cm. B, Nodulation of *lot1* roots to which wild-type shoots had been grafted. Bar, 1 cm. C, Nodulation of wild-type roots to which *lot1* shoots had been grafted. Bar, 1 cm. D, Number of mature nodules. The means and SDs are presented ($n$ = 10, 14, 5, and 17 for *lot1*/*lot1*, WT/*lot1*, *lot1*/WT, and WT/WT, respectively). Different letters above the bars indicate significant differences at $P < 0.05$ according to the $t$ test.

*alb1* (Imaizumi-Anraku et al., 1997). In these other mutants, many bumps or empty nodules are formed and infection thread development is markedly arrested. Linkage analysis also indicated that *LOT1* is distinct from both *CRINKLE* and *ALB1* (Y. Ooki, M. Hayashi, and M. Kawaguchi, unpublished data). The phenotype of *lot1* is similar to that of *Ljsym73*, which

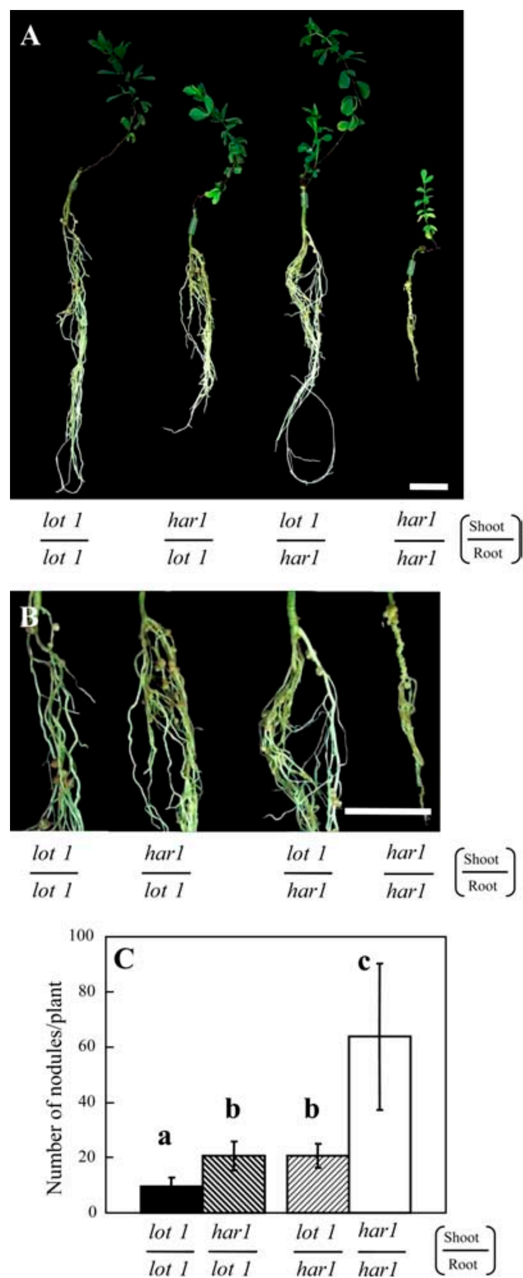


**Figure 6.** Grafting experiments with the *lot1* and *har1-4* mutants. A, Plants grown for 6 weeks after grafting and 5 wpi of *M. loti* Tono. Bar, 2 cm. B, Enlarged root portions. Bar, 2 cm. C, Number of mature nodules. The means and SDs are presented ($n$ = 22, 12, 12, and 10 for *lot1*/*lot1*, *har1*/*lot1*, *lot1*/*har1*, and *har1*/*har1*, respectively). Different letters above the bars indicate significant differences at $P < 0.01$ according to the $t$ test.

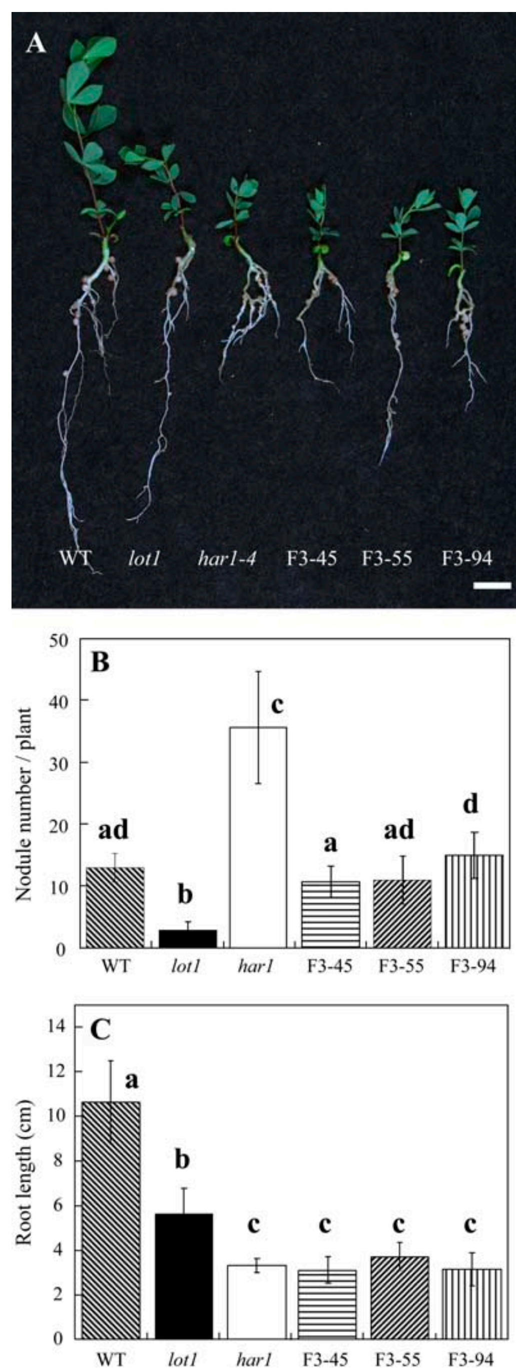


**Figure 7.** Phenotype of putative double mutants of *lot1* and *har1-4*. A, The appearance of the putative double mutants F3-45, F3-55, and F3-94. The wild type (Gifu B-129) and the single mutants are also shown for comparison. The plants were grown for 4 weeks after inoculation of *M. loti* Tono. Bar, 1 cm. B and C, Nodule number and root length, respectively, of the plants. The means and SDs are presented ($n$ = 10, 15, 15, 15, 14, and 12 for the wild type, *lot1*, *har1-4*, F3-45, F3-55, and F3-94, respectively). Different letters above the bars indicate significant differences at $P < 0.01$ according to the $t$ test.

nodulates at very low frequency and exhibits normal mycorrhizal colonization (Kawaguchi et al., 2002). However, crossing of *lot1* with *Ljsym73* indicated that they are not allelic (M. Banba, M. Yoshikawa, M. Hayashi, and M. Kawaguchi, unpublished data). It is worth noting that *lot1* does not belong to any of the four basic categories of nodulation mutants: $Nod^-$, $Nod^{2+}$, $Hist^-$, and $Fix^-$.

The results of grafting experiments with the *lot1* mutant and wild type suggested that the root genotype mainly determines low nodulation phenotype of the *lot1* mutant (Fig. 5). This is in sharp contrast to *har1* mutants, in which the shoot genotype determines the hypernodulation phenotype systemically. Besides the *HAR1* autoregulation pathway, Postma et al. (1988) described a pea hypernodulation mutation that was determined by the root genotype. A root meristem-derived factor that controls nodulation of soybean has also been reported (Caetano-Anollés et al., 1991). It is possible that the *LOT1* gene is involved in these root-derived local regulations of nodule formation. Unlike nodulation, the wavy trichome morphology of the *lot1* mutant was determined by the shoot genotype. Therefore, we speculate that the *LOT1* gene is expressed not only in roots but also in shoots, where it acts in trichome formation and growth regulation.

The effects of nitrate on plant morphogenesis are complicated. Lateral roots of Arabidopsis, for example, show very contrasting responses to high concentrations of nitrate (Casimiro et al., 2003; Lopez-Bucio et al., 2003). When plants are grown on a medium with a uniformly high nitrate supply, lateral root elongation is inhibited throughout the root system. On the other hand, when a section of a primary root grown on low nitrate is exposed to a high nitrate supply, localized stimulation of lateral root elongation occurs. In the former case, the systemic inhibition is thought to be due to sufficient nitrogen metabolites. In the latter case, the signal is thought to be nitrate itself (Casimiro et al., 2003; Lopez-Bucio et al., 2003). Since most hypernodulation mutants of legumes show nitrate tolerance, the relationship between nitrate inhibition of nodule formation and autoregulation of the nodule number has long been pointed out (Delves et al., 1986; Caetano-Anollés and Gresshoff, 1991). In most cases, the availability of nitrogen per se does not seem to repress the new nodule formation, but preexisting nodules do so systemically. However, the control mechanism may be complicated as in the case of Arabidopsis lateral roots. In this work, *lot1* showed higher sensitivity to exogenous nitrate than the wild type (Fig. 3). As described above, *lot1* seems to be involved in local regulation of root nodule formation. Thus, *lot1* may exhibit aberrant nitrogen sensing in roots, causing the low nodulation phenotype. The inoculation of a *nifH*-defective strain of *M. loti* onto *lot1* roots resulted in a smaller increase of nodule number than that in the wild type (Table III). Therefore, it is still possible that *lot1* also has a defect in the recognition of unknown signals from preexisting nodules other than nitrate.

We found that the low nodulation phenotype of *lot1* is not caused by overexpression of the *HAR1* gene. We next designed grafting experiments with *lot1* and *har1-4* mutants. If *LOT1* and *HAR1* act in the same genetic pathway, one can expect that either the *lot1* phenotype or the *har1-4* phenotype is observed after grafting. For example, Delves et al. (1986) reported that the *har1/nark* hypernodulation phenotype of soybean was completely suppressed by a root-expressed nonnodulation mutation when hypernodulation shoots were grafted onto nonnodulation roots. In that case, it is highly likely that the causal gene for nonnodulation acts downstream of the *HAR1/NARK* gene in the same regulation pathway. Interestingly, however, grafting of *har1-4* shoots onto *lot1* roots resulted in an intermediate number of nodules, showing an additive effect of the two mutations (Fig. 6). The phenotype of the putative double mutants of *lot1* and *har1* supported this observation (Fig. 7). These findings strongly indicate that *LOT1* and *HAR1* determine distinct control pathways, *LOT1* and *HAR1* exhibiting augmentative and suppressive effects, respectively, on the nodule number.

What is the mechanism underlying the low nodulation by *lot1*? In this regard, it is noteworthy that ACC, AVG, nor STS showed any significant effect on the nodule number of grown-up *lot1* seedlings, although *lot1* showed the normal triple response just after germination (Fig. 4). These results are in contrast to those for some pea mutants showing a reduced nodule number that are hypersensitive to ethylene (Fearn and LaRue, 1991) or overproducers of ethylene (Lee and LaRue, 1992). The plant hormone ethylene regulates a variety of functions in plant growth and development. In Arabidopsis, ethylene modulates plant responses through common upstream pathways, including receptor-CTR1 complexes (Gao et al., 2003), EIN2 (Alonso et al., 1999), etc., and specialized downstream pathways involving the EIN3/EIL family (Solano et al., 1998) and ethylene-responsive element binding proteins (Riechmann and Meyerowitz, 1998). It is possible that a nodulation-specific downstream pathway is constitutively activated in the *lot1* mutant of *L. japonicus*. In this sense, *lot1* seems to be in symmetry with *sickle* of *M. truncatula* that is insensitive to ethylene (Penmetsa and Cook, 1997). On the other hand, the normal triple response suggests that the common upstream pathways and the other downstream pathways except the nodulation-specific one are functioning well in the *lot1* mutant. This working hypothesis would explain the low nodulation phenotype of *lot1*, although we cannot rule out other mechanisms at present. The moderate dwarfism of the mutant may also be related to the conceivable constitutive ethylene response.

In summary, we isolated a hitherto-unknown low nodulation mutant, designated as *lot1*. Positional cloning of the *LOT1* gene in the future will provide new insights into the homeostatic control of symbiotic root nodule formation.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

*Lotus japonicus* Gifu B-129 was used as the parental line for mutation and as the wild-type control in other experiments. As a crossing partner, *L. japonicus* Miyakojima MG-20 was used (Kawaguchi et al., 2001). Unless otherwise stated, seeds were scarified, surface sterilized, and allowed to germinate on 0.8% agar plates under sterile conditions. The plants were kept dark at 25°C for 2 d and then subjected to greening for 5 d in a controlled-environment growth chamber (Sanyo, Tokyo) with a 16-h-day/8-h-night cycle at 25°C and a light intensity of 260 $\mu E\ s^{-1}\ m^{-2}$ with 60% humidity. Then, up to 25 seedlings were transferred to a Magenta jar containing 150 mL of vermiculite supplied with 125 mL of Broughton and Dilworth (B & D) medium (Broughton and Dilworth, 1971). A whole (1 cm diameter) was made at the center of the lid and sealed with MilliSeal (Millipore, Billerica, MA). The modified lid was set on the jar, and the seedlings were grown under the above conditions. After 2 to 3 weeks, the lid was removed just before the shoots reached to it. When M1 plants were grown to collect M2 seeds, they were grown on Power Soil (Kreha Chemical Industry, Tokyo) in an air-conditioned greenhouse at 25°C with 60% humidity.

### Microbial Strains and Inoculation

*Mesorhizobium loti* Tono was isolated by M. Kawaguchi (Kawaguchi et al., 2002) and used as a standard symbiont. *M. loti* MAFF303099 was also used as a wild-type control in some experiments. *M. loti* BNO2, which constitutively expresses GFP, was prepared by K. Saeki (to be published elsewhere) from *M. loti* JRL501 (Imaizumi-Anraku et al., 1997), which is a spontaneous nalidixic acid-tolerant derivative of MAFF303099. *M. loti* MAFF303099 derivative ML001 carrying pGD499 (*nodB:lacZ*; Ditta et al., 1985), which constitutively expresses the *lacZ* reporter gene, was provided by Dr. K. Minamisawa, Tohoku University, Japan. Another *M. loti* MAFF303099 derivative that lacks the *nifH* gene was established by J. Maruya and K. Saeki (to be published elsewhere). The *M. loti* strains and *Rhizobium etli* CE3 were grown at 28°C for 2 d with shaking in yeast extract-mannitol medium (Vincent, 1970) in the presence of appropriate antibiotics. One week after transplantation of the germinated seedlings onto vermiculite, $2.6 \times 10^{10}$ bacterial cells per Magenta jar were inoculated to the seedlings. A soil inoculum containing spores and hyphae of the arbuscular fungus *Glomus mosseae* was a kind gift from Dr. K. Nagashima, Idemitsu Kosan, Tokyo. One-week-old seedlings of *L. japonicus* were transplanted into 38-mL glass tubes (2.2 cm diameter $\times$ 10 cm high) containing vermiculite and modified Hornum nutrient solution (Handberg and Stougaard, 1992) with a lowered $NaH_2PO_4$ concentration of 250 $\mu$M. Then, 2 g of the soil inoculant was added to each plant. The plants were watered with 5 mL of the modified Hornum solution at 5-d intervals.

### Mutagenesis and Screening

Seeds of *L. japonicus* Gifu B-129 were scarified, shaken gently in water for 2 h and in 0.4% (w/v) ethyl methanesulfonate (Sigma, St. Louis) for 6 h at room temperature, and then rinsed more than 8 times with water. After germination, M1 plants were grown to maturity as described above, and the resulting M2 seeds were individually harvested to obtain a seed family. About 17 seeds from each M2 seed family were sterilized, germinated, inoculated with *R. etli* CE3, a heterologous symbiont (Banba et al., 2001), and then grown for 5 weeks on vermiculite containing nitrogen-free B & D medium in sterile Magenta jars. Vermiculite was carefully removed from the plant roots, and then gross changes in the nodule number and morphology were assessed for about 11,500 M2 seedlings. The resulting putative mutants were transplanted into Magenta jars containing vermiculite and half-strength Gamborg B5 medium (Wako, Osaka) to collect M3 seeds and to check the heritability of the M2 phenotypes. A number of mutants showing a nodulation deficiency, an increased shoot number, round-shaped leaves, etc. were isolated (Y. Ooki, unpublished data). Fortuitously, *R. etli* scarcely forms nodules on *lot1* roots (Table III), which is probably why we could discover the mutant among them. In turn, our screening system is not suitable for isolation of hypernodulating mutants. Actually, *har1-4* inoculated with *R. etli* CE3 formed a much lower number of nodules than in the case with *M. loti* (Table III).

### Microscopy of the Nodule Formation Process and Arbuscular Mycorrhizal Colonization

The morphology of root hairs and their deformation by *M. loti* were examined by the following two methods. When the deformation was observed within 12 h after inoculation of *M. loti*, Fåhraeus slides were used (Fåhraeus, 1957; Heidstra et al., 1994; Niwa et al., 2001). Two-day-old seedlings were prepared in vertically positioned agar plates and then transplanted into Fåhraeus slides. The roots were left to stand in $10^8$ cells/mL of *M. loti* Tono and examined at hourly intervals by bright-field microscopy. When the deformation was examined at 2 d after inoculation, the seedlings were incubated with the above density of *M. loti* in vertically positioned agar plates (Bonfante et al., 2000).

The numbers of infection threads and nodule primordia were determined with *M. loti* BNO2, which expresses GFP constitutively. One-week-old seedlings were inoculated with $6.6 \times 10^9$ cells/plant *M. loti* BNO2 in Magenta jars containing vermiculite and nitrogen-free B & D medium. At 1, 3, 5, or 7 dpi, infection threads and nodule primordia were visualized and counted as to green fluorescence under a Nikon ECLIPSE E600 microscope (Nikon, Tokyo). Excitation and detection were carried out at 490 nm and 520 nm, respectively.

The entire nodule-formation process was monitored by inoculation of *M. loti* ML001 harboring pGD499. The symbiont was inoculated onto 1-week-old seedlings as described above. After appropriate periods, whole roots were fixed and stained for $\beta$-galactosidase activity as described by Tansengco et al. (2003). Then, the infection threads and nodule primordia were counted under bright-field optics.

For assessment of *G. mosseae* colonization, roots were cleared with 10% KOH and then stained with 0.05% trypan blue in lactoglycerol (Phillips and Hayman, 1970). The stained roots were examined under a light microscope.

### Acetylene Reduction Assay

The acetylene reduction assay was carried out as described previously (Banba et al., 2001).

### Grafting Experiments

Grafting was performed as described by Nishimura et al. (2002a) using plastic tubes (diameter, 0.79 mm).

### Real-Time RT-PCR

Total RNA was extracted from shoots under symbiotic conditions, shoots under nonsymbiotic conditions, noninfected roots, and mature nodules using an RNeasy plant mini kit (Qiagen, Hilden, Germany). Each RNA preparation was reverse transcribed with oligo(dT) and Superscript II (Invitrogen, Carlsbad, CA), and then subjected to real-time PCR with specific primer pairs and SYBR Green I according to the manufacturer's instructions (Real Time RT-PCR Core kit; TaKaRa BIO, Otsu, Japan) using a Smart Cycler system (Cepheid, Sunnyvale, CA). The forward and reverse primers for *HAR1* and the $\beta$-actin gene were 5′-GATACCCCTTGACAAGTGTCATC-3′ and 5′-GTTGTTTCACTTCTCACAATCTAGG-3′, and 5′-GCATTGTTGGTCGTCCTCGT-3′ and 5′-TGTGCCTCATCCCCAACATA-3′, respectively. For *HAR1*, the reaction mixture was heated at 95°C for 30 s and then subjected to PCR cycles of 95°C for 10 s, 52°C for 20 s, and 72°C for 10 s, the resulting fluorescence being monitored. For the constitutive $\beta$-actin gene (Matamoros et al., 2003), the mixture was heated at 95°C for 30 s, and then subjected to PCR cycles of 95°C for 10 s, 62°C for 20 s, and 72°C for 10 s. Heat dissociation curves showed that a single PCR product was amplified for each gene. Melting temperatures were 86.3°C and 85.3°C for the PCR products of *HAR1* and the $\beta$-actin gene, respectively.

### Preparation of Putative Double Mutants of *lot1* and *har1*

To generate double mutant lines having mutations in both *LOT1* and *HAR1* genes, a *lot1* homozygote was crossed with a *har1-4* homozygote. The $F_1$ plants were allowed to self, and 15 plants homozygous for the *har1-4* allele were selected from the resulting $F_2$ plants making use of a CAPS marker. The $F_2$ plants were naturally self-crossed, and the resulting $F_3$ plants were inoculated with *M. loti* Tono. Three lines that show different nodulation from that of the *har1* single mutant, F3-45, F3-55, and F3-94, were further characterized.

Confirmation of their genotype is now under way by crossing them to the wild type for segregation of the two parental phenotypes.

## Linkage Analyses

The *lot1* mutant, which was derived from *L. japonicus* Gifu B-129, was crossed with *L. japonicus* Miyakojima MG-20, the resulting $F_1$ plants were self-crossed, and 32 $F_2$ plants with the mutant phenotype were used for subsequent analysis. To map the *lot1* locus roughly, simple sequence repeat markers in the genetic linkage map of *L. japonicus* (Hayashi et al., 2001) were selected, and then bulked segregant analyses involving agarose gel electrophoresis using 3% NuSieve 3:1 agarose or 3% Metaphor agarose (Cambrex, East Rutherford, NJ) were performed.


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