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

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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* (Schauer et al., 1998; Szczygłowski 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 *sunr* 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), DMII (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 Giftu 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.
According to the means and SDs are presented (Materials and Methods). The means and SDs are presented (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...
Each value for $P$ was significantly lower than that for the wild type at $P < 0.01$ according to the $t$ test.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>dpi</th>
<th>Wild Type</th>
<th>lot1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of infection threads</td>
<td>3</td>
<td>17.7 ± 6.1</td>
<td>2.8 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>68.4 ± 20.0</td>
<td>29.2 ± 16.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>153 ± 39.2</td>
<td>51.9 ± 21.5</td>
</tr>
<tr>
<td>Number of nodules and primordia</td>
<td>3</td>
<td>7.3 ± 1.4</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>Successful nodulation (%)</td>
<td></td>
<td>21</td>
<td>4</td>
</tr>
</tbody>
</table>

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 $P$ was significantly lower than that for the wild type at $P < 0.01$ according to the $t$ test.

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 $M. loti$ 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/mark 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.

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.

<table>
<thead>
<tr>
<th></th>
<th>$\mu$mol h$^{-1}$ plant$^{-1}$</th>
<th>$\mu$mol h$^{-1}$ g$^{-1}$ (fresh nodule weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.12 ± 0.49 a</td>
<td>17.4 ± 2.1 a</td>
</tr>
<tr>
<td>lot1</td>
<td>0.20 ± 0.09 b</td>
<td>16.2 ± 4.6 a</td>
</tr>
</tbody>
</table>
Chromosomal Mapping

Using the F2 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 (Schauers et al., 1998; SzczYGowski 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; Fenmetsa and Cook, 1997; Wopereis et al., 2000; Nishimura et al., 2002a, 2002c; Tansengco et al., 2003). It is thought that most nodulation-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; SzczYGowski 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).

**Figure 3.** Effects of varying concentrations of nitrate on nodule formation by lot1 and the wild type. Seedlings of 7 d after germination were inoculated with M. loti MAFF 303099 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.

<table>
<thead>
<tr>
<th>KNO3 (mM)</th>
<th>Number of nodules/seedling</th>
<th>lot1</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.0 a</td>
<td>w</td>
<td>x</td>
</tr>
<tr>
<td>0.1</td>
<td>3.0 a</td>
<td>w</td>
<td>x</td>
</tr>
<tr>
<td>0.3</td>
<td>4.0 a</td>
<td>w</td>
<td>x</td>
</tr>
<tr>
<td>0.5</td>
<td>5.0 a</td>
<td>w</td>
<td>x</td>
</tr>
<tr>
<td>1.0</td>
<td>6.0 a</td>
<td>w</td>
<td>x</td>
</tr>
<tr>
<td>2.0</td>
<td>7.0 a</td>
<td>w</td>
<td>x</td>
</tr>
<tr>
<td>3.0</td>
<td>8.0 a</td>
<td>w</td>
<td>x</td>
</tr>
<tr>
<td>5.0</td>
<td>9.0 a</td>
<td>w</td>
<td>x</td>
</tr>
<tr>
<td>10</td>
<td>10 a</td>
<td>w</td>
<td>x</td>
</tr>
</tbody>
</table>

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

A nifH-defective mutant of *M. loti* (ΔnifH; 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.

<table>
<thead>
<tr>
<th>Rhizobia</th>
<th>Number of Nodules</th>
<th>lot1</th>
<th>har1-4</th>
<th>Gifu B-129</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. loti</em> MAFF 303099</td>
<td>3.3 ± 1.5 a</td>
<td>NDa</td>
<td>8.1 ± 1.8 a</td>
<td></td>
</tr>
<tr>
<td>ΔnifH</td>
<td>5.8 ± 2.9 b</td>
<td>ND</td>
<td>26.3 ± 4.2 b</td>
<td></td>
</tr>
<tr>
<td><em>M. loti</em> Tono</td>
<td>2.2 ± 0.8 a</td>
<td>23.4 ± 7.3 a</td>
<td>6.8 ± 1.7 a</td>
<td></td>
</tr>
<tr>
<td><em>R. etli</em> CE3</td>
<td>0.2 ± 0.4 c</td>
<td>13.1 ± 4.3 b</td>
<td>7.3 ± 2.2 a</td>
<td></td>
</tr>
</tbody>
</table>

*aND. Not determined. bAbout 17% of the lot1 mutant inoculated with *R. etli* CE3 formed only a single nodule, the others not forming any nodules.*
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

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**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 μM ACC or 5 μM STS, and then the mature nodules were counted. C, Effect of 1 μM 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.

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**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/har1, 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 nodule mutation: Nod-, Nod2+, Hist-, and Fix-.

The results of grafting experiments with the lot1 mutant and wild type suggested that the root genotype mainly determines low nodule 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-Anolle´s 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-Anolle´s 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 nodule 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 HARI gene. We next designed grafting experiments with lot1 and har1-4 mutants. If LOT1 and HARI 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 non-nodulation 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 HARI/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 HARI determine distinct control pathways, LOT1 and HARI 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 (Penneta 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., 2003). 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 μmol m⁻² s⁻¹ 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 Dillworth (B & D) medium (Broughton and Dillworth, 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 collected to grow M2 seeds, they were grown on Power Soil (Krea 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 (Iimazumi-Anraku et al., 1997), which is a spontaneous naldixic acid-tolerant derivative of MAFF303099. M. loti MAFF303099 derivative ML001 carrying pGD499 (modBlacZ; 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 × 10⁷ 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 × 10 cm high) containing vermiculite and modified Hornum nutrient solution (Handberg and Stougaard, 1992) with a lowered NaH₂PO₄ concentration of 250 μM. 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 methane sulfonate (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, and then grown for 5 weeks 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 β-actin gene were 5'-GATACCCCTTGTCAAGTGCTATC-3' and 5'-GTTCTT- TTCACCCTTCACAACTTACAG-3', and 5'-GCATTGTGTTGCTCCTGTG-3' and 5'-TGTCGCTTCACTCCACACA-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 β-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 β-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 F₁ plants were allowed to self, and 15 plants homozygous for the har1-4 allele were selected from the resulting F₂ plants making use of a CAPS marker. The F₂ plants were naturally self-crossed, and the resulting F₃ 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.

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 examined within 12 h after inoculation of M. loti, Fähraeus slides were used (Fähraeus, 1957; Heidstra et al., 1994; Nivea et al., 2001). Two-day-old seedlings were prepared in vertically positioned agar plates and then transplanted into Fähraeus slides. The roots were left to stand in 10⁴ 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 (Bonante 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 × 10⁶ cells/plant of 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 β-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 β-actin gene were 5'-GATACCCCTTGTCAAGTGCTATC-3' and 5'-GTTCTTTACCCCTTCACAACTTACAG-3', and 5'-GCATTGTGTTGCTCCTGTG-3' and 5'-TGTCGCTTCACTCCACACA-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 β-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 β-actin gene, respectively.
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 $l$ot1 mutant, which was derived from $L. \text{ japonicus}$ Gifu B-129, was crossed with $L. \text{ japonicus}$ Miyakojima MG-20, the resulting F$_2$ plants were self-crossed, and 32 F$_2$ plants with the mutant phenotype were used for subsequent analysis. To map the $l$ot1 locus roughly, simple sequence repeat markers in the genetic linkage map of $L. \text{ japonicus}$ (Hayashi et al., 2001) were selected, and then linkage 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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**LITERATURE CITED**


Fähræus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J Gen Microbiol 16: 374–381


