**plenty, a Novel Hypernodulation Mutant in *Lotus japonicus***

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Nitrogen fixation in nodules that contain symbiotic rhizobial bacteria enables legumes to thrive in nitrogen-poor soils. However, this symbiosis is energy consuming. Therefore, legumes strictly control nodulation at both local and systemic levels. Mutants deficient in such controls exhibit a range of phenotypes from non-nodulation to hypernodulation. Here, we isolated a novel hypernodulation mutant from the *M*2 progeny derived from *Lotus japonicus* MG-20 seeds mutagenized by irradiation with a carbon ion beam. We named the mutant 'plenty' because it formed more nodules than the wild-type MG-20. The nodulation zone in the *plenty* mutant was wider than that in the wild type, but not as enhanced as those in other previously reported hypernodulation mutants such as *har1*, *klv* or *tml* of *L. japonicus*. Unlike these hypernodulation mutants, the *plenty* mutant developed nodules of the same size as MG-20. Overall, the *plenty* mutant exhibited a unique phenotype of moderate hypernodulation. However, a biomass assay indicated that this unique pattern of hypernodulation was a hindrance to host plant growth. The *plenty* mutant displayed some tolerance to external nitrates and a normal triple response to ethylene. Grafting experiments demonstrated that the root of *plenty* was responsible for its hypernodulation phenotype. Genetic mapping indicated that the *PLENTY* gene was located on chromosome 2.

**Keywords:** Biomass • Ion beam irradiation • Legume–Rhizobium symbiosis • *Lotus japonicus* • Nitrate • Root-determined hypernodulation.

**Abbreviations:** ACC, 1-aminocyclopropane-1-carboxylate; AON, autoregulation of nodulation; dCAPS, derived cleaved amplified polymorphic sequence; *har1*, hypernodulation aberrant root formation 1; *klv*, klavier; NF, nod factor; SSR, simple sequence repeat; *tml*, too much love.

**Introduction**

Legumes and rhizobia interact in a form of symbiotic mutualism, the basis of which is the formation of nodules, plant organs specific to the symbiosis. In the nodules, rhizobia fix nitrogen for use by the plant, and this symbiosis makes it possible for legumes to thrive on nitrogen-poor soils where other plant species generally struggle to survive. Rhizobial nitrogen fixation in nodules (symbiotic nitrogen fixation) is a major asset for the survival strategy of the plants and also a potentially valuable resource for maximizing crop yields. Therefore, this symbiosis has long been studied in crop legumes such as soybeans and peas both out of intrinsic biological interest and for its agricultural utility. Isolation and characterization of symbiotic mutants have become one of the main approaches to investigating the phenomenon. For example, mutants that fail to form nodules (non-nodulating mutants) have been studied to identify factors necessary for their formation (Endre et al. 2002, Stracke et al. 2002, Madsen et al. 2003, Oldroyd and Long 2003, Radutoiu et al. 2003, Ane et al. 2004, Levy et al. 2004, Mitra et al. 2004, Imaizumi-Anraku et al. 2005, Kalo et al. 2005, Smit et al. 2005, Kanamori et al. 2006, Saito et al. 2007).

The process of nodulation starts from signaling interactions between the plant and its rhizobial partner. In response to flavonoids secreted from the plant, the rhizobia secrete a signal compound called nod factor (NF) that is detected by NF receptors on the plant root hairs. Thereafter, the NF signaling pathway induces a series of events in the plant root epidermis and cortex. In the root epidermis, root hair cells undergo morphological changes, and swell, branch and curl to trap rhizobia. Trapped rhizobia are forwarded to the epidermal cell layer through infection threads formed by the host plant. An infection thread is a structure that grows and invaginates through a root hair cell, and functions as a loading route. In the cortex tissue, dedifferentiated cortical cells start to divide and differentiate into nodule primordia. When an infection thread has carried rhizobia to a nodule primordium, the rhizobia are packed into it by endocytosis and grow as bacteroids. The model legume *Lotus japonicus* develops a determinate type of nodules in which cell divisions in the nodule primordia eventually cease and the cells then elongate to achieve the final nodular structure.

Host plants use considerable energy in the nodulation process described above. In addition, the plants have to expend...
energy in nourishing the rhizobia in the nodules to enable them to fix nitrogen, and in transportation of the fixed nitrogen to other tissues. From the standpoint of energy consumption, it is crucial to the plants to maintain the appropriate scale of nodules. Therefore, plants are assumed to have negative regulatory systems to control nodulation.

The \textit{Glycine max} \textit{nts1} mutant \textit{plenty} \textit{har1} (Krussell et al. 2002, Nishimura et al. 2002a) turned out to be an ortholog of \textit{HAR1} (Searle et al. 2003). Split root experiments showed that prior nodulation inhibits subsequent nodulation, suggesting that nodulation is suppressed by a systemic negative feedback loop (Pierce and Bauer 1983, Kosslik and Bohlool 1984). Such negative feedback regulation is called autoregulation of nodulation (AON), and investigation of the \textit{nts1} mutant of \textit{G. max} using a split root system indicated that AON is impaired in the \textit{nts1} mutant (Olsson et al. 1989). Reciprocal grafting between wild-type plants and \textit{har1} mutants demonstrated that the shoot of \textit{har1} was responsible for hypernodulation (Krussell et al. 2002, Nishimura et al. 2002a), and it is thought that \textit{HAR1}/\textit{NTS1} in the shoot mediates presumptive AON signals between the root and the shoot (Okamoto et al. 2009). In \textit{L. japonicus}, another shoot-determined hypernodulating mutant \textit{klavier} (\textit{klv}) (Oka-Kira et al. 2005), and a root-determined hypernodulating mutant \textit{too much love} (\textit{tml}) (Magori et al. 2009) are also considered to be defective in AON.

In addition to systemic negative regulation via long-distance signaling, plants also have local regulation via short-distance signaling. Susceptibility to infection by rhizobia is limited to the expansion zone of the roots (Bhuvaneswari et al. 1981). The positions at which nodule organogenesis occurs are generally opposite the xylem poles and are regulated by ethylene (Heidstra et al. 1997). Ethylene can also inhibit infection thread formation by reducing the number of root hair cells (Oldroyd et al. 2001). The \textit{sickle} mutant of \textit{M. truncatula} has lost regulation by ethylene and shows a hypernodulation phenotype (Pemetsa and Cook 1997). At the infection sites, the various levels of controls work coordinately to repress production of excess nodules. Failure of this coordination would allow thousands of rhizobia to enter the root cells before autoregulatory suppression could be stimulated.

The appropriate level of nodulation is only established by the harmonious interaction of local and short-distance signaling as well as systemic and long-distance signaling. Hypernodulation mutants are invaluable for dissecting this complex web of negative regulatory systems. Here, we screened for symbiotic mutants and isolated a novel hypernodulation mutant that we named \textit{plenty}. The characteristics of this mutant are described here.

### Results

#### Screening for symbiotic mutants and isolation of a novel hypernodulation mutant

The first identified hypernodulation mutant in \textit{L. japonicus} was \textit{har1}, which was found in the Gifu B-129 ecotype (Wopereis et al. 2000, Krussell et al. 2002, Nishimura et al. 2002a). We subsequently found two other mutants, \textit{klv} (Oka-Kira et al. 2005) and \textit{tml} (Magori et al. 2009), in the MG-20 ecotype. We performed a further screen of \textit{M.} \textit{sativum} seeds irradiated with 80 Gy of carbon ion (C\textsuperscript{60}). A visual examination of 7,017 \textit{M.} \textit{sativum} lines identified 12 lines with defective nodulation, either hypernodulation or no nodulation. Two lines showed hypernodulation and breeding tests showed that the phenotypes were inherited. Allelism tests indicated that one of the lines was novel (Table 1). This mutant was crossed to the parental MG-20, and \textit{F\textsubscript{1}} plants were found to exhibit wild-type nodulation. We screened 300 \textit{F\textsubscript{2}} progeny derived from self-pollinated \textit{F\textsubscript{1}} plants and found that 67 displayed hypernodulation while the remaining 233 showed wild-type nodulation. The segregation pattern conformed to the 3:1 ratio expected of a recessive mutation ($\chi^2 = 1.138, P>0.05$). The mutant was named \textit{plenty} because it formed more nodules than the wild type but not as many as classical hypernodulation mutants.

#### Comparison of nodule numbers and distribution in \textit{plenty} and MG-20

A comparison of nodule numbers in \textit{plenty} and wild-type plants 3 weeks after inoculation with \textit{Mesorhizobium loti} showed that the former had between three and five times more nodules (Fig. 1A). The relative size of the nodulation zone, defined as the length of the nodule-forming region compared with the length of the primary root, was approximately four times larger in the mutant than in the wild type (Fig. 1B). While these

<table>
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<tr>
<th>Nodule number</th>
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<td>\textit{F\textsubscript{1}} plants (female $\times$ male)</td>
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<tr>
<td>plenty $\times$ MG-20</td>
<td>3.2 $\pm$ 1.9</td>
</tr>
<tr>
<td>plenty $\times$ har1-7</td>
<td>4.6 $\pm$ 1.9</td>
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<tr>
<td>plenty $\times$ klv</td>
<td>4.0 $\pm$ 0.7</td>
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<tr>
<td>plenty $\times$ tml</td>
<td>6.9 $\pm$ 1.6</td>
</tr>
<tr>
<td>Parents</td>
<td></td>
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<tr>
<td>MG-20</td>
<td>5.8 $\pm$ 2.1</td>
</tr>
<tr>
<td>plenty</td>
<td>14.3 $\pm$ 3.5</td>
</tr>
<tr>
<td>har1-7</td>
<td>31.1 $\pm$ 9.7</td>
</tr>
<tr>
<td>klv</td>
<td>26.9 $\pm$ 7.2</td>
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<tr>
<td>tml</td>
<td>25.9 $\pm$ 5.0</td>
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\footnote{Six-day-old seedlings were inoculated with \textit{M. loti} MAFF30–3099 and nodules were counted at 21 d after inoculation. Values are means $\pm$ SD.}
two aspects of nodule formation differed significantly between plenty and MG-20, the size of the nodules was indistinguishable in the two genotypes (Supplementary Fig. S1A). The phenotypic differences in nodule characteristics between the mutant and wild type were still apparent at 6 weeks after inoculation: plenty plants had approximately four times more nodules than MG-20 plants, while nodule sizes were similar (Supplementary Fig. S1B).

With regard to other aspects of the plenty phenotype, we noticed that the plants had a compact root architecture (Fig. 1C). In order to assess whether this root morphology was constitutive in plenty, we examined the root architecture of plants grown in the absence of rhizobia. MG-20 and plenty seedlings were grown for 3 weeks without infection of M. loti in soil soaked with B&D nutrient solution containing 1mM KNO₃. Root lengths were measured and numbers of lateral roots were counted. The morphological changes in the roots of plenty were found to be constitutive: both the primary root and the lateral roots were approximately 40% shorter than those of MG-20 (Table 2). Although plenty produced a larger number of lateral roots than MG-20, the total length of these roots was shorter than in MG-20 (Table 2). Primary root lengths were not significantly different between MG-20 and plenty at 1 week of cultivation (29.1 and 25.8 mm, respectively). However, the subsequent growth rate of the primary root was distinguishable between MG-20 and plenty. In addition, the primary root of plenty was wider than that of the wild type (Supplementary Fig. S2). The compact root architecture of plenty was still evident at 6 weeks of cultivation (data not shown). As described above for M. loti-infected plants, the shoots of uninfected plenty plants were generally shorter than those of the wild type, perhaps as a result of shorter internodes (Fig. 1C). Other than this, there were no obvious differences between the shoots in plenty and wild-type plants.

**Triple response of the plenty mutant to ethylene**

The sickle mutant of M. truncatula displayed a hypernodulation phenotype and ethylene insensitivity (Penmetsa and Cook 1997). The sickle mutant forms nodules in a dense manner in a limited root zone, a somewhat similar phenotype to that of plenty. As the triple response in sickle is known to be abnormal, we sought to determine whether the altered pattern of nodulation in plenty involved a change in sensitivity to ethylene by treating the plants with 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene (Penmetsa and Cook 1997). Wild-type and plenty seedlings were grown in the dark for a week on agar plates containing 0, 1, 10 or 100 µM ACC. We found that the plenty plants showed a normal triple response. As the ACC concentration rose, plenty seedlings

**Fig. 1** Nodulation phenotype of the mutant plenty. After germination, wild type (MG-20) and mutant plenty seedlings were inoculated with M. loti MAFF30-3099 in vermiculite soaked with B&D nutrient solution containing 1mM KNO₃ and grown for 3 weeks. (A) The number of nodules per plant. (B) The nodulation zone as a proportion of root length in MG-20 and plenty plants. The nodulation zone was defined as the distance between the first and last nodules on the primary root. Values are means ± SD. **P < 0.01, Student’s t-test. n = 7. (C) Three-week-old MG-20 (left) and plenty (right). (D, E) Close-up view of the nodules. (D) MG-20 and (E) plenty. Scale bar = 1 cm (C) or 5 mm (D, E).

**Table 2** Root properties of 3-week-old wild-type and plenty plants

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<tr>
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<th>Primary root</th>
<th>Lateral root</th>
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<tr>
<td></td>
<td>Length (cm)</td>
<td>Number</td>
</tr>
<tr>
<td>MG-20</td>
<td>10.5 ± 1.2</td>
<td>19.0 ± 2.9</td>
</tr>
<tr>
<td>plenty</td>
<td>6.7 ± 0.6**</td>
<td>23.4 ± 3.8**</td>
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*Plants were grown for 3 weeks after germination in closed plant boxes packed with vermiculite soaked with B&D nutrient solution containing 1mM nitrate. Values are means ± SD.

**P < 0.01, Student’s t-test. n = 5.**
increasingly exhibited suppressed elongation of hypocotyls and roots, radial swelling of hypocotyls and an exaggerated angle of the apical hooks (Fig. 2). These responses indicate that the plenty mutant is not defective in ethylene sensitivity.

**Biomass of plenty mutants in relation to nodule formation**

Nodule formation and plant growth are closely related; hypernodulation mutants generally show clear growth retardation. In contrast, such growth retardation was unclear in the moderate hypernodulation mutant plenty. If the mutation has no severe effect on growth, then a greater number of nodules than the wild type might be within an acceptable range in a biological sense. Hence, we examined the relationship between biomass accumulation and nodule formation in plenty. We measured dry weights of shoots, roots and nodules from MG-20 and plenty plants grown with or without *M. loti* inoculation and at different KNO₃ concentrations (0, 5 or 10 mM) for 3 or 6 weeks. The plants were given a supplement of KNO₃ every 4 d.

In the absence of rhizobia, there were no significant differences in the total dry weights of MG-20 and plenty under all tested conditions (Fig. 3A–F). Thus, plenty plants showed almost identical biomass production to MG-20 when free from nodulation.

In stark contrast, however, there were clear differences in plant growth in the presence of rhizobia, especially under conditions of nitrate deficiency. In plants grown with 0 mM KNO₃ the dry weights of plenty shoots and roots were lower than those of MG-20 at both 3 and 6 weeks (Fig. 3G, J). The dry weights of plenty shoots were approximately 75% of those of MG-20 at 3 and 6 weeks (Fig. 3G). A greater difference was found for root biomass: the dry weights of plenty roots were 54% of those of MG-20 at 3 weeks and 58% at 6 weeks (Fig. 3J). In contrast, the nodule biomass of plenty was almost twice as large as that of MG-20 at 3 and 6 weeks (Fig. 3M).

When external nitrate was abundant (at 10 mM KNO₃), the dry weights of plenty shoots and roots did not differ significantly from those of MG-20 (Fig. 3I, L). At 5 mM KNO₃, there was a significant difference in root biomass at 6 weeks (Fig. 3K). Even under high nitrogen conditions, the nodule biomass of plenty was greater than that of MG-20: approximately six times more at 5 mM nitrate and nine times more at 10 mM nitrate (Fig. 3N, O).

Regardless of the cultivation conditions, the plenty mutants showed a compact appearance with smaller shoots and root architecture (data not shown).

**plenty showed partial nitrate tolerance to increased external nitrate concentration**

Legumes are able to utilize gaseous nitrogen through symbiotic nitrogen fixation. They defray the energy cost of this process using photosynthetic products. Thus, when the soil is rich in nitrates or ammonium, legumes strive to suppress nodulation and symbiotic nitrogen fixation. The suppression of nodulation in soils abundant in nitrogen sources was first recorded almost 150 years ago (Rautenberg and Kühn 1864). External nitrates also affect the growth of nodules that have already formed (Fujikake et al. 2002, Fujikake et al. 2003).

We found that nodule biomass in plenty declined with increasing nitrate concentration (Fig. 3M–O). This decline could result from a decrease in the number of nodules, repression of the growth of the nodules or a combination of both factors. To determine the cause, we counted the number of nodules and measured their diameters. In MG-20 plants, the fall in nodule biomass was related to a decrease in nodule numbers as well as nodule diameters; this effect was present at both 3 and 6 weeks (Fig. 4A, B, E, F). In contrast, at 3 weeks, plenty showed a slightly increased number of nodules at higher nitrate concentrations (Fig. 4A, C). At 6 weeks, however, nodule numbers of plenty decreased (Fig. 4B, D). Nodule diameters of plenty decreased at both 3 and 6 weeks (Fig. 4E, F).

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**Fig. 2** Ethylene sensitivity of the plenty mutant. Wild-type and plenty seedlings were grown in the dark for a week on vertical agar plates containing 0, 1, 10 or 100 µM of the ethylene precursor, 1-aminocyclopropane-1-carboxylate (ACC). (A) Relative lengths of hypocotyls. The length of seedlings treated with 0 µM ACC was standardized as 100%. Values are means ± SE. n = 5. (B) Plenty seedlings displayed a clear dose-dependent response to ACC similar to wild-type seedlings. Scale bar = 1 cm.
**Fig. 3** Biomass assay. Wild-type and *plenty* plants were grown under different conditions with (+) or without (−) *M. loti* inoculation, and sampled at 3 and 6 weeks. Shoots and roots from each plant were dried separately and weighed. (A–F) Dry weights of shoots and roots in the absence of rhizobial inoculation. (G–O) Dry weights of shoots, roots and nodules after rhizobial inoculation. (A, D, G, J, M) 0 mM nitrate. (B, E, H, K, N) 5 mM nitrate. (C, F, I, L, O) 10 mM nitrate. Values are means ± SD. *P < 0.05; **P < 0.01 (Student’s t-test). n = 5.
Nitrate tolerance and initiation of nodule formation in plenty

As described above, the number of nodules in plenty at 3 weeks after inoculation with rhizobia did not decrease at high concentrations of nitrate (Fig. 4A, C). We therefore sought to investigate the initiation of nodule formation in plenty at different concentrations of KNO₃. To this end, we examined infection thread formation and nodule organogenesis. As the infection threads act as loading routes for rhizobia, they can be observed by staining the densely packed bacteria. Mesorhizobium loti NZP2235 constitutively expresses the lacZ reporter gene, and are stained for β-galactosidase activity. We sowed seeds in soil soaked with B&D nutrient solution containing different concentrations of nitrate and a suspension of M. loti NZP2235, and 10-day-old infected roots were stained for β-galactosidase activity. The numbers of infection threads and nodule primordia in seedlings were counted. We found that MG-20 seedlings showed a drastic decrease in the number of infection threads and nodule primordia with the increase in nitrate concentrations. The plenty seedlings likewise showed a decrease with increased nitrate concentrations, but the numbers of both infection threads and nodule primordia were always greater than in MG-20 (Fig. 5A, B). In the plenty seedlings, nodule organogenesis appeared to be affected more strongly than infection thread formation by the higher nitrate concentrations. Overall, at the time of initiation of nodule formation, plenty responded to higher nitrate concentrations, though the responsiveness of plenty was lower than that of MG-20 (Fig. 5C, D).
Nodulation in the plenty mutant is controlled by the roots

Grafting experiments showed that the shoots and not the roots are responsible for the loss of control of nodule formation in har1 (Krusell et al. 2002). Similarly, in klv, the shoot determines hypernodulation (Oka-Kira et al. 2005). In contrast to these two mutants, the root controls nodulation in tml (Magori et al. 2009).

In order to assess how nodule formation is determined in plenty, we carried out reciprocal grafting experiments on 3-day-old MG-20 and plenty seedlings in the following combinations: MG-20 (scion)/plenty (rootstock) and plenty (scion)/MG-20 (rootstock); and MG-20/MG-20 and plenty/plenty as controls.

After grafting surgery, we transplanted the successful grafts into soil soaked with B&D nutrient solution containing 1 mM KNO₃, and inoculated them with M. loti a few days later. The number of nodules was counted 3 weeks later. We found that nodule numbers only increased when the root was provided by the plenty mutant (Fig. 6A). We therefore conclude that the root but not the shoot determines the high number of nodules in plenty.

We also undertook other grafting experiments to investigate the interaction of plenty with the har1 mutant. The following graft combinations were compared: har1 (scion)/plenty (rootstock), har1 (scion)/har1 (rootstock) and har1 (scion)/MG-20 (rootstock). The har1/plenty combination showed an intriguing additivity in the number of nodules (Fig. 6B). It is therefore possible that PLENTY functions locally in a different signaling pathway from that of HAR1.

Mapping of the plenty mutation to L. japonicus chromosome 2

To determine the chromosomal location of the plenty mutation, we performed a linkage analysis using simple sequence repeat (SSR) markers covering the six chromosomes of L. japonicus. This analysis indicated that the mutation was located on the long arm of chromosome 2 between the markers TM0002 and TM0324. The range was further narrowed to between TM0545 and TM0099. The analyses indicated that the PLENTY locus was strongly linked with TM0370 (Fig. 7).

Discussion

We have described here a novel hypernodulation mutant, plenty, that was identified in L. japonicus MG-20 following mutagenesis by carbon ion beam irradiation. The mutation was called ‘plenty’ as its phenotype involves excessive root nodules. The mutant was not allelic with other L. japonicus hypernodulation mutants, har1, klv or tml, or astray, the enhanced-nodulating mutant of L. japonicus (Nishimura et al. 2002b, Nishimura et al. 2002c). Although plenty shows some similarities to previously reported hypernodulation mutants,
it also shows some unique characteristics. For instance, plenty has a short primary root with an increased number of short lateral roots. This root architecture resembles that of har1, except that har1 has a higher number of shorter lateral roots than plenty (data not shown). The latter shows a superficial resemblance to astray or sickle with regard to the pattern of nodulation, but the details differ. The plenty mutant formed nodules in a wider root zone than astray or sickle, but never approached the root tip. This pattern also contrasts with that of an AON-impaired mutant such as har1. In AON-impaired mutants, nodule formation continues as the roots elongate, so that nodules almost entirely cover the roots. Furthermore, the plenty mutant formed fewer nodules than AON-impaired mutants. The most distinctive feature of plenty is that it formed nodules of a normal size; other hypernodulation mutants form small nodules. With regard to this aspect of nodule size, the plenty model is truly novel. Overall, the plenty mutant is unlike all previously reported hypernodulating mutants and displays a unique nodulation phenotype, suggesting that it forms a new category of hypernodulation mutation.

We found here that external nitrate could inhibit the growth of nodules (Fig. 4E, F). This effect has also been observed in soybeans (Fujikake et al. 2002, Fujikake et al. 2003). In addition, the reduction in size of nodules due to external nitrate cancelled the negative impact of excessive nodule numbers on plant growth (Figs. 3H, I, 4A, B). Thus, even if nodule numbers are high, the total effect of the nodules can be restrained by nodule growth inhibition reducing the size of the nodules. In contrast, nodules grow and gain weight in nitrate-deficient conditions. Excessive nodules in plenty gained weight and became a hindrance to growth of the plenty shoots and roots (Fig. 3G, J, M). The pattern of plenty hypernodulation is inappropriate for plant growth; only under external nitrate-deficient conditions as often occur in the natural environment does nodulation provide a valuable contribution to survival in legumes. The pattern of plenty hypernodulation also suggested that both the numbers and sizes of nodules are important, as together they both have an impact on the growth of the plants. In this respect, dry weights proved to be a precise index for evaluating the total scale of nodule formation. We do not know yet whether legumes have any external nitrate-independent internal mechanism(s) that inhibits nodule growth. That is, do legumes have a mechanism through which they can detect an excess of nodules and can selectively stop distribution of photosynthates to the extra nodules? On the assumption that such an internal mechanism(s) does not exist, any deviation from the appropriate number of nodules should be prevented because even a slight excess of nodules could be a great hindrance to host plant growth, as seen in plenty.

Generally, in mutants with smaller nodule numbers per plant, the size and biomass per nodule tend to increase relative to the wild type. In contrast, the size and biomass per nodule tend to decrease in mutants with increased nodule numbers. However, such a tendency was not observed in plenty. At the same time, the nodule biomass of plenty did not increase...
in proportion to the number and size of nodules. The plenty mutant formed 3–5 times more nodules of the same size as the wild type, but the total biomass of nodules in plenty was only twice as large as that of MG-20. Thus, the weight per nodule in plenty is not as great as in the wild type. One possible reason is that the histological structure of nodules is different between the wild type and plenty. Nodules of plenty might have lower cell density or more highly vacuolated uninfected cells than the wild type.

Biomass assay indicated another intriguing aspect of the plenty mutant. In the absence of rhizobia, plenty plants were comparable with the wild-type plants in biomass production (Fig. 3A–F). However, the total length of roots was significantly reduced in the plenty mutants (Table 2). Since the root width of plenty was already wider than that of the wild type at 1 week of cultivation (Supplementary Fig. 52), the shorter roots of plenty were possibly compensated by increased radial expansion. Longitudinal cell elongation might be abnormal in plenty, and the same type of compensation might cause shorter internodes of plenty shoots because the plenty shoots with shorter internodes were thicker than the wild type by visual inspection. Cytological observations will be needed to examine whether or not the plenty mutation also affects cell proliferation.

Although the plenty mutant always formed a larger number of nodules than the wild type, it did not lose the ability to respond to external nitrates. After 6 weeks of cultivation, the ability of plenty to respond to external nitrates appeared indistinguishable from that of the wild type (Fig. 4D). At 3 weeks, however, the relative number of nodules increased in plenty (Fig. 4C). Since the only difference in response of plenty was found at the earlier cultivation interval, the PLENTY gene might have a more important role in juvenile plants.

When the external nitrate concentration was high, plenty showed a reduction in infection thread formation and in nodule organogenesis (Fig. 5). Nevertheless, the absolute number of both infection threads and nodules did not decrease to the wild-type level. This suggests that the effects of the external nitrate concentration on the initiation of nodulation could only partially cancel the effect of the plenty mutation. If PLENTY functions in multiple negative signaling cascades and some of the cascades in plenty can be rescued by the nitrate inhibition pathway(s), it is possible to understand why external nitrate inhibition only partially cancels the effect of the plenty mutation.

One of the important aspects in characterization of a hypernodulation mutant is determining which organ, the shoot or the root, is responsible for the hypernodulation phenotype. Our grafting experiments here showed that the root of plenty was responsible for hypernodulation (Fig. 6A). This raises the question of whether PLENTY is one of the components that function in the HAR1-mediated AON pathway, or is a local factor working in a different pathway from that of HAR1-mediated AON. Besides, if plenty were defective in AON, we would expect that roots would have many more nodules over their entire lengths. However, one possible reason why plenty has a moderately enhanced nodulation zone might be because it is a relatively weak mutation. Further investigations will be needed to reach a definitive answer on the question of whether PLENTY is a local factor independent of HAR1-mediated systemic regulation. We are now generating plenty/har1 double mutants in order to perform genetic analyses to elucidate the relationship between PLENTY and HAR1.

The region in which the PLENTY gene is located also contains other genes associated with nodulation, namely NIN, NUP133, NFR1, ALB1, LOT1, NUP85, and NSP2 (Sandal et al. 2006). These are positive factors that function in NF signaling. PLENTY is the first gene positioned in this region that acts as a negative factor. This leads us to speculate that PLENTY is a negative regulator that appeared along with positive controllers during the evolution of symbiosis. Cloning the gene will enable further analyses of PLENTY, which should shed light on the mechanism(s) of negative regulation of nodulation.

## Materials and Methods

### Plant materials

The plenty mutant was isolated by screening M3 seedlings obtained from L. japonicus MG-20 seeds mutagenized by carbon ion beams. The details of the ion beam irradiation have been reported previously (Oka-Kira et al. 2005, Magori et al. 2009). For allelism tests and grafting, we used har1-7, klv and tml mutants on an MG-20 background. For mapping, Gifu B-129 was crossed to plenty.

### Plant and bacterial growth conditions

Seeds were scarified with sandpaper, sterilized with 25% sodium hypochlorite solution and rinsed with enough sterilized water for overnight imbibition. Seeds were sown in sterilized vermiculite soaked with B&D nutrient solution (Broughton and Dilworth 1971). Seedlings were grown under a light/dark cycle of 16 h day/8 h night in a Biotron LH-300 (Nihon-ika Co. Ltd.) with a light intensity of 150 μmol photons m⁻² s⁻¹ at 22°C. The bacterial strains M. loti MAFF30-3099 and NZP2235 were used. NZP2235 is a strain expressing the lacZ reporter gene under a hemA promoter. Bacteria were cultured in YEM at 28°C for 48 h, and then collected and resuspended in B&D nutrient solution. The suspension was used to soak vermiculite to inoculate bacteria onto seedlings.

### Nodulation assay

The number of nodules was counted under a microscope at 3 and 6 weeks of cultivation. Nodules and nodule primordia of >0.2 mm diameter were counted. For the infection threads assay, roots were removed from plants at 10 d after inoculation, fixed for 1 h with 1.25% glutaraldehyde in 0.1 M sodium...
phosphate buffer (pH 7.2) by vacuum infiltration, washed twice in 0.1 M sodium phosphate and stained overnight at room temperature in 0.1 M sodium phosphate containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 0.08% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Non-specific staining was removed by washing the roots in 70% alcohol solution for 5 min. The roots were then observed under bright-field microscopy (Olympus BX50).

**Bioassay**

Twelve different growth conditions were established: with or without *M. loti* inoculation, three different nitrate concentrations (0, 5 and 10 mM) and two different cultivation times (3 and 6 weeks). Plants in each treatment group received a supplement containing the appropriate concentration of KNO$_3$ every 4 d. The shoots, roots and nodules of the plants were selected F2 plants and PCR was performed using the primers selected for rootstocks being covered by a piece of moistened filter paper separated by a water film. After surgery, the grafts were grown vertically for 2 d (16 h light/8 h dark); seeds for scions were incubated in the dark for 24 h and grown vertically for 24 h (16 h light/8 h dark); seeds for rootstocks were incubated in the dark for 2 d and grown vertically for 24 h; seeds for scions were incubated in the dark for 7 d. For the nitrate sensitivity assay, plants were generally grown in soil soaked with B&D nutrient solution containing 0, 5 or 10 mM KNO$_3$.

**Grafting**

The grafting technique essentially followed that described by Magori et al. (2009) but with a few modifications. In brief, seeds for rootstocks were incubated in the dark for 24 h and grown vertically for 2 d (16 h light/8 h dark); seeds for scions were incubated in the dark for 2 d and grown vertically for 24 h (16 h light/8 h dark). Any excess water was wiped from the graft junction to ensure that the scion and rootstock were not connected by a water film. After surgery, the grafts were grown vertically on moistened filter papers in plastic plates, with the rootstocks being covered by a piece of moistened filter paper to avoid their tips drying. Success or failure of each graft was observed under bright-field microscopy (Olympus BX50).

**Mapping**

The mapping populations were generated from a cross between *plenty* and Gifu B-129. F1 plants were selfed and F2 seeds were collected. F2 plants were inoculated with *M. loti* and grown under standard conditions. Plants exhibiting the *plenty* phenotype were selected by visual screening and used for the linkage and mapping analyses. Genomic DNA was extracted from the selected F2 plants and PCR was performed using the primers for the following SSR and dCAPS (derived cleaved amplified polymorphic sequence) markers: TM0002, TM0324, TM0545, TM0099, TM0031 and TM0370. PCR products were resolved on non-denaturing 30% acrylamide gels.

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**References**


