Enhanced Nodulation and Nitrogen Fixation in the Abscisic Acid Low-Sensitive Mutant *enhanced nitrogen fixation1* of *Lotus japonicus*[1][OA]

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The phytohormone abscisic acid (ABA) is known to be a negative regulator of legume root nodule formation. By screening *Lotus japonicus* seedlings for survival on an agar medium containing 70 μM ABA, we obtained mutants that not only showed increased root nodule number but also enhanced nitrogen fixation. The mutant was designated *enhanced nitrogen fixation1* (*enf1*) and was confirmed to be monogenic and incompletely dominant. The low sensitivity to ABA phenotype was thought to result from either a decrease in the concentration of the plant's endogenous ABA or from a disruption in ABA signaling. We determined that the endogenous ABA concentration of *enf1* was lower than that of wild-type seedlings, and furthermore, when wild-type plants were treated with abamine, a specific inhibitor of 9-cis-epoxycarotenoid dioxygenase, which results in reduced ABA content, the nitrogen fixation activity of abamine-treated plants was elevated to the same levels as *enf1*. We also determined that production of nitric oxide in *enf1* nodules was decreased. We conclude that endogenous ABA concentration not only regulates nodulation but also nitrogen fixation activity by decreasing nitric oxide production in nodules.

Many legumes establish nitrogen-fixing root nodules following reciprocal signal exchange between the plant and rhizobia (Hayashi et al., 2000; Hirsch et al., 2003). The host plant produces chemical compounds, frequently flavonoids, which induce rhizobial *nod* genes, whose products are involved in the synthesis and secretion of Nod factor. Perception of this chitolipooligosaccharide by the host plant results in the triggering of a signal transduction cascade that leads to root hair deformation and curling and subsequent cortical cell divisions, which establish the nodule primordium. The rhizobia enter the curled root hair cell and nodule primordial cells through an infection thread. Eventually, the rhizobia are released into nodule cells, enclosed within a membrane, and differentiate into nitrogen-fixing bacteroids that reduce atmospheric nitrogen into ammonia. In return, the host plant supplies photosynthetic products, to be used as carbon sources, to the rhizobia (Zuanazzi et al., 1998; Hayashi et al., 2000).

The host plant is known to be important for regulating the number of nodules established on its roots. For example, hypernodulating mutants such as *nitrate-tolerant symbiotic1* (*nts1*; *Glycine max*), *hypernodulation aberrant root formation1* (*har1*; *Lotus japonicus*), *super numeric nodules* (*sunn*; *Medicago truncatula*), and *symbiosis29* (*sym29*; *Pisum sativum*) disrupt the balance between supply and demand by developing excessive root nodules (Oka-Kira and Kawaguchi, 2006). Grafting experiments demonstrated that leaf tissue is a principal source of the systemic signals contributing to the autoregulation of nodulation (Pierce and Bauer, 1983; Kosslak and Bohlool, 1984; Krusell et al., 2002; Nishimura et al., 2002b; van Brussel et al., 2002; Searle et al., 2003; Schnabel et al., 2005). The *Nts1*, *Har1*, *Sunn*, and *Sym29* genes encode a receptor-like kinase similar to CLAVATA1, which regulates meristem cell number and differentiation (Krusell et al., 2002; Nishimura et al., 2002a; Searle et al., 2003; Schnabel et al., 2005).

Phytohormones are also known to regulate nodulation (Hirsch and Fang, 1994). For example, ethylene is a well-known negative regulator of nodulation, influ-

encing the earliest stages from the perception of Nod factor to the growth of infection threads (Nukui et al., 2000; Oldroyd et al., 2001; Ma et al., 2003). The ethylene-insensitive mutant *sickle1* (*skl1*) of *M. truncatula* has a hypernodulating phenotype (Penmetsa and Cook, 1997). *Skl1* is homologous to *Ethylene insensitive2* of Arabidopsis (*Arabidopsis thaliana*), which is part of the ethylene-signaling pathway (Alonso et al., 1999; Penmetsa et al., 2008). In contrast, cytokinin is a positive regulator of nodulation. The cytokinin-insensitive mutant *hyperinfected1* (loss of function) of *L. japonicus* and the *spontaneous nodule formation2* (gain of function) mutants of *M. truncatula* provide genetic evidence demonstrating that cytokinin plays a critical role in the activation of nodule primordia (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007).

Abscisic acid (ABA), added at concentrations that do not affect plant growth, also negatively regulates nodulation in some legumes (Phillips, 1971; Cho and Harper, 1993; Bano et al., 2002; Bano and Harper, 2002; Suzuki et al., 2004; Nakatsukasa-Akune et al., 2005; Liang et al., 2007). Recently, *M. truncatula* overexpressing *abscisic acid insensitive1-1*, a gene that encodes a mutated protein phosphatase of the type IIC class derived from Arabidopsis and that suppresses the ABA-signaling pathway (Leung et al., 1994; Hagenbeek et al., 2000; Gampala et al., 2001; Wu et al., 2003), was shown to exhibit ABA insensitivity as well as a hypernodulating phenotype (Ding et al., 2008).

In this study, we isolated a *L. japonicus* (Miyakojima MG20) mutant that showed an increased root nodule phenotype and proceeded to carry out its characterization. This mutant, named *enhanced nitrogen fixation1* (*enf1*), exhibits enhanced symbiotic nitrogen fixation activity. Most legume nitrogen fixation activity mutants, such as *ineffective greenish nodules1* (*ign1*), *stationary endosymbiont nodule1*, and *symbiotic sulfate transporter1* (*sst1*), are Fix$^-$ (Suganuma et al., 2003; Krusell et al., 2005; Kumagai et al., 2007).

RESULTS

Isolation of the *enf1* Mutant

To obtain ABA-insensitive or low-sensitive mutants of *L. japonicus*, we treated Miyakojima MG20 with ethyl methanesulfonate to induce base substitutions randomly in the genome. M3 seeds were sown on an agar-solidified medium containing 70 μM ABA, a concentration that inhibits the germination of wild-type MG20 seeds. M4 plants obtained by the screening were inoculated with rhizobia (*Mesorhizobium loti* MAFF303099), and the number of nodules per plant was counted 35 d after inoculation (DAI). Mutant *No. 12* not only formed more root nodules than did the wild-type MG20 plants but, surprisingly, also exhibited increased nitrogen fixation activity per plant. Both mutant phenotypes were stably inherited in the M4 and M5 generations. Back-crossing mutant *No. 12* to wild-type MG20 yielded 153 F2 progeny, from which a line that showed the highest nitrogen fixation activity and numerous nodules per plant was derived. This line was designated *enf1*.

Symbiotic *enf1* Phenotypes

Figure 1 illustrates the nodulation phenotypes of MG20 and *enf1* *L. japonicus* at 28 DAI. The number of nodules formed on *enf1* roots was approximately 1.7 times greater than that of MG20; concomitantly, the weight of nodules per *enf1* plant also increased slightly (Figs. 1 and 2, A and B). In general, hypernodulating mutants tend to form smaller nodules than wild-type plants (Wopereis et al., 2000), and this is also the case for the *enf1* mutant. Surprisingly, however, the nitrogen fixation activity per *enf1* plant was elevated 1.8 times over that of the wild-type plants (Fig. 2C). Moreover, based on the fact that nitrogen fixation activity per unit of *enf1* nodule weight was also increased 1.7 times (Fig. 2D), we concluded that the increased nitrogen fixation activity was not solely due to the enhanced number of root nodules.

ABA is known to inhibit the early stages of nodulation. Therefore, we investigated infection thread formation in *enf1* from 4 to 12 DAI. Although infection pockets were detected (4 DAI) earlier in MG20 root hairs compared with the *enf1* mutant (data not shown),

Figure 1. Symbiotic and growth phenotypes of the *L. japonicus* *enf1* mutant. *M. loti*-inoculated plants were grown in pots containing vermiculite, which was watered with B&D medium. Wild-type MG20 (left) and *enf1* (right) *L. japonicus* plants 28 DAI are shown. Bar = 1 cm.
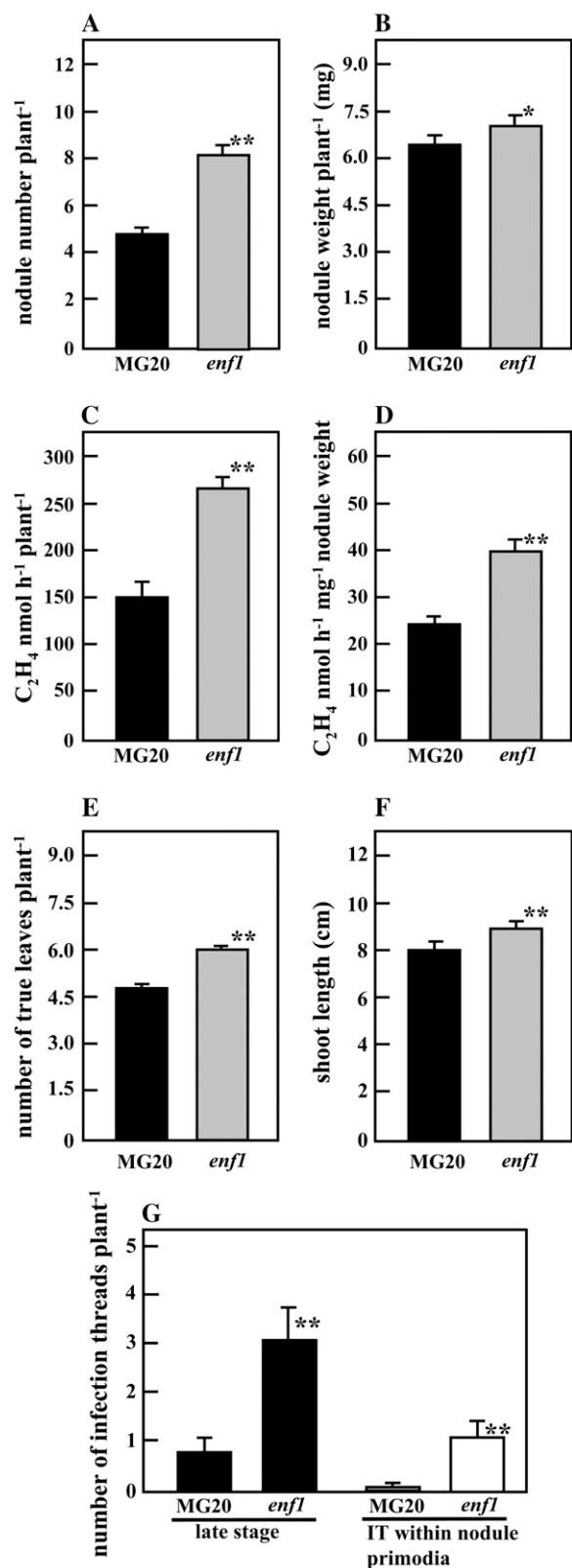
Figure 2. Analysis of *enf1* symbiotic phenotypes. *M. loti*-inoculated plants were grown for 28 d in pots containing vermiculite and watered with B&D medium (A–F) or grown on B&D agar-solidified (1.5%, w/v) medium for 12 d (G). The plants on the agar plates were inoculated with

elongated infection threads were more common in *enf1* root hairs at later stages of development (8–12 DAI; black bars in Fig. 2G). Furthermore, infection threads were detected in nodule primordia more frequently in *enf1* compared with MG20 (white bars in Fig. 2G).

Nonsymbiotic *enf1* Phenotypes

In addition to the symbiotic phenotypes just described, *enf1* had more true leaves and exhibited greater shoot length at 28 DAI than the MG20 plants (Fig. 2, E and F). To confirm that *enf1*'s vigorous growth was due to an increase in nitrogen supplied from nodules, MG20 and *enf1* plants without rhizobial inoculation were grown in the presence (10 mM KNO_3) or absence of nitrogen. Figure 3, A and B, illustrates data derived from MG20 and *enf1* plants 21 d after sowing (DAS). Surprisingly, under conditions of nitrogen sufficiency or nitrogen inefficiency in the absence of rhizobia, *enf1* shoot length was statistically lower than that of MG20 (Fig. 3, C and D), a result completely opposite to that observed for plants inoculated with *M. loti* (Fig. 2F). These results indicate that rhizobial inoculation of the *enf1* mutant has a greater effect on the shoot growth than it does on wild-type plant.

The number of true leaves in *enf1* plants was also increased (usually by one) compared with MG20 plants under both (+)nitrogen and (−)nitrogen conditions (Fig. 3, E and F), which was similar to the result observed for inoculated plants (Fig. 2E).

Because ABA is known to affect lateral root formation in *L. japonicus* (Suzuki et al., 2004), the number of lateral roots of 24-d-old (24 DAS) MG20 and *enf1* were counted. Following inoculation with *M loti*, only a few lateral roots were observed, so that a significant difference between MG20 and *enf1* could not be detected (data not shown). However, the number of lateral roots developed on uninoculated *enf1* was significantly decreased compared with MG20 without *M. loti* (Fig. 3G).

Low Sensitivity of *enf1* to ABA

Because *enf1* was isolated as an ABA low-sensitivity mutant, we tested seed germination on an agar medium containing 70 μM ABA. Two independent experiments of 40 seeds each demonstrated that *enf1* seeds germinated better than the wild-type seeds (MG20 average = 6.25%, *enf1* average = 17.5%). No difference
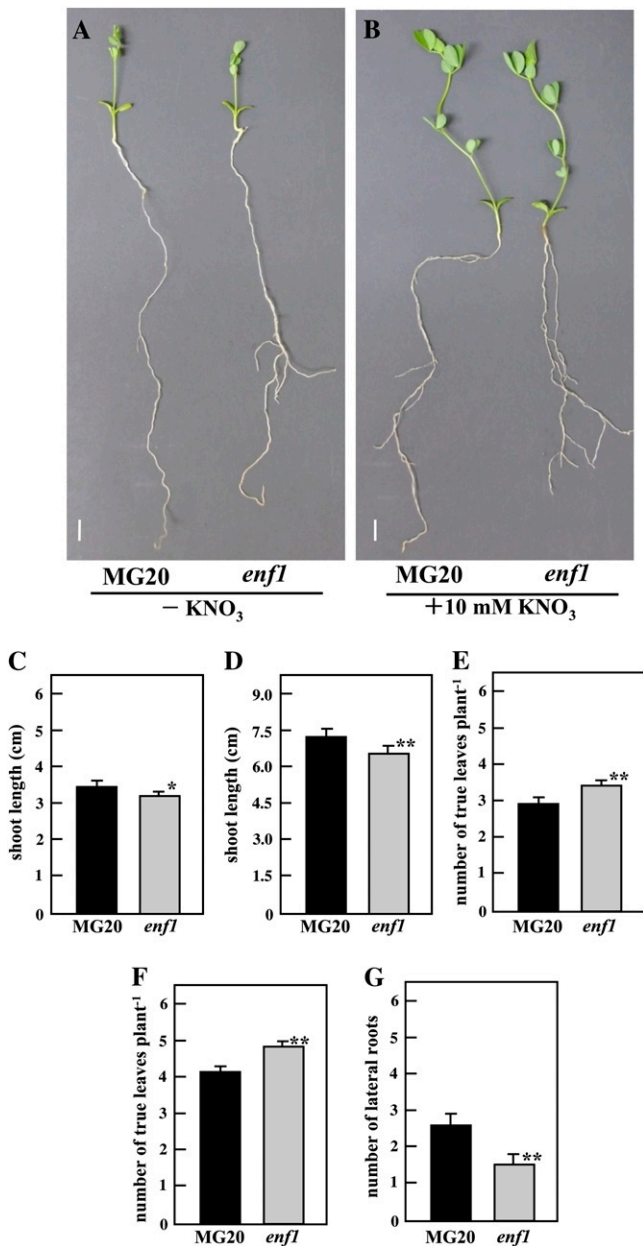
Figure 3. Nonsymbiotic phenotypes of the *enf1* mutant. A to F, Plants were grown for 21 d in vermiculite-filled pots supplied with B&D medium with or without 10 mM KNO$_3$. G, Plants were grown on B&D agar-solidified (1.5%, w/v) medium. A, MG20 (left) and *enf1* (right) grown with no nitrogen. B, MG20 (left) and *enf1* (right) grown with 10 mM KNO$_3$. C and D, Shoot length resulting from growth without KNO$_3$ (C) or with 10 mM KNO$_3$ (D). E and F, Number of true leaves per plant in medium without KNO$_3$ (E) or with 10 mM KNO$_3$ (F). G, Number of lateral roots per plant at 24 DAS. At least 30 plants were used in each experiment. Error bars indicate SE, and statistical significance is indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, by Student's *t* test).

was observed in seed germination between MG20 and *enf1* in agar medium without ABA (data not shown).

We next examined whether ABA had any toxicity effects on the growth of MG20 and *enf1*. When *M. loti*-inoculated MG20 plants were grown in the presence of

0.5 μM ABA for 17 d, most of the young leaves at the shoot tip turned brown and shriveled (78.7%; 48 of 61 plants). In contrast, although some yellowing of *enf1* leaves occurred, the shoot tip showed very little dieback (9.6%; five in 52 plants; Fig. 4). These results strongly suggest that *enf1* is not as sensitive to exogenous ABA as is the wild-type MG20.

Low Endogenous Concentration of ABA in *enf1*

We measured the endogenous ABA concentration of *enf1* at 21 DAI and found that it was four or 2.5 times lower in nodulated roots and shoots, respectively, compared with wild-type nodulated roots and shoots (Fig. 5).

Analysis of *enf1* in Long-Term Growth Experiments with *M. loti*

We found little difference between the *enf1* mutant and wild-type *L. japonicus* in terms of seed set rate, pod length, and the number of seeds per pod at 98 DAI (Table I). However, the fresh and dry weights of 100 seeds of *enf1* were significantly larger than the comparable number of wild-type seeds, suggesting that
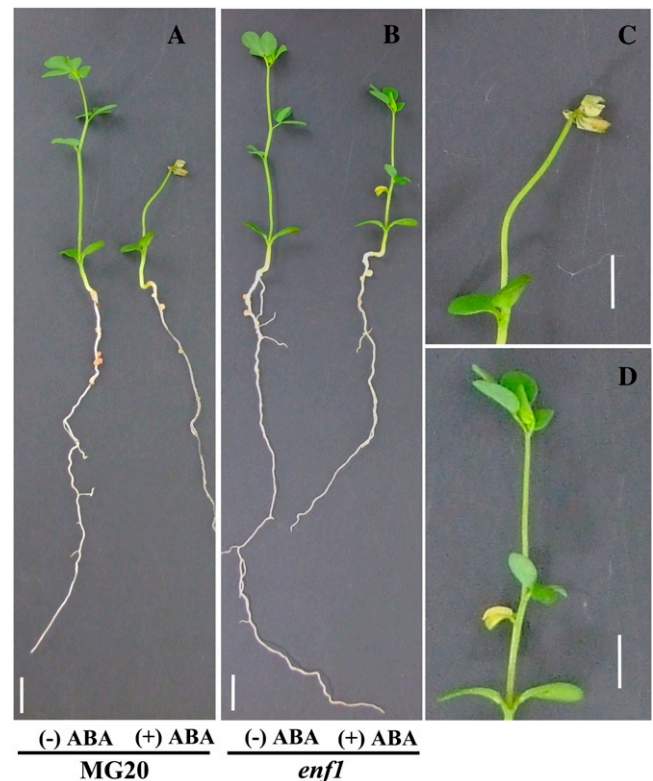

Figure 4. Effects of 0.5 μM ABA on the growth of *enf1*. A, MG20 at 17 DAI with *M. loti*. B, *enf1* at 17 DAI with *M. loti*. Shown are untreated MG20 and *enf1* (left) and treated with 0.5 μM ABA (right). C and D, Shoot tips of MG20 (C) and *enf1* (D) following 0.5 μM ABA treatment. Bars = 1 cm.
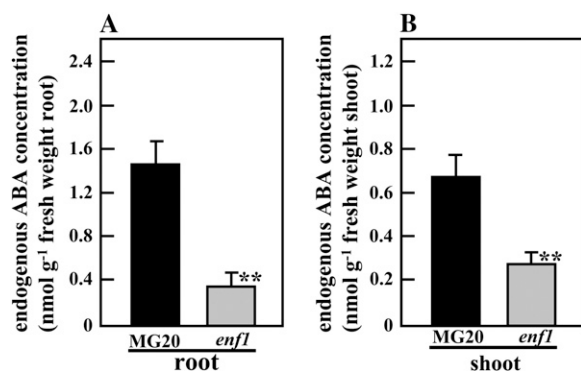
Figure 5. Endogenous concentration of ABA in *enf1*. *M. loti*-inoculated plants were grown for 21 d on vermiculite-filled pots supplied with B&D medium. Endogenous concentrations of ABA in MG20 and *enf1* roots (A) and shoots (B) are shown. The data represent averages ± SE of six independent experiments derived from eight different plants. Statistical significance is indicated by asterisks (** $P < 0.01$, by Student's *t* test).

enf1 may result in increased seed yield (Table I). Because the nitrogen fixation activity per *enf1* plant was elevated 1.8 times over that of the wild-type plants, we measured seed nitrogen content. As shown in Table I, the nitrogen content per dry weight of *enf1* seeds was significantly larger than that of MG20. Based on seed dry weight, the total nitrogen content per seeds was calculated to be increased more than 18%.

Growth at 72 DAS of *enf1* mutant and wild-type plants in field soil-filled pots was also analyzed. Although no *M. loti* inoculum was added, root nodules were formed by indigenous rhizobia on all plants tested. As shown in Table II, the number of root nodules, their dry weight, the dry weight of entire plants including pods and seeds, as well as total nitrogen content of the *enf1* mutant were significantly larger than the comparable parameters for MG20. These results suggest that a larger amount of nitrogen is fixed in the *enf1* mutant than in wild-type plants.

We also determined in long-term growth experiments that *M. loti*-inoculated *enf1* plants flowered

significantly later than the wild-type plants: 54.4 ± 1.1 versus 62.1 ± 0.8 DAI (Table III). The flowering time of *enf1* plants grown in 10 mM KNO₃ without *M. loti* inoculation was also investigated. Under these conditions, *enf1* showed a significantly earlier flowering time compared with wild-type plants (i.e. 50.9 ± 1.4 versus 58.6 ± 1.7 DAS; Table III).

enf1 Is Monogenic and Shows Incomplete Dominance

To confirm that the described phenotypes were due to a mutation in a single gene, a segregation analysis of 152 F2 progeny derived from the back-crossed MG20 and *No. 12 L. japonicus* was carried out using a histogram analysis of root nodule number and nitrogen fixation activity. The mean value of the F1 progeny was found to be in the middle range. Similarly, the mean value of the F2 group was also in the middle of the averages of MG20 and *enf1* (data not shown). These results suggested that *enf1* was incompletely dominant, but it was difficult to distinguish a segregation ratio of 1:2:1 between *enf1* and MG20 because the differences observed were smaller than those shown by other symbiotic mutants (Nishimura et al., 2002a; Suganuma et al., 2003; Krusell et al., 2005; Oka-Kira et al., 2005; Kumagai et al., 2007).

To determine an accurate segregation ratio, we created histograms for root nodule number and nitrogen fixation activity (Fig. 6). Thirty-eight MG20 plants and the same number of *enf1* plants were examined, because if the mutation is the result of incomplete dominance, the expected number of the MG20 type or of the *enf1* type in an F2 progeny consisting of 152 plants should be 38. First, the number of overlaps between the F2 progeny and *enf1* or MG20 types was determined. This number was defined as the experimental value (Table IV). The experimental value of the intermediate type was obtained by subtracting the experimental value of the MG20 and *enf1* types from the number of F2 progeny (e.g. for root nodule number, 152 − [37 + 34] = 81). Thus, the segregation ratio based on nodule number for the different types was MG20:intermediate:*enf1* = 37:81:34 (χ^2 = 0.78; Table IV,

Table I. *Growth parameters of wild-type (MG20) and enf1 L. japonicus*

Indicated parameters of MG20 and *enf1* were analyzed at 98 DAI. The data represent averages ± SE of 28 (MG20) or 33 (*enf1*) individual plants, except for total nitrogen of seeds. The data of total nitrogen of seeds represent averages ± SE of six independent experiments derived from 50 different seeds. Statistical significance is indicated by asterisks (** $P < 0.01$, by Student's *t* test).

Parameter	MG20	*enf1*
No. of flowers per plant	11.4 ± 0.6	10.7 ± 0.6
No. of pods per plant	6.7 ± 0.3	6.5 ± 0.4
Seed set (%)	65.7 ± 2.6	69.2 ± 3.0
Pod length (mm)	30.1 ± 0.4	30.6 ± 0.4
No. of seeds per pod	21.4 ± 0.5	22.7 ± 0.5
Fresh weight of 100 seeds (mg)	116.8 ± 2.4	130.4 ± 1.4**
Dry weight of 100 seeds (mg)	108.8 ± 2.2	122.2 ± 1.3**
Nitrogen content of seeds (μg mg^{-1} dry weight)	58.4 ± 0.7	61.8 ± 0.7**

Table II. *Growth and symbiotic parameters of wild type (MG20) and enf1 L. japonicus*

Uninoculated plants were grown for 72 d using field soil-filled pots supplied with water in the Saga University greenhouse (average temperature was 24.7°C). Nodules were formed by indigenous rhizobia on the roots of all plants tested. For total nitrogen content, entire plants, including pods and seeds, were analyzed. The data represent averages $\pm$ SE of five individual plants. Statistical significance is indicated by asterisks (* $P < 0.05$, by Student's *t* test).

Parameter	MG20	*enf1*
No. of root nodules per plant	155.0 ± 14.3	253.4 ± 30.9*
Dry weight of nodules (mg plant^{-1})	29.9 ± 4.9	55.2 ± 6.9*
Dry weight of entire plant (mg plant^{-1})	3,640.9 ± 213.0	4,310.8 ± 192.2*
Total nitrogen content (mg plant^{-1})	85.2 ± 2.6	108.5 ± 8.9*

top). For nitrogen fixation activity, the segregation ratio was MG20:intermediate:*enf1* = 33:84:35 ($\chi^2 = 1.74$; Table IV, bottom). These results closely fit the expected value of 1:2:1; thus, we conclude that the mutation resulting in *enf1* is monogenic and incompletely dominant.

Effect of Endogenous ABA on Nitrogen Fixation Activity

Because *enf1* had a low endogenous ABA concentration, we hypothesized that the decrease in ABA concentration caused the elevation of nitrogen fixation activity. To test this hypothesis, we treated wild-type plants at 28 DAI with 20 μM abamine, a specific inhibitor of ABA synthesis (Han et al., 2004). After a 3-d treatment period, acetylene reduction activity was measured. Such short treatment periods of abamine are not expected to induce new nodule development. In MG20 roots treated with abamine, the endogenous concentration of ABA decreased to about one-fourth of that of control plants, and at the same time, the acetylene reduction activity per nodule weight was significantly up-regulated (Fig. 7). These results strongly suggest that the decrease in endogenous ABA concentration in *enf1* was responsible for the increased levels of nitrogen fixation activity. On the other hand, plants treated with 0.5 μM ABA or with both ABA and abamine showed no difference in nitrogen fixation activity from the control plants.

Expression Analysis of Nitrogen Fixation Activity-Related Genes

To investigate the mechanism whereby nitrogen fixation activity was elevated in *enf1*, we analyzed the relative expression of several already identified nitrogen fixation activity-related genes in *enf1*-derived nodules at 28 DAI. We found no significant difference in the amount of relative expression of either *LjLbs*, the three class 2 hemoglobin (Hb) genes of *L. japonicus* (Nagata et al., 2008), or *nifH*, a rhizobial gene encoding nitrogenase (Shimoda et al., 2009), compared with wild-type plants (Fig. 8). Moreover, the amount of relative expression of other genes including *LjHb1*, one of the class 1 Hb genes of *L. japonicus* (Uchiumi et al.,

2002; Shimoda et al., 2009), of *Ign1*, a gene encoding a protein that consists of ankyrin repeats with transmembrane regions (Kumagai et al., 2007), of *Sst1*, a gene encoding the nodule-specific sulfuric acid transporter (Krusell et al., 2005), and of *LjGlu1*, a gene coding for β-1,3-glucanase (Suzuki et al., 2008), was not significantly different from that observed in wild-type nodules (Fig. 8).

Production of Nitric Oxide in *enf1* Nodules

In plants, nitric oxide (NO) is a key component of the ABA signaling pathway (García-Mata and Lamattina, 2002; Neill et al., 2002). NO serves as a signal molecule that induces plant defense, whereas symbiotic rhizobia induce transient NO production in the roots of host plants (Durner et al., 1998; Dordas et al., 2003; Nagata et al., 2008). Furthermore, inhibition of nitrogenase activity by NO production in nodules results in decreased nitrogen fixation activity (Trinchant and Rigaud, 1982). Recently, *L. japonicus* overexpressing *LjHb1*, a gene that encodes a class 1 Hb that exhibits an extremely high affinity for oxygen, demonstrated enhanced nitrogen fixation activity by decreasing NO production (Shimoda et al., 2009).

NO production in root nodules formed by *enf1* at 21 and 28 DAI was examined using the fluorescent dye diaminofluorescein-FM (DAF-FM), a NO-specific detector, and relative fluorescence unit (RFU) values were estimated. The RFU values of *enf1* nodules at 21 DAI were clearly decreased compared with that of

Table III. *Flowering time of MG20 and enf1*

In long-term growth experiments, flowering time was investigated. The data represent averages $\pm$ SE. Statistical significance is indicated by asterisks (** $P < 0.01$, by Student's *t* test).

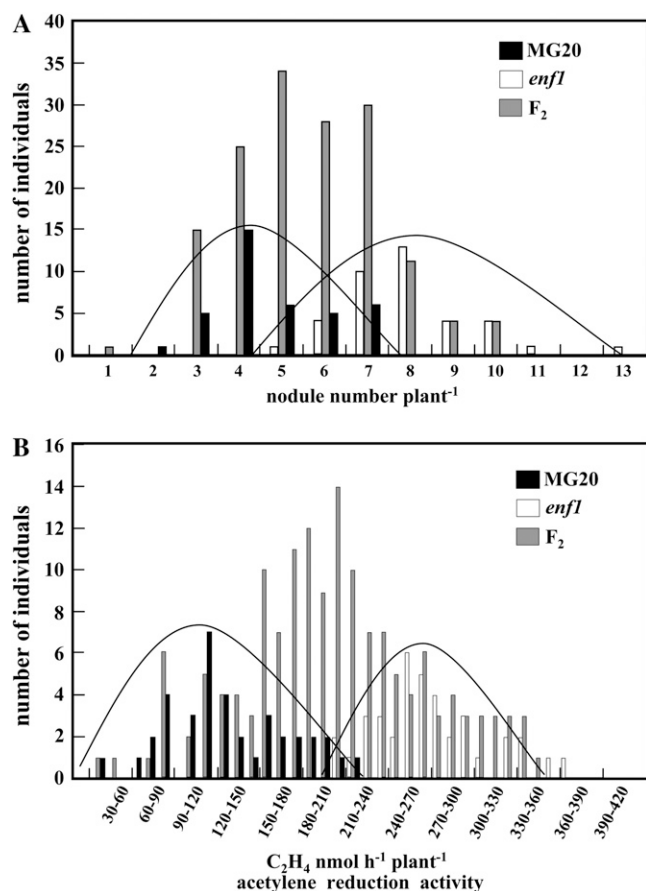|	Flowering Time
$-KNO_3$ with *M. loti*	
MG20 ($n = 33$)	54.4 ± 1.1[a]
enf1 ($n = 36$)	62.1 ± 0.8[a] **
$+KNO_3$ without *M. loti*	
MG20 ($n = 36$)	58.6 ± 1.7[b]
enf1 ($n = 37$)	50.9 ± 1.4[b] **

[a]DAI.   [b]DAS.

Figure 6. Histograms of MG20, *enf1*, and F2 progeny. Histograms of nodule number per plant (A) and of acetylene reduction activity per plant (B) are shown. Black bars indicate MG20, white bars indicate *enf1*, and gray bars indicate F2 progeny.

MG20; this trend was more obvious at 28 DAI (Fig. 9, A and B). Moreover, the effect of reduced ABA concentration caused by treatment with abamine on NO production was analyzed. When nodules formed on the roots of 28-d-old plants were treated, the RFU value of the *enf1* mutant was almost the same for (−) abamine and (+)abamine-treated plants, whereas the

RFU value of abamine-treated MG20 plants was significantly reduced compared with nontreated MG20 (Fig. 9C). These results strongly suggest that decreased production of NO caused by the low concentration of ABA in *enf1* nodules was responsible for the increase in nitrogen fixation activity.

DISCUSSION

In this study, we isolated a mutant designated *enf1* that exhibited decreased sensitivity to exogenous ABA and that was also reduced in endogenous ABA concentration. We found that the *enf1* mutant not only produced more root nodules but also showed elevated nitrogen fixation activity.

In our earlier work, we provided evidence showing that ABA was a negative regulator of *L. japonicus* nodulation (Suzuki et al., 2004). High endogenous ABA levels inhibited nodule number, whereas low concentrations promoted root nodule formation. As described herein, endogenous ABA levels were lower in *enf1 L. japonicus* compared with wild-type plants (Fig. 5); concomitantly, nodule numbers increased to 1.7 times greater than those of the wild type (Fig. 2A). Based on this correlation, we conclude that the increase in root nodule number in *enf1* is caused by the lowered concentration of endogenous ABA compared with wild-type plants. ABA is believed to regulate early nodulation stages negatively by inhibiting Nod factor signaling, bacterial infection, and nodule initiation (Miwa et al., 2006; Ding et al., 2008). On the other hand, *M. loti*-inoculated *enf1* mutants had more infection sites and developed more infection threads than wild-type roots (Fig. 2G), strongly suggesting a correlation between decreased endogenous ABA levels and the increased occurrence of infection events. We conclude that the earliest stages of nodule development are not as strongly inhibited in *enf1* as they are in wild-type MG20.

Other phenotypic differences from wild-type plants, such as shoot length and number of true leaves, can also be attributed to a reduction in en-

Table IV. *Genetic analysis of F2 progeny of MG20 and enf1 L. japonicus*

Segregation analyses were carried out using histograms for nodule number and nitrogen fixation activity. The χ^2 tests were carried out using experimental values (segregation ratio) based on nodule number and nitrogen fixation activity.

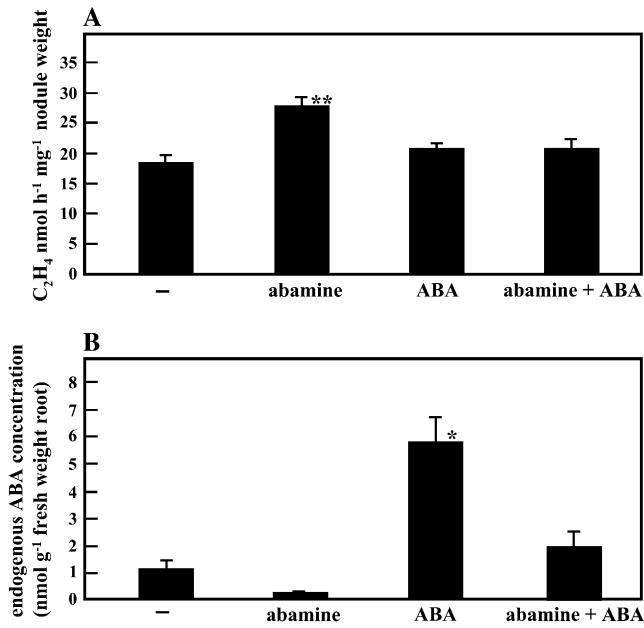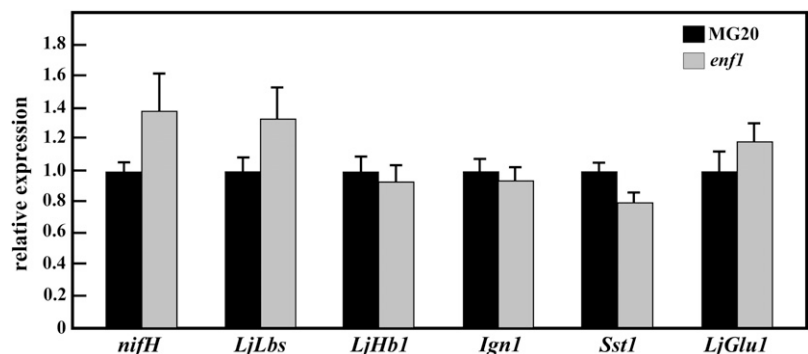|	Experimental Value	Expected Value	χ^2	P
Root nodule no.				
MG20 type	37	38		
enf1 type	34	38	0.78	0.68
MG20 × *enf1* intermediate	81	76		
Nitrogen fixation activity				
MG20 type	33	38		
enf1 type	35	38	1.74	0.42
MG20 × *enf1* intermediate	84	76		

Figure 7. Effects of ABA concentration on nitrogen fixation activity. *M. loti*-inoculated plants were grown for 21 d on vermiculite-filled pots supplied with B&D medium. Plant roots at 28 DAI were treated with 0.5 μM ABA, 20 μM abamine, both ABA and abamine, or were untreated (B&D medium control) for 3 d. A, Acetylene reduction activity per nodule weight. B, ABA concentration in root. At least 15 plants were used in acetylene reduction assay. Four different plants were used for measurement of ABA concentration, and three repeats were performed. Error bars indicate the SE, and the significance of differences between untreated control and treated values was determined by the two-tailed multiple *t* test with Bonferroni correction following ANOVA (three comparisons in four groups): * $P < 0.05$, ** $P < 0.01$.

dogenous ABA content. Overall, *enf1* shoot length was less than that of MG20 grown in 10 mM KNO_3 or without nitrogen (Fig. 3, C and D). However, *enf1* shoot length increased compared with that of the wild-type controls following inoculation with *M. loti* (Fig. 2F). Such a result may be explained by the fact that nodules on *enf1* mutant roots supply additional nitrogen to the plants. Alternatively, the results may be explained by the lowered ABA concentration caused by the mutation, with a concomitant change in the levels of other growth factors, which also affect

nodulation. The most likely candidate would be GA_3, which is involved in nodulation (Maekawa et al., 2009) but also in controlling shoot length (Ueguchi-Tanaka et al., 2005) and flowering (Blazquez and Weigel, 2000). Based on the evidence on hand, we favor the first hypothesis.

L. japonicus is a model legume used to elucidate the physiological functions of leguminous crops, for example, soybean (*Glycine max*), using molecular biology techniques. If this knowledge is to be applied from the model plant to crop species, it is important to investigate yield in long-term growth experiments after inoculation with a rhizobial symbiont. Although some yield parameters were the same for both *enf1* and wild-type plants, both the dry weight and nitrogen content of 100 seeds and entire *enf1* plants were significantly larger compared with those of wild-type seeds and plants (Tables I and II), strongly suggesting that the *enf1* mutation has the potential to bring about increased yield. The augmentation of the weight and nitrogen content of the *enf1* plants most likely reflects the increased nitrogen supplied by the additional *enf1* nodules and the concomitant increase in nitrogen fixation activity.

We hypothesize that the increase in nitrogen fixation activity of *enf1* results from the decrease in endogenous concentration of ABA. An earlier paper reported that application of 100 μM ABA to roots inhibited nitrogen fixation activity in *P. sativum* (Gonzalez et al., 2001). Because this concentration of ABA may have adverse growth effects, we applied 0.5 μM ABA and 20 μM abamine (an inhibitor of 9-cis-epoxycarotenoid dioxygenase, an ABA synthesis gene). No negative effects following a 3-d treatment on wild-type *L. japonicus* growth on agar medium were observed. Wild-type plants treated with abamine had a reduced endogenous ABA concentration in roots, to about one-fourth of the level of control plants. However, nitrogen fixation activity was elevated to about 170% over the nontreated controls (Fig. 7). This result phenocopies *enf1*, which shows decreased endogenous ABA concentration as well as elevated nitrogen fixation activity. Applying 0.5 μM ABA did not result in a further increase in nitrogen fixation activity, even though the endogenous ABA concentrations are presumed to increase (Fig. 7).

Figure 8. Relative amounts of nitrogen fixation-related gene transcripts in *enf1* nodules at 28 DAI. Transcript amounts of *nifH*, *LjLbs*, *LjHb*, *Ign1*, *Sst1*, and *LjGlu1* were normalized against *sigA* (used as an internal control for *nifH*) and *ATP synthase* (internal control for *LjLbs*, *LjHb*, *Ign1*, *Sst1*, and *LjGlu1*) transcripts. The mean value of expression in wild-type plants was set to 1. The data represent averages ± SE of three independent experiments of nodules derived from five different plants.
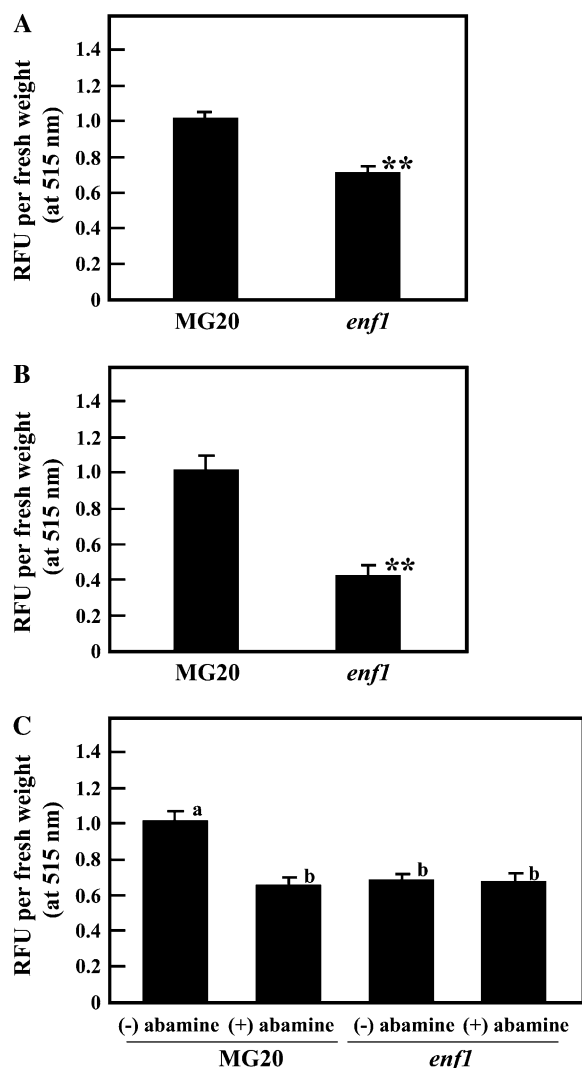
Figure 9. NO production in nodules. A and B, Quantification of NO produced in nodules of MG20 and *enf1* at 21 DAI (A) and 28 DAI (B). C, Quantification of NO in nodules that were treated with abamine. Nodules on the root of 28-d-old plants were treated with 20 μM abamine for 3 d. RFU values per nodule fresh weight at 515 nm, normalized against MG20 plants, are shown. The data represent averages $\pm$ SE of three independent experiments derived from nodules of six to eight plants. In A and B, statistical significance is indicated by asterisks (** $P < 0.01$, by Student's *t* test). In C, the significance of differences among four groups was determined by the two-tailed multiple *t* test with Bonferroni correction following ANOVA (six comparisons in four groups), and the different letters refer to significant differences at $P < 0.01$.

To get a better understanding of how endogenous ABA controls nitrogen fixation activity, we looked at the expression of various markers for nitrogen fixation such as *nifH* and *LjLbs* (Shimoda et al., 2009) in *enf1* nodules at 28 DAI and found no significant difference in expression for either gene (Fig. 8). The expression levels in other nitrogen fixation activity-related genes, such as *Ign1* (Kumagai et al., 2007), *Sst1* (Krusell et al., 2005), *LjHb1* (Shimoda et al., 2009), and *LjGlu1* (Suzuki

et al., 2008), also showed no significant differences (Fig. 8). Thus, these genes are unlikely to be downstream of the pathway affected by the *enf1* mutation.

NO is known as a strong inhibitor of nitrogen fixation activity (Trinchant and Rigaud, 1982) as well as a signal component in the ABA signaling pathway (García-Mata and Lamattina, 2002; Neill et al., 2002). The decreasing concentration of endogenous ABA in *enf1* was correlated with elevated nitrogen fixation activity without enhanced *nifH* gene expression (Figs. 2D and 8) and with reduced NO production in the root nodule (Fig. 9). This result phenocopies *L. japonicus* overexpressing *LjHb1*, which shows decreased NO production as well as increased nitrogen fixation activity without elevated *nifH* gene expression (Shimoda et al., 2009). Therefore, we conclude that the increased nitrogen fixation activity in *enf1* was due to the decrease in NO production by root nodules. Moreover, ABA is known to negatively regulate early nodulation stages. Our results demonstrate that the decreased endogenous ABA levels of *enf1* resulted in an increase in nodule number as a consequence of more infection events, in this way enhancing nitrogen fixation activity.

Other reports link ABA and nodulation in the studies of mutants such as *lateral root organ-defective* (*latd*), *sensitivity to ABA-1* (*sta-1*), and *Beyma* (Liang et al., 2007; Ding et al., 2008; Biswas et al., 2009). Although *latd*, *Beyma*, and *enf1* showed reduced or less sensitivity to ABA, sensitivity to ABA in *sta-1* differed depending on developmental processes. For lateral root formation, *sta-1* was less sensitive to ABA than *enf1*, and the number was reduced compared with control plants in both mutants (Fig. 3G). Although the emergence of lateral roots increased, in *latd* mutants, the number of elongated roots decreased because of a root apical meristem defect (Bright et al., 2005). In terms of nodulation and nodule function, *enf1* plants exhibit advantageous phenotypes (i.e. enhanced nodule number and nitrogen fixation), whereas the other mutants show negative phenotypes. The reduced nodulation of *sta-1* is the result of a hypersensitivity to ABA, the development of nodules in *latd* is arrested due to the nodule meristem abortion, and nitrogen fixation activity in *latd* and *Beyma* are decreased. Overall, because the regulation of root nodule formation by ABA is likely to be polygenic, the identification of genes responsible for this regulation is essential for understanding the involvement of ABA.

Until now, the majority of symbiotic mutants that have been described represent loss of or defects in root nodule formation (Schauser et al., 1999; Stracke et al., 2002; Radutoiu et al., 2003; Imaizumi-Anraku et al., 2005; Murray et al., 2007). Many of these mutants induce nodules that are Fix⁻ (Suganuma et al., 2003; Krusell et al., 2005; Kumagai et al., 2007). Although reports of mutants that show increased root nodule number (Penmetsa and Cook, 1997; Nishimura et al., 2002b; Oka-Kira et al., 2005; Magori et al., 2009) or spontaneous root nodule formation (Tirichine et al., 2006a, 2006b, 2007) exist, mutations whereby nitrogen

fixation activity is elevated without deleterious effects on plant growth and development have been limited. One exception is the *L. japonicus root-determined hypernodulation1* (*rdh1*) mutant, which also exhibits a hypernodulation and enhanced nitrogen fixation phenotype (Ishikawa et al., 2008). However, the *RDH1* gene is unlikely to be the same as the *ENF1* gene, because the phenotype of *rdh1* is inherited in a monogenic, recessive manner, whereas *enf1* is monogenic but incompletely dominant. Moreover, except for the nodulation phenotype, no other differences were observed in growth traits between *rdh1* and wild-type plants (Ishikawa et al., 2008).

In this report, we have shown that mutating the *ENF1* gene leads to an elevation of nitrogen fixation activity and to enhanced biomass production without accompanying adverse growth effects. Therefore, this gene should be an important target for molecular breeding. To determine the identity of the *ENF1* gene, we have initiated mapping experiments by employing backcrossed F2 plants of *enf1* and Gifu. Our future work will identify the gene responsible for these positive growth effects.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Lotus japonicus Miyakojima MG20 mutants were generated by ethyl methanesulfonate treatment. Seeds that germinated on agar medium containing 70 μM ABA, which inhibits wild-type seed germination, were selected as ABA low-sensitivity candidates from a total of 4,000 M3 seeds. The *No. 12* mutant line, which displayed increased nodule number and nitrogen fixation activity, was isolated from these candidates. The *No. 12* mutant was then backcrossed to MG20 to investigate the mutant phenotypes in greater detail. From 153 F2 progeny, a mutant designated *enf1*, which displayed the highest nitrogen fixation activity and nodule number per plant, was isolated.

Seeds were surface-sterilized by immersion in sodium hypochlorite (2% [v/v] containing 0.1% [v/v] Tween 20) for 20 min and rinsed several times with sterile distilled water. After overnight imbibition, the swollen seeds were sown on vermiculite-filled pots that were watered with B&D medium (nitrogen-deficient; Broughton and Dilworth, 1971) with or without 10 mM KNO$_3$ or with or without 1.0×10^7 cells mL^{-1} *Mesorhizobium loti* MAFF303099 (Keele et al., 1969; Kaneko et al., 2000; Saeki and Kouchi, 2000), which had been grown in yeast mannitol liquid medium (Keele et al., 1969). In this method, the stage of plant for inoculation was day 1. The plants were grown at 24°C under 16-h-light/8-h-dark conditions at a light intensity of 150 μmol m^{-2} s^{-1}. Seeds were also sown on 0.8% (w/v) agar medium, and the plates were incubated at 24°C in the dark. After 3 d, germinated seedlings were transplanted onto B&D medium containing 1.5% (w/v) agar and concomitantly inoculated with rhizobia at a concentration of 1.0×10^7 cells per plant. The plants were grown at 24°C under 16-h-light/8-h-dark conditions at a light intensity of 150 μmol m^{-2} s^{-1}.

Measurement of Dry Weights and Total Nitrogen Contents

Plant samples were dried at 80°C for 3 d and then weighed. Nitrogen content of seeds was measured by micro-Kjeldahl and indophenol methods (Cataldo et al., 1974). For the analysis of total nitrogen content, soil obtained from a field within Saga University was used without sterilization. Imbibed seeds were sown on field soil-filled Wagner pots (16 cm diameter $\times$ 19 cm height) and supplied with water, and the plants were grown for 72 d in the Saga University greenhouse (average temperature was 24.7°C) without additional rhizobial inoculation.

Measurement of ABA Concentration of *enf1*

Plants were grown for 21 d in vermiculite-filled pots supplied with B&D medium, inoculated with *M. loti*, and after harvest frozen rapidly in liquid nitrogen. ABA concentration was measured by the Phytodetek ABA enzyme immunoassay test kit (Agdia) as described previously (Suzuki et al., 2004).

Acetylene Reduction Assay

A plant was grown for 28 d in a vermiculite-filled pot supplied with B&D medium, inoculated with *M. loti*, placed in a 34-mL test tube, which was covered with a rubber serum cap, and degassed for 30 s. Acetylene that was diluted five times was injected in the tube, which was then incubated in a growth chamber at 25°C. After 2 h of incubation, the amount of ethylene formed was determined by gas chromatography (Suzuki et al., 2008).

Observation of Sensitivity of Plants to ABA Treatment

To study germination rate, seeds imbibed for 2 h were sown on 0.8% (w/v) agar medium containing 70 μM ($\pm$)ABA [(2-cis,4-trans)-5-(1-hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-2,4-pentadienoic acid; Sigma Aldrich]. The plates were placed upside down and incubated at 24°C in the dark. After 3 d, the rate of seed germination was measured using root emergence as the criterion for germination. To study plant responses to exogenous ABA, seeds were sown on vermiculite-filled pots supplied with B&D medium containing 0.5 μM ABA, which had been inoculated with *M. loti*. The degree of leaf yellowing/browning and shoot tip shriveling was measured 17 DAI.

Observation of Infection Threads Using Fluorescence Microscopy

Seeds were sown on 0.8% (w/v) agar medium and incubated at 24°C in the dark. After 3 d, germinated seedlings were transplanted onto 1.5% (w/v) agar-solidified B&D medium or 1% (w/v) Phytagar-solidified one-quarter-strength Hoagland medium (Machlis and Torrey, 1956) without nitrogen and inoculated with *M. loti* strain NZP2037 (Scott et al., 1996) carrying the GFP plasmid pHC60 (Cheng and Walker, 1998). The seedlings were then grown at 24°C under 16-h-light/8-h-dark conditions at a light intensity of 100 μmol m^{-2} s^{-1} for 4 to 12 d. The roots of plants were rinsed three times with water and observed by fluorescence microscopy. Wavelengths for excitation and emission were 460 to 495 and over 510 nm, respectively.

ABA and Abamine Treatment

Plants were grown for 28 d on vermiculite-filled pots supplied with B&D medium that had been inoculated with *M. loti*. Roots were treated 28 DAI with 0.5 μM ABA and 20 μM abamine, with abamine alone, with ABA alone, or with B&D medium (control) for 3 d. Abamine is an inhibitor of 9-cis-epoxycarotenoid dioxygenase (Han et al., 2004).

Isolation of Total RNA from *L. japonicus*

Plants were quick-frozen in liquid N$_2$ and stored at –80°C. Total RNA was prepared using the Plant Total RNA Extraction miniprep system (Viogene). DNaseI treatment was performed using deoxyribonuclease RT-Grade (Wako). RNA was then precipitated by ethanol in the presence of ethachinmate (Wako) as a carrier and was resuspended in RNase-free water.

Analysis of Gene Expression by Real-Time Reverse Transcription-PCR

To quantify the relative amount of transcripts derived from nitrogen fixation-related genes, real-time reverse transcription (RT)-PCR was performed in the One Step SYBR PrimeScript RT-PCR Kit (TaKaRa). The enzymatic reactions were performed using a LightCycler 1.5 Instrument (Roche). After the RT reaction at 42°C for 5 min followed by heating at 95°C for 10 s, the target gene was amplified in 40 cycles of 95°C for 5 s and 60°C for 20 s. The sequences of primers were as follows: for *MlnifH*, 5'-TCCAAGCTCATC-CACTTCGTG-3' and 5'-AGTCCGGCGCATACTGGATTA-3'; for *MlsigA*,

5′-GCCCTCTGCTCGACCTTTCC-3′ and 5′-AGCATCGCCATCGTGTCCTC-3′; for *LjHb1*, 5′-CCTTTGGAGGAGAACCCCAA-3′ and 5′-AGACTGCTGATT-CACAAGTCATG-3′; for *Ign1*, 5′-TGCATTAACCAGAGACCACAA-3′ and 5′-GTGAACGTCTTTCTTAATCTGAGTA-3′; for *LjLbs*, 5′-TCTGGRCCYAM-GCAYAGTC-3′ and 5′-CRTCRCGWGTCAGTSCAAAA-3′; for *LjSst1*, 5′-TCT-TGGACTGGTCTTCCTTGCT-3′ and 5′-TGGTCTCTTGTTCCTCACGTGT-3′; for *LjGlu1*, 5′-GCTGTCGGTAACGAAGTTCC-3′ and 5′-TCATAGCCGGG-AGAACTGAC-3′; for *LjATPsyn*, 5′-ACATGCTTGCACCATACCAA-3′ and 5′-TCCCCAACTCCAGCAAATAC-3′.

The *MlsigA* gene for nodules and *LjATPsyn* for *L. japonicus* were used for normalizing the results of real-time RT-PCR (Nakagawa and Kawaguchi, 2006; Shimoda et al., 2009).

Quantification of NO by Fluorescence Spectrophotometry

A stock solution of DAF-FM (7 mM in dimethyl sulfoxide) was diluted 1,000-fold in water before use. Detached root nodules of *L. japonicus* at 21 and 28 DAI were soaked in the DAF-FM solution for 1 min. To analyze the effect of abamine on the production of NO, nodules on the roots of 28-d-old plants were covered by filter paper containing 20 μM abamine for 3 d. Then, the detached nodules were soaked in DAF-FM solution for 1 min. The RFU of the DAF-FM solution was measured using a NanoDrop ND-3300 fluorospectrometer (NanoDrop Technologies). The wavelengths for excitation and emission were 470 and 515 nm, respectively.

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