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Enhanced nodulation and nitrogen fixation in the ABA low-sensitive mutant enf1 (enhanced nitrogen fixation 1) of Lotus japonicus

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ABSTRACT

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 *enf1* (enhanced nitrogen fixation 1) 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 (NCED), which results in reduced ABA content, the N fixation activity of abamine-treated plants was elevated to the same levels as *enf1*. We also determined that production of nitric oxide (NO) in *enf1* nodules was decreased. We conclude that endogenous ABA concentration not only regulates nodulation, but also nitrogen fixation activity by decreasing NO production in nodules.
INTRODUCTION

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 chitooligosaccharide 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 nts1 (Glycine max), har1 (Lotus japonicus), sunn (Medicago truncatula), and sym29 (Pisum sativum L.) 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; Kossak and Bohlool, 1984; Krusell et al., 2002;
Nishimura et al., 2002a; 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 (CLV1), which regulates meristem cell number and differentiation (Krusell et al., 2002; Nishimura et al., 2002b; 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, influencing 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, *skl1* of *Medicago truncatula*, has a hypernodulating phenotype (Penmetsa and Cook, 1997). *Skll* is homologous to *Ein2* of Arabidopsis, 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 *hit1* (loss-of-function) of *Lotus japonicus* and the *snf2* (gain-of-function) mutants of *Medicago 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, *Medicago truncatula* overexpressing *abi1-1*, 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 *Lotus japonicus* (Miyakojima MG20) mutant that showed an increased root nodule phenotype and proceeded to carry out its characterization. This mutant, named *enf1* (enhanced nitrogen fixation 1) exhibit enhanced symbiotic N fixation activity. Most legume N fixation activity mutants, such as *ign1*, *senl* and *sstl*, 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 *Lotus japonicus*, we treated Miyakojima MG20 with EMS to induce base substitutions randomly in the genome. M$_3$ seeds were sown on an agar-solidified medium containing 70 µM ABA, a concentration that inhibits the germination of wild-type MG20 seeds. M$_4$ plants obtained by the screening were inoculated with rhizobia (*Mesorhizobium loti* MAFF303099), and the number of nodules per plant was counted 35 days after inoculation (DAI). Plant No. 12 not only formed more root nodules than did the wild-type MG20 plants, but surprisingly it also exhibited increased nitrogen fixation activity per plant. Both mutant phenotypes were stably inherited in the M$_4$ and M$_5$ generation. Back-crossing mutant No. 12 to wild-type MG20 yielded 153 F$_2$ progeny from which a line that showed the highest nitrogen fixation activity and numerous nodules per plant was derived. This line was designated *enf1* (enhanced nitrogen fixation 1).

Symbiotic enf1 phenotypes

Figure 1 illustrates the nodulation phenotypes of MG20 and *enf1* *L. japonicus* 28 DAI. The number of nodules formed on *enf1* roots was approximately 1.7 times greater than that of MG20, and concomitantly, the weight of nodules per *enf1* plant also increased slightly (Fig. 1, Fig. 2A, 2B). 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 N 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 N fixation activity per unit of enf1 nodule weight was also increased 1.7 times (Fig. 2D), we concluded that the increased N 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 (IT) formation in enf1 from 4 to 12 DAI. Although infection pockets were detected (4 DAI) earlier in MG20 root hairs compared to the enf1 mutant (data not shown), elongated ITs were more common in enf1 root hairs at later stages of development (8-12 DAI) (black bars in Fig. 2G). Furthermore, ITs were detected in nodule primordia more frequently in enf1 compared to MG20 (white bars in Fig. 2G).

Non-symbiotic 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. 2E, 2F). To confirm that enf1’s vigorous growth was due to an increase in N supplied from nodules, MG20 and enf1 plants without rhizobial inoculation were grown in the presence (10 mM KNO₃) or absence of N. Figures 3A and 3B illustrate data derived from MG20 and
enf1 plants 21 Days After Sowing (DAS). Surprisingly, under conditions of N sufficiency or N inefficiency in the absence of rhizobia, enf1 shoot length was statistically lower than that of MG20 (Fig. 3C, 3D), 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 to MG20 plants under both +N and –N conditions (Fig. 3E, 3F), 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-day-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 to 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 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 days, most of the young leaves at the shoot tip turned brown and shranked (78.7%; 48 of 61 plants). In contrast, although some yellowing of enf1 leaves occurred, the shoot tip showed very little die-back (9.6%; 5 in 52 plants) (Fig. 4A to 4D). 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 4 or 2.5 times lower in nodulated roots and shoots, respectively compared to wild-type nodulated roots and shoots (Fig. 5A, 5B).

**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 weight of 100 seeds of enf1 was significantly larger than the
comparable number of seeds of wild-type, suggesting that enf1 may result in increased seed yield (Table I). Because the N fixation activity per enf1 plant was elevated 1.8 times over that of the wild-type plants, we measured seed N content. As shown in Table I, the N content per dry weight of enf1 seeds was significantly larger than that of MG20. Based on seed dry weight, the total N 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 N content of the enf1 mutant was significantly larger than the comparable parameters for MG20. These results suggest that 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 ± 6.1 versus 62.1 ± 4.7 DAI (Table III, top panel). 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 early flowering time compared to wild-type plants, i.e. 50.9 ± 8.7 versus 58.6 ± 10.4 DAS (Table III, bottom panel).
**enfl 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 F\(_2\) 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 F\(_1\) progeny was found to be in the mid-range. Similarly, the mean value of the F\(_2\) group was also in the middle of the averages of MG20 and *enfl* (data not shown). These results suggested that *enfl* was incompletely dominant, but it was difficult to distinguish a segregation ratio of 1:2:1 between *enfl* and MG20 because the differences observed were smaller than those shown by other symbiotic mutants (Nishimura et al., 2002b; 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 a histogram for root nodule number and N fixation activity (Fig. 6A, 6B). Thirty-eight MG20 plants and the same number of *enfl* plants were examined because if the mutation is the result of incomplete dominance, the expected number of the MG20 type or of the *enfl* type in an F\(_2\) progeny consisting of 152 plants should be 38. First, the number of overlaps between the F\(_2\) progeny and *enfl* 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 *enfl* types from the number of F\(_2\) 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:enfl = 37:81:34 (χ²=0.78) (Table IV, top panel). For N fixation activity, the segregation ratio was MG20:intermediate:enfl = 33:84:35 (χ²=1.74) (Table IV, bottom panel). These results closely fit the expected value of 1:2:1, and thus we conclude that the mutation resulting in enfl is monogenic and incompletely dominant.

**Effect of endogenous ABA on N fixation activity**

Because enfl had a low endogenous ABA concentration, we hypothesized that the decrease in ABA concentration caused the elevation of N 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 three day-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, the acetylene reduction activity (ARA) per nodule weight was significantly up-regulated (Fig. 7A, 7B). These results strongly suggest that the decrease in endogenous ABA concentration in enfl was responsible for the increased levels of N fixation activity. On the other hand, plants treated with 0.5 µM ABA or with both ABA and abamine showed no difference in N fixation activity from the control plants.
Expression analysis of N fixation activity-related genes

To investigate the mechanism whereby N fixation activity was elevated in enf1, we analyzed the relative expression of several already identified N fixation activity-related genes in enf1-derived nodules 28 DAI. We found no significant difference in the amount of relative expression of either LjLbs, the three class 2 Hb genes of L. japonicus (Nagata et al., 2008) or nifH, a rhizobial gene encoding nitrogenase (Shimoda et al., 2009) compared to 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 (Trichant and Rigaud, 1982). Recently, *L. japonicus* overexpressing *LjHb1*, a gene that encodes a class1 hemoglobin 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 *enfl* 21 DAI and 28 DAI was examined by using the fluorescent dye DAF-FM, an NO specific detector, and relative fluorescent unit (RFU) values were estimated. The RFU values of *enfl* nodules 21 DAI were clearly decreased compared to that of MG20; this trend was more obvious at 28 DAI (Fig. 9A, 9B). 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-day-old plants were treated, the RFU value of the *enfl* mutant was almost the same for (-) abamine and (+) abamine-treated, whereas, the RFU value of abamine-treated MG20 plants was significantly reduced compared to non-treated MG20 (Fig. 9C). These results strongly suggest that decreased production of NO caused by the low concentration of ABA in *enfl* nodules was responsible for the increase in N 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 N 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 to wild-type plants (Fig. 5A, 5B), and concomitantly, nodule numbers increased to 1.7 times greater than those of 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 to 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 endogenous ABA content. Overall, *enf1* shoot length was less than that of MG20 grown in 10 mM KNO₃ or without N (Fig. 3C, 3D). However, *enf1* shoot length increased compared to 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 N to the plants. Alternatively, the results may be explained by the lowered ABA concentration caused by the mutation with a concomitant changes in the levels of other growth factors, which also affect nodulation. The most likely candidate would be gibberellic acid, 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, by 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 N content of 100 seeds and entire *enf1* plants were significantly larger compared than those of wild-type seeds and plants (Table I, II), strongly suggesting the *enf1* mutation has the potential to bring about increased yield.
The augmentation of the weight and N content of the enf1 plants most likely reflects the increased N supplied by the additional enf1 nodules and the concomitant increase in N fixation activity.

We hypothesize that the increase in N 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 N fixation activity in pea (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 NCED, an ABA synthesis gene). No negative effects following a 3-day 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, N fixation activity was elevated to about 170% over the non-treated controls (Fig. 7A, 7B). This result phenocopies enf1, which shows decreased endogenous ABA concentration as well as elevated N fixation activity. Applying 0.5 µM ABA did not result in a further increase in N fixation activity even though the endogenous ABA concentrations are presumed to increase (Fig. 7A, 7B).

To get a better understanding of how endogenous ABA controls N 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 N
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.

Nitric oxide (NO) is known as a strong inhibitor of N fixation activity (Trinchant and Rigaud, 1982), as well as a signal component in ABA signaling pathway (García-Mata and Lamattina, 2002; Neill et al., 2002). The decreasing concentration of endogenous ABA in *enf1* was correlated with elevated N fixation activity without enhanced *nifH* gene expression (Fig. 8, Fig. 2D) 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 N fixation activity without elevated *nifH* gene expression (Shimoda et al., 2009). Therefore, we conclude that the increased N 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 N fixation activity.

Other reports link ABA and nodulation in the studies of mutants such as *latd, 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 as is *enf1*, and the number was reduced compared to 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 represents 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., 2002a; Oka-Kira et al., 2005; Magori et al., 2009) or spontaneous root nodule formation exist (Tirichine et al., 2006a, 2006b; Tirichine et al., 2007), mutations whereby N fixation activity is elevated without
deleterious effects on plant growth and development have been limited. One exception is the *L. japonicus 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 *enfl* 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 N 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 F$_2$ plants of *enfl* 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 EMS 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 N fixation activity, was isolated from these candidates. The No. 12 mutant was then back-crossed to MG20 to investigate the mutant phenotypes in greater detail. From 153 F2 progeny, a mutant, designated enf1, which displayed the highest N 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 (N-deficient) (Broughton and Dilworth 1971) with or without 10 mM KNO3, or with or without 1.0 x 107 cells/mL M. loti MAFF303099 (Kaneko et al., 2000; Saeki and Kouchi, 2000; Keele et al., 1969), 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 m² 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 days, germinated seedlings were transplanted onto B & D medium containing 1.5% (w/v) agar and concomitantly inoculated with rhizobia at a concentration of $1.0 \times 10^7$ 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 N content**

Plant samples were dried at 80°C for three days, and then weighed. N content of seeds was measured by micro-Kjeldahl and indophenol methods (Cataldo et al., 1974). For the analysis of total N content, soil obtained from field within Saga University was used without sterilization. Imbibed seeds were sown on field soil-filled Wagner pots (size: 16 cm diameter x 19 cm height), supplied with water, and the plants were grown for 72 days 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 days in vermiculite-filled pots supplied with B & D medium and 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 Inc.; Elkhart, IN, U.S.A) as previously described (Suzuki et al., 2004).
Acetylene Reduction Assay

A plant grown for 28 days in a vermiculite-filled pot supplied with B & D medium and inoculated with *M. loti*, was placed in a 34 mL test tube, which was covered with a rubber serum cap, and degassed for 30 s. Acetylene that was diluted 5 times was injected in the tube, which was then incubated in a growth chamber at 25°C. After 2 h 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 (±)-abscisic acid [(2-cis,4-trans)-5-(1-hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-2,4-pentadienoic acid] (ABA) (Sigma Aldrich) The plates were placed upside-down and incubated at 24°C in the dark. After 3 days, 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 shrivelting 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 days, germinated seedlings were transplanted onto 1.5% (w/v) agar-solidified B & D medium or 1% (w/v) Phytagar-solidified 1/4 strength Hoagland’s medium (Machlis and Torrey, 1956) without nitrogen and inoculated with *M. loti* strain NZP2037 (Scott et al., 1996) carrying the GFP-plasmid pH60 (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 days. The roots of plants were rinsed three times with water and observed under 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 days on vermiculite-filled pots supplied with B & D medium, which had been inoculated with *M. loti*. Roots were treated 28 DAI with 0.5 µM ABA and 20 µM abamine, with abamine alone, or ABA alone, or with B & D medium (control), for 3 days. Abamine is an inhibitor of 9-cis-epoxycarotenoid dioxygenase (NCED) (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
by 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 RT-PCR**

To quantify the relative amount of transcripts derived from N fixation-related genes, real-time RT-PCR was performed in One Step SYBR® PrimeScript™ RT-PCR Kit (TaKaRa). The enzymatic reactions were performed using a LightCycler® 1.5 Instrument (Roche). After the reverse transcription 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, 60°C for 20 s. The sequences of primers were as follows: for *MlnifH*, 5'-TCCAAGCTCATCCACTTCGTG-3' and 5'-AGTCCGCGCATACTGGATTA-3'; for *MlsigA*, 5'-GCCCTCTGCTCGACCTTTCC-3' and 5'-AGCATCGCCATCGTGTCCCTC-3'; for *LjHb1*, 5'-CCTTTGGAGGAGAACCCCAA-3' and 5'-AGACTGCTGATTCAAAAGTCATG--3'; for *Ign1*, 5'-TGCATTAAACCAGAGACCACAA-3' and 5'-GTGAACGTCTTTTCTTTATCTGAGTACTC-3'; for *LjLbs*, 5'-TCTGGRCCYAMGCAYAGTC-3' and 5'-CRTCRCGWGTCAGTSCAAAA-3'; for *LjSst1*, 5'-TCTTGGACTGGTCTTCCTTGCT-3' and 5'-TGGTCTCTTGTTCCTCACGTGT-3'; for *LjGlu1*, 5'-
GCTGTCGGTAAACGAAATTCC-3’ and 5’- TCATAGCCGAGGAACCTGAC-3’; for
LjATPsyn, 5’-ACATGCTTGCACCATAACCA-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 dimethylsulfoxide) was diluted 1,000-fold in water before use. Detached root nodules of L. japonicus 21 DAI 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-day-old plants were covered by filter paper containing 20 µM abamine for 3 days. 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, Inc., Wilmington, DE, U.S.A.). The wavelengths for excitation and emission were 470 and 515 nm, respectively.
ACKNOWLEDGMENTS

We thank Professor Tadao Asami (University of Tokyo) for providing the abamine. *Lotus japonicus* Miyakojima MG20 seeds were provided by the National Bio-Resource Project (NBRP) of MEXT, Japan.
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**FIGURE LEGENDS**

**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. Bar =1 cm.

**Figure 2.** Analysis of *enf1* symbiotic phenotypes.

*M. loti*-inoculated plants were grown for 28 days in pots containing vermiculite and were watered with B & D medium (A to F) or were grown on B & D agar-solidified (1.5% w/v) medium for 12 days (G). The plants on the agar plates were inoculated with *M. loti* NZP2037 carrying plasmid pHC60 (G).

A, Nodule number per plant. B, Nodule weight per plant. C and D, Acetylene reduction activity (ARA) per plant (C), and per nodule weight (D). E, Number of true leaves per plant. F, Shoot length. G, The number of infection threads per plant at 12 DAI. Black bars indicate MG20, gray bars indicate *enf1* (A to F). Black bar indicates later stages of infection thread formation, whereas the white bar refers to the initiation of nodule formation (G). At least 40 plants (A to F) and 17 to 20 plants (G) were examined in each experiment. Error bars are given as standard error (A to G). Statistical significance is indicated by *(P<0.05; student’s t-test) or **(P<0.01; student’s t-test).

**Figure 3.** Non-symbiotic phenotypes of the *enf1* mutant.
A to F, Plants were grown for 21 days in vermiculite-filled pots supplied with B & D medium, with or without 10 mM KNO₃. G, Plants were grown on B & D agar-solidified (1.5% w/v). A, MG20 (left) and enf1 (right) grown with no nitrogen. B, MG20 (left) and enf1 (right) grown with 10 mM KNO₃. C and D, Shoot length resulting for growth without KNO₃ (C), or with 10 mM KNO₃ (D). E and F, Number of true leaves per plant in medium without KNO₃ (E), or with 10 mM KNO₃ (F). Number of lateral roots per plant at 24 DAS (G). At least 30 plants were used in each experiment. Error bars indicate standard error, and statistical significance is indicated by *(P<0.05; student’s t-test) or **(P<0.01; student’s t-test).

Figure 4. Effects of 0.5 μM ABA on the growth of enf1.

A, MG20 17 DAI with M. loti. B, enf1 17 DAI with M. loti. Untreated MG20 and enf1 (A and B; left) and treated with 0.5 μM ABA (A and B; right). C and D, Shoot tips of MG20 (C) and enf1 (D) following 0.5 μM ABA treatment. Bar = 1cm.

Figure 5. Endogenous concentration of ABA in enf1.

M. loti-inoculated plants were grown for 21 days on vermiculite-filled pots supplied with B & D medium. A and B, Endogenous concentrations of ABA in MG20 and enf1 roots (A) and shoots (B). The data represent the average ± standard error of 6 independent experiments derived from 8 different plants. Statistical significance is
indicated by **(P<0.01; student’s t-test).

**Figure 6. Histograms of MG20, enf1, and F2 progeny.**

A and B, Histograms of nodule number per plant (A) and of ARA per plant (B). Black bar indicates MG20 and the white indicates enf1; the gray bar indicates F2 progeny.

**Figure 7. Effects of ABA concentration on nitrogen fixation activity.**

*M. loti*-inoculated plants were grown for 21 days on vermiculite-filled pots supplied with B & D medium. Plant roots 28 DAI were treated with 0.5 µM ABA, 20 µM abamine, with both ABA and abamine, or were untreated (B & D medium control), respectively, for 3 days. A, ARA 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 3 repeats were performed. Error bars indicate the standard error, 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.

**Figure 8. Relative amounts of nitrogen fixation-related gene transcripts in enf1 nodules 28 DAI.**

Transcript amounts of *nifH, LjLbs, LjHb, Ign1, Sst1* and *Ljglu1*, respectively 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 the average ± standard error of 3 independent experiments of nodules derived from 5 different plants.

**Figure 9. NO production in nodules.**

A and B, Quantification of nitric oxide produced in nodules of MG20 and *enf1* at 21DAI (A) and at 28 DAI (B). C, Quantification of nitric oxide in nodules that were treated with abamine. Nodules on the root of 28-days-old plants were treated with 20 µM abamine for 3 days. Relative fluorescent units (RFU) per nodule fresh weight at 515 nm, normalized against MG20 plants, are shown. The data represent the average ± standard error of 3 independent experiments derived from nodules of 6 to 8 plants. A and B, Statistical significance is indicated by **(*P*<0.01; student’s t-test). 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.
Table 1. *Growth parameters of wild type (MG20) and enf1 of Lotus japonicus*

Indicated parameters of MG20 and enf1 were analyzed at 98 DAI. The data represent the average ± standard error of 28 (MG20) or 33 (enf1) individual plants, except for total N of seeds. The data of total N of seeds represent the average ± standard error of 6 independent experiments derived from 50 different seeds. Statistical significance is indicated by **(*P*<0.01; student's t-test).

<table>
<thead>
<tr>
<th></th>
<th>MG20</th>
<th>enf1</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of flowers plant$^{-1}$</td>
<td>11.4 ± 0.6</td>
<td>10.7 ± 0.6</td>
</tr>
<tr>
<td>number of pods plant$^{-1}$</td>
<td>6.7 ± 0.3</td>
<td>6.5 ± 0.4</td>
</tr>
<tr>
<td>seed set (%)</td>
<td>65.7 ± 2.6</td>
<td>69.2 ± 3.0</td>
</tr>
<tr>
<td>pod length (mm)</td>
<td>30.1 ± 0.4</td>
<td>30.6 ± 0.4</td>
</tr>
<tr>
<td>number of seeds pod$^{-1}$</td>
<td>21.4 ± 0.5</td>
<td>22.7 ± 0.5</td>
</tr>
<tr>
<td>fresh weight of 100 seeds (mg)</td>
<td>116.8 ± 2.4</td>
<td>130.4 ± 1.4**</td>
</tr>
<tr>
<td>dry weight of 100 seeds (mg)</td>
<td>108.8 ± 2.2</td>
<td>122.2 ± 1.3**</td>
</tr>
<tr>
<td>N content of seeds (µg mg$^{-1}$ dry weight)</td>
<td>58.4 ± 0.7</td>
<td>61.8 ± 0.7**</td>
</tr>
</tbody>
</table>
Table II. \textit{Growth and symbiotic parameters of wild type (MG20) and enf1 of Lotus japonicus}

Uninoculated plants were grown for 72 days using field soil-filled pots supplied with water in the Saga University green house (average temperature was 24.7°C). Nodules were formed by indigenous rhizobia on the root of all plants tested. For total N content, entire plants, including pods and seeds, were analyzed. The data represent the average ± standard error of 5 individual plants. Statistical significance is indicated by *(P<0.05; student's t-test).

<table>
<thead>
<tr>
<th></th>
<th>MG20</th>
<th>enf1</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of root nodules plant$^{-1}$</td>
<td>155.0 ± 14.3</td>
<td>253.4 ± 30.9*</td>
</tr>
<tr>
<td>dry weight of nodules (mg plant$^{-1}$)</td>
<td>29.9 ± 4.9</td>
<td>55.2 ± 6.9*</td>
</tr>
<tr>
<td>dry weight of entire plant (mg plant$^{-1}$)</td>
<td>3640.9 ± 213.0</td>
<td>4310.8 ± 192.2*</td>
</tr>
<tr>
<td>total N content (mg plant$^{-1}$)</td>
<td>85.2 ± 2.6</td>
<td>108.5 ± 8.9*</td>
</tr>
</tbody>
</table>
Table III. Flowering time of MG20 and enf1

In long-term growth experiments, flowering time was investigated. The data represent the average ± standard error. There is a significant difference between different letters at $P < 0.01$ by t-test analysis.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Flowering time (DAI)</th>
<th>Flowering time (DAS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-KNO$_3$ with <em>M. loti</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG20 (n = 33)</td>
<td>54.4 ± 1.1$^a$</td>
<td></td>
</tr>
<tr>
<td><em>enf1</em> (n = 36)</td>
<td>62.1 ± 0.8$^b$</td>
<td></td>
</tr>
<tr>
<td>+KNO$_3$ without <em>M. loti</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG20 (n = 36)</td>
<td>58.6 ± 1.7$^a$</td>
<td></td>
</tr>
<tr>
<td><em>enf1</em> (n = 37)</td>
<td>50.9 ± 1.4$^b$</td>
<td></td>
</tr>
</tbody>
</table>
Table IV. *Genetic analysis of F2 progeny of MG20 and enf1 of Lotus japonicus*

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

<table>
<thead>
<tr>
<th>root nodule number</th>
<th>experimental value</th>
<th>expected value</th>
<th>$\chi^2$ value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG20 type</td>
<td>37</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>enf1 type</td>
<td>34</td>
<td>38</td>
<td>0.78</td>
<td>0.68</td>
</tr>
<tr>
<td>MG20 x enf1 intermediate</td>
<td>81</td>
<td>76</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>nitrogen fixation activity</th>
<th>experimental value</th>
<th>expected value</th>
<th>$\chi^2$ value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG20 type</td>
<td>33</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>enf1 type</td>
<td>35</td>
<td>38</td>
<td>1.74</td>
<td>0.42</td>
</tr>
<tr>
<td>MG20 x enf1 intermediate</td>
<td>84</td>
<td>76</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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. Bar =1 cm.
Figure 2. Analysis of enf1 symbiotic phenotypes.
*M. loti*-inoculated plants were grown for 28 days in pots containing vermiculite and were watered with B & D medium (A to F) or were grown on B & D agar-solidified (1.5% w/v) medium for 12 days (G). The plants on the agar plates were inoculated with *M. loti* NZP2037 carrying plasmid pH60 (G).

A, Nodule number per plant. B, Nodule weight per plant. C and D, Acetylene reduction activity (ARA) per plant (C), and per nodule weight (D). E, Number of true leaves per plant. F, Shoot length. G, The number of infection threads per plant at 12 DAI. Black bars indicate MG20, gray bars indicate enf1 (A to F). Black bar indicates later stages of infection thread formation, whereas the white bar refers to the initiation of nodule formation (G). At least 40 plants (A to F) and 17 to 20 plants (G) were examined in each experiment. Error bars are given as standard error (A to G). Statistical significance is indicated by *($P<0.05$; student’s t-test) or **($P<0.01$; student’s t-test).
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A, Nodule number per plant. B, Nodule weight per plant. C and D, Acetylene reduction activity (ARA) per plant (C), and per nodule weight (D). E, Number of true leaves per plant. F, Shoot length. G, The number of infection threads per plant at 12 DAI. Black bars indicate MG20, gray bars indicate *enf1* (A to F). Black bar indicates later stages of infection thread formation, whereas the white bar refers to the initiation of nodule formation (G). At least 40 plants (A to F) and 17 to 20 plants (G) were examined in each experiment. Error bars are given as standard error (A to G). Statistical significance is indicated by *(P<0.05; student’s t-test) or **(P<0.01; student’s t-test).
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A to F, Plants were grown for 21 days 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). 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 for 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). Number of lateral roots per plant at 24 DAS (G). At least 30 plants were used in each experiment. Error bars indicate standard error, and statistical significance is indicated by *(P<0.05; student’s t-test) or **(P<0.01; student’s t-test).
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*M. loti*-inoculated plants were grown for 21 days on vermiculite-filled pots supplied with B & D medium. A and B, Endogenous concentrations of ABA in MG20 and *enfI* roots (A) and shoots (B). The data represent the average ± standard error of 6 independent experiments derived from 8 different plants. Statistical significance is indicated by **((P<0.01; student’s t-test)).
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