Control of Nodule Number by the Phytohormone Abscisic Acid in the Roots of Two Leguminous Species

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The effects of the phytohormone abscisic acid (ABA) on plant growth and root nodule formation were analyzed in *Trifolium repense* (white clover) and *Lotus japonicus*, which form indeterminate and determinate nodules, respectively. In *T. repense*, although the number of nodules formed after inoculation with *Rhizobium leguminosarum* bv. *trifolii* strain 4S (wild type) was slightly affected by exogenous ABA, those formed by strain H1(pC4S8), which forms ineffective nodules, were dramatically reduced 28 days after inoculation (DAI). At 14 and 21 DAI, the number of nodules formed with the wild-type strain was decreased by exogenous ABA. In *L. japonicus*, the number of nodules was also reduced by ABA treatment. Thus, exogenous ABA inhibits root nodule formation after inoculation with rhizobia. Observation of root hair deformation revealed that ABA blocked the step between root hair swelling and curling. When the ABA concentration in plants was decreased by using abamine, a specific inhibitor of 9-*cis*-epoxycarotenoid dioxygenase, the number of nodules on lateral roots of abamine-treated *L. japonicus* increased dramatically, indicating that lower-than-normal concentrations of endogenous ABA enhance nodule formation. We hypothesize that the ABA concentration controls the number of root nodules.

Keywords: Abscisic acid — Autoregulation — *Lotus japonicus* — Nodulation — Symbiosis — *Trifolium repense*.

Abbreviation: DAI, days after inoculation.

Introduction

Leguminous plants are capable of establishing nitrogen-fixing symbiosis with soil bacteria generally known as rhizobia. In this symbiosis, compatible rhizobia and plant partners recognize each other through the exchange of chemical signals (Hirsch et al. 2003). Host plants produce compounds that act as inducers of the bacterial *nod* gene, whose products are involved in the synthesis and secretion of Nod factor. In turn, the Nod factor signal triggers a series of host responses, culminating in the development of a symbiotic organ on the plant, the root nodule, in which rhizobia convert atmospheric nitrogen to nitrogen-containing compounds (Zuanazzi et al. 1998, Hayashi et al. 2000).

Despite the beneficial aspects of this symbiosis, rhizobial infection is regulated by the host plant. One mechanism for controlling infection by compatible rhizobia is feedback regulation (or autoregulation) of nodule development, leading to restriction of nodulation zones. Experiments on split-root plants have shown that autoregulation is controlled systemically (Pierce and Bauer 1983, Kosslak and Bohlool 1984, van Brussel et al. 2002). In addition, hypernodulating mutants have been found in several leguminous species, such as *nts1*, *har1*, and *sym29* derived from soybean (*Glycine max*), *Lotus japonicus*, and pea (*Pisum sativum*), respectively. These mutants are characterized by excessive growth of either nodules (symbiotic phenotype) or lateral roots (non-symbiotic phenotype). Split-root and grafting experiments with these mutants have shown that leaf tissue is the principal source of the systemic signal(s) contributing to autoregulation of nodulation. Recently, it was reported that the *nts1*, *har1*, and *sym29* genes encode a receptor-like kinase similar to CLAVATA (*CLV1*) (Nishimura et al. 2002, Krusell et al. 2002, Searle et al. 2003).

The other mechanism for control of nodule number is negative regulation by the gaseous plant hormone ethylene (Nukui et al. 2000, Oldroyd et al. 2001, Ma et al. 2003). A study of the *sickle* mutant of *Medicago truncatula*, which is insensitive to ethylene, revealed that the ethylene signaling system negatively regulates rhizobial infection (Penmetsa and Cook 1997).

Abscisic acid (ABA) is a plant hormone involved in regulating the physiology, growth, and development of higher plants (Spollen et al. 2000). It is involved in mediating responses to environmental stresses (Shinozaki and Yamaguchi-Shinozaki 2000), notably, stomatal closure (McAinsh et al. 1990, MacRobbie 1995) and the activities of many stress-related genes (Urao et al. 1993), and it has a role in developmental processes such as seed maturation and dormancy (Baker et al. 1988, Dure et al. 1989). Several studies have shown that ABA affects root nodule formation (Phillips 1971, Cho and Harper 1993, Bano et al. 2002, Bano and Harper 2002). In soybean, although exogenous ABA actually decreases nodule number in both the wild type and a supernodulation mutant, it also adversely affects plant growth (Cho and Harper 1993, Bano et al. 2002, Bano

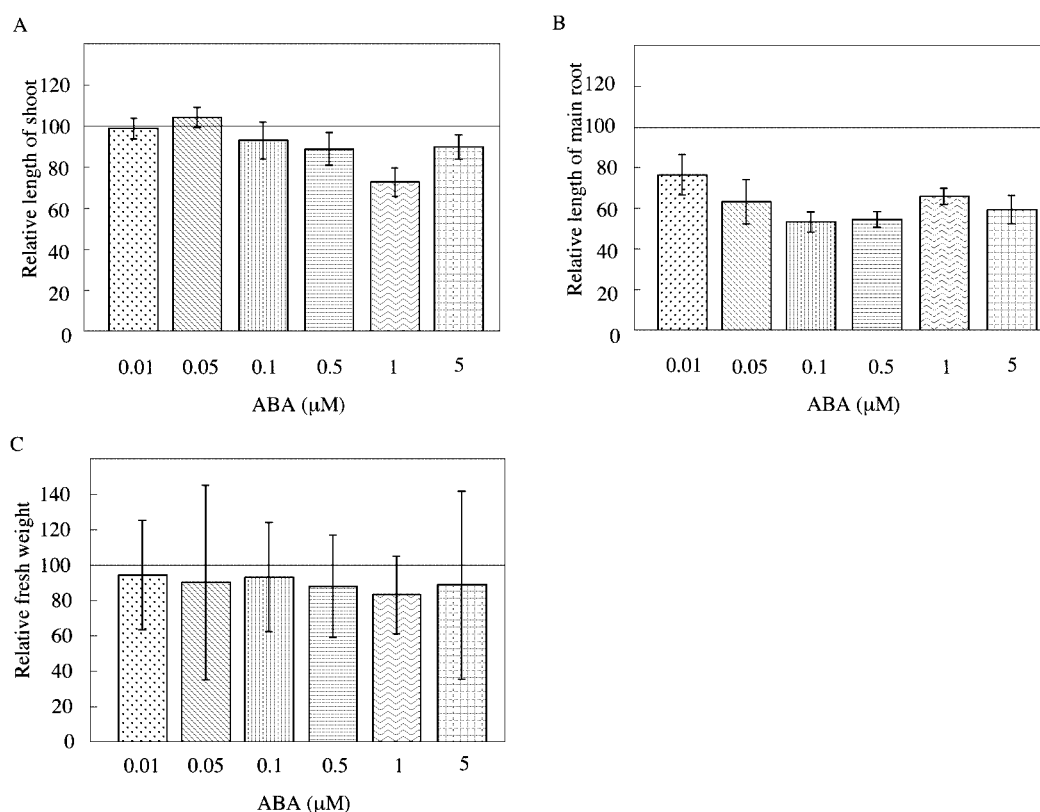[3] Corresponding author: E-mail, azuki@sci.kagoshima-u.ac.jp; Fax, +81-99-285-8163.

Fig. 1 Effects of various concentrations of exogenous ABA on the growth of *T. repense* 28 DAI with *R. leguminosarum* bv. *trifolii* strain H1(pC4S8). The values shown are normalized relative to the mean value in plants grown in the absence of ABA, which was set to 100. At least 50 seedlings were used in each experiment. Error bars indicate standard deviations. (A) Shoot lengths. The normalized mean value of 100 is equivalent to 28.6 mm. (B) Lengths of main root. The normalized mean value of 100 is equivalent to 33.9 mm. (C) Fresh weight. The normalized mean value of 100 is equivalent to 16.2 mg.

and Harper 2002). Therefore, two other leguminous species were selected for the present study.

Our previous study (Suzuki et al. 2001) showed that *Rhizobium leguminosarum* bv. *trifolii* strain H1(pC4S8) can make nodules on the roots of white clover (*Trifolium repense*), although this strain cannot fix nitrogen. The number of nodules per plant formed by strain H1(pC4S8) is 3–4 times the number formed by the wild-type strain (4S). The increased nodule number in strain H1(pC4S8) occurs because plasmid pC4S8 contains only *nod* genes derived from the sym plasmid (pRt4Sa) of *R. leguminosarum* bv. *trifolii* strain 4S as an insert. Therefore, strain H1(pC4S8) has no ability to fix nitrogen. These facts suggest that the level of signal molecule for autoregulation might be limited in the case of this strain. Thus, this clover symbiosis system should be a promising tool for studying the control mechanism of root nodule number. *L. japonicus* is a well-known leguminous plant model. The great advantage of this species is its well-studied molecular genetics. In this study, we therefore investigated the effects of changes in endogenous ABA levels on root nodule formation using these two leguminous species.

Results

Effect of ABA on the growth of legumes

To investigate the effects of ABA on the growth of a host legume, white clover, which forms an indeterminate-type nodule, various concentrations of ABA were added to Fåhraeus agar medium. Twenty-eight days after inoculation (DAI) with H1(pC4S8) rhizobium, the shoot length, root length, and fresh weight of the plants were measured (Fig. 1). Shoot growth was very slightly affected, depending on the concentration of ABA (Fig. 1A). Root lengths were clearly affected by ABA (Fig. 1B): at 0.01 μM ABA, root length was reduced to 80% of that in control plants, and at 0.05–5.0 μM ABA, root lengths were reduced to about 60% of the root length in control plants. The fresh weight of the whole plant was not affected by ABA at the concentrations tested (Fig. 1C). When we inoculated white clover with the wild-type strain (4S), the effects of ABA on plant growth showed the same trends (data not shown).

After inoculation with *Mesochizobium loti*, *L. japonicus* forms determinate root nodules. To analyze the effects of ABA on the growth of this plant, germinated seeds were transferred to a vermiculite/perlite mix containing B&D medium. In con-
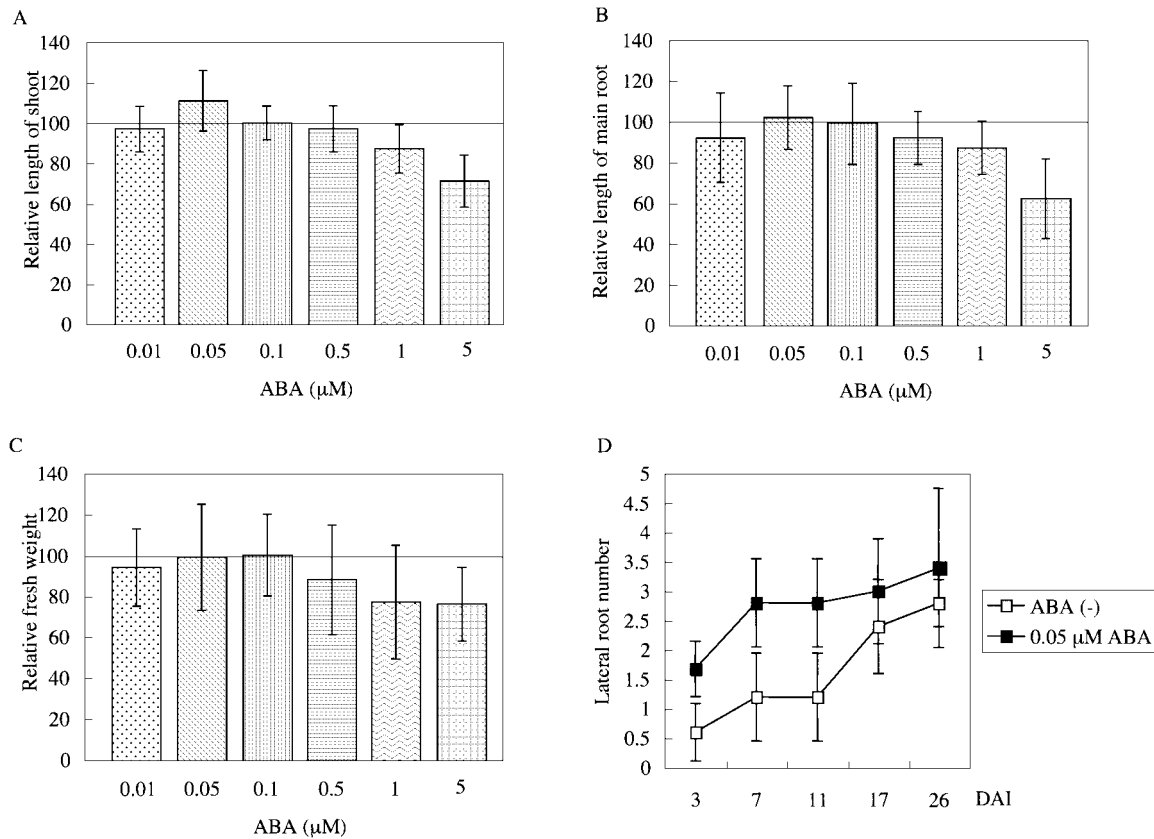
Fig. 2   Effects of various concentrations of exogenous ABA on the growth of *L. japonicus* 28 DAI with *M. loti* MAFF303099. The values shown are normalized relative to the mean value in plants grown in the absence of ABA. At least 50 seedlings were used in each experiment. Error bars indicate standard deviations. (A) Shoot lengths. The normalized mean value of 100 is equivalent to 44.6 mm. (B) Lengths of main root. The normalized mean value of 100 is equivalent to 82.8 mm. (C) Fresh weight. The normalized mean value of 100 is equivalent to 60.7 mg. (D) Number of lateral roots at various DAI of plants treated with 0.05 μM ABA.

trast to the results obtained with white clover, none of the growth parameters (shoot length, main root length, or fresh weight of the whole plants) of ABA-treated *L. japonicus* plants were affected by low concentrations (0.01–0.5 μM) of ABA (Fig. 2A, B, C). The number of lateral roots increased after inoculation, and the total number of lateral roots was higher in the presence of 0.05 μM ABA than in the absence of ABA (Fig. 2D).

Effects of ABA on root nodule formation

We analyzed whether ABA affects indeterminate-type root nodule formation in white clover (Fig. 3). Twenty-eight DAI with wild-type strain 4S or with mutant H1(pC4S8) rhizobia, the number of root nodules was counted under a binocular microscope. With strain 4S, the nodule number was only slightly reduced by ABA. In contrast, with H1(pC4S8), ABA caused a drastic reduction in nodule number, and the extent of the reduction depended on the concentration of ABA. The number of root nodules formed by strain H1(pC4S8) was reduced to about 70% of that in control plants at 0.01 and 0.05 μM ABA. At higher ABA concentrations (0.10–5.0 μM),

the nodule numbers were markedly reduced, to almost the same level as with the wild-type rhizobium strain.

In the absence of ABA, the difference in the number of nodules formed between strains 4S and H1(pC4S8) is recognizable 14 DAI (Suzuki et al. 2001). Thus, in the present study, we also examined the effects of higher ABA concentrations (0.10–1.0 μM) on root nodule formation in white clover on days 14, 21, and 28 after inoculation (Fig. 3C). With H1(pC4S8) rhizobium, the number of nodules was low on all days after inoculation and at all concentrations of ABA tested. In the wild-type strain, although the nodule number was almost the same as in the absence of ABA 28 DAI, nodulation was apparently suppressed at earlier stages of germination. Moreover, the extent of the decrease in nodule number on each day depended on the concentration of ABA. These results show that nodule formation is affected by ABA treatment for both the H1(pC4S8) mutant and the wild-type strain 4S.

In *L. japonicus*, the effects of ABA on root nodule formation by *M. loti* were analyzed in plants grown on Fåhraeus agar plates. The nodule number was clearly reduced, depending on the concentration of ABA (Fig. 4).
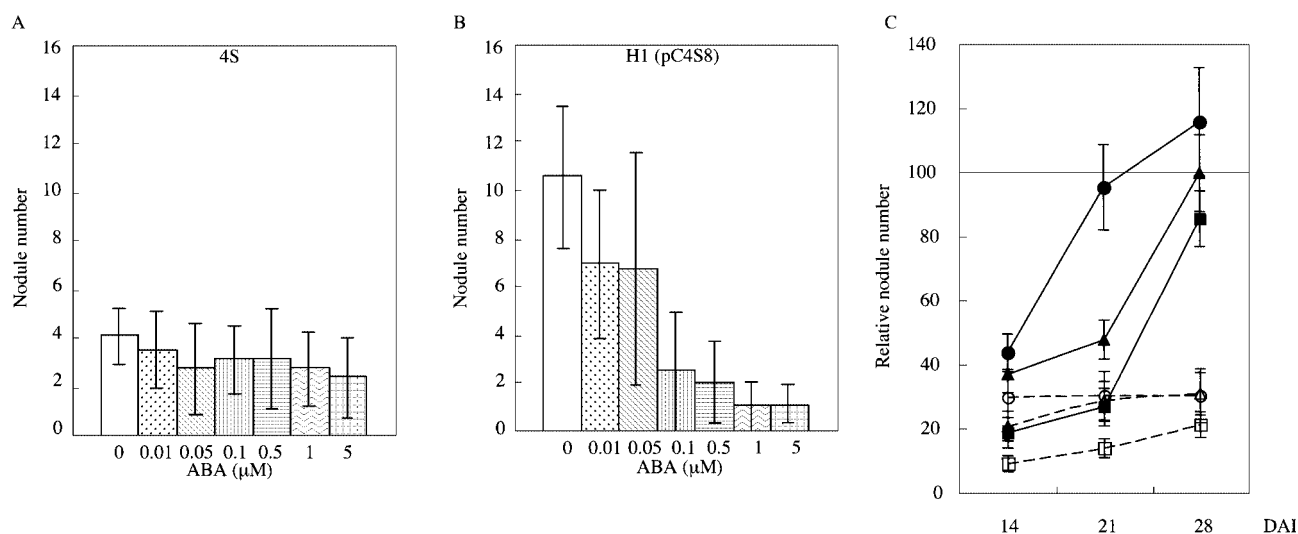
Fig. 3 Average numbers of nodules on roots of ABA-treated *T. repense* after inoculation with *R. leguminosarum* bv. *trifolii* strain 4S or H1(pC4S8). At least 40 seedlings were used in each experiment. Error bars indicate standard deviations. (A) Twenty-eight DAI with wild-type strain 4S. (B) Twenty-eight DAI with strain H1(pC4S8). (C) Numbers of nodules formed on roots of *T. repense* in the presence of ABA as a function of DAI. The values shown are normalized relative to the mean value for plants grown in the absence of ABA. At least 40 seedlings were used in each experiment. Black symbols, wild-type rhizobium (strain 4S); open symbols, H1(pC4S8) mutant rhizobium. Circles, 0.1 μM ABA; triangles, 0.5 μM ABA; squares, 1.0 μM ABA. In strain 4S, normalized mean values of 100 are equivalent to 1.6, 2.8, and 3.4, for 14 DAI, 21 DAI, and 28 DAI, respectively. In strain H1(pC4S8), normalized mean values of 100 are equivalent to 2.2, 5.1, and 7.8, for 14 DAI, 21 DAI, and 28 DAI, respectively.

The effect of ABA on root hair swelling and curling and infection thread formation in white clover

The proportion of swollen root hairs was apparently increased at all of the tested stages after the addition of ABA. On the other hand, that proportion was decreased in the absence of ABA (Fig. 5A). In contrast, the proportions of curling root hairs and root hairs containing infection threads were decreased by the treatment with ABA (Fig. 5B, C). In the absence of ABA, the proportion of curling root hairs increased with days after inoculation. Examples of white clover root hairs showing curling, in roots of untreated plants, is shown in Fig. 5E, and examples of swelling, in treated plants, are shown in Fig. 5F and 5G.

Effects of abamine treatment on the growth of L. japonicus

Abamine is an inhibitor of 9-*cis*-epoxycarotenoid dioxygenase (NCED), which functions in the indirect pathway of ABA synthesis (Asami et al. 2003) and has been used to decrease in vivo levels of ABA. The level of endogenous ABA of leaves decreased, as expected, as a function of abamine concentration (Fig. 6A). The endogenous ABA level of roots of plants treated with 10 μM abamine also decreased compared with control plants (Fig. 6B). Moreover, this abamine effect was completely reversed by the addition of 0.05 μM ABA (Fig. 6B). Although shoot length, fresh weight, and the number of lateral roots were not affected by abamine, the root length was slightly affected (Fig. 6C). The total number of root nodules in plants treated with 10 μM abamine was about 1.5 times the number in control plants. Abamine affected the number of nod-

ules mainly on lateral roots: the number of nodules on lateral roots was about three times that on control roots in the presence of 10 μM abamine (Fig. 6D). Moreover, the effect of abamine treatment on increased nodule number was completely reversed by the addition of ABA (Fig. 6E). Thus, on the lateral roots, the number of nodules was drastically increased when the concentration of endogenous ABA was low.

Discussion

In this study, the effects of ABA on root nodule formation in the legumes *T. repense* and *L. japonicus*, which make indeterminate and determinate types of nodules, respectively, were examined. This is probably the first report of an analysis of the effects of ABA on root nodule formation in both species. The doubling time of the rhizobium strains used in this study was not affected by the addition of 5 μM ABA (data not shown).

T. repense inoculated with the hypernodulation mutant rhizobium, *R. leguminosarum* bv. *trifolii* strain H1(pC4S8) and cultured in the presence of ABA for 28 d had fewer nodules than plants cultured in the absence of ABA; the amount of the reduction in nodule number depended on the ABA concentration (Fig. 3). In contrast, nodule formation by the wild-type rhizobium strain 4S was only slightly affected. At 0.10–5.0 μM ABA, the nodule number after inoculation with strain H1(pC4S8) was almost same as that in the wild-type strain 4S. These results suggest that ABA inhibits excessive root nodule formation.

Strain H1(pC4S8) rhizobium makes ineffective nodules on the roots of white clover, but it makes 3–4 times as many nod-
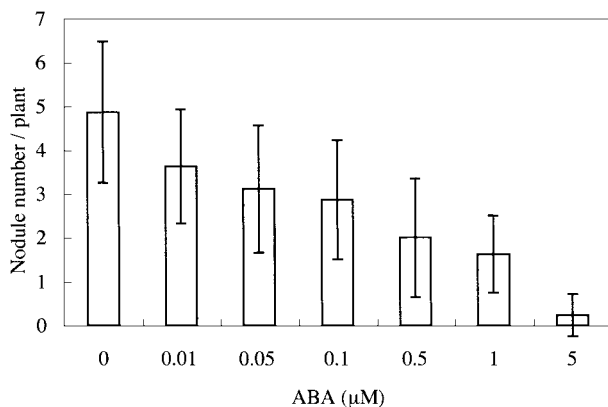
Fig. 4 Effect of ABA on the number of nodules formed on roots of *L. japonicus* 28 DAI with *M. loti*. At least 36 seedlings were used in each experiment. Error bars indicate standard deviations.

ules as does the wild-type strain 4S, suggesting that the level of signal molecule for autoregulation might be limited. The difference in nodule number between strain 4S and H1(pC4S8) can be observed 14 DAI (Suzuki et al. 2001), suggesting that the decrease in nodule number occurs earlier than 28 DAI. Therefore, we analyzed the effects of ABA on root nodule formation before 28 d and found (Fig. 3C) that the nodule number was drastically decreased by ABA not only with strain H1(pC4S8) but also with strain 4S. In other words, exogenous ABA inhibits normal root nodule formation by the wild-type strain 4S as early as 2–3 weeks after inoculation. After that, no additional inhibition of normal root nodule formation occurred, although the production of excessive numbers of root nodules by H1(pC4S8) was decreased by ABA.

At which step of infection does ABA have an inhibitory affect? To answer this question, phenotypic changes in the root hairs of white clover, inoculated with wild type strain 4S with or without ABA, were observed (Fig. 5). When no rhizobium was inoculated, no root hair deformation occurred either with or without ABA (data not shown). In control plants (without ABA), the proportion of swollen root hairs was almost the same at all three stages (3, 7, and 14 days after inoculation) after inoculation with rhizobium, but the proportion of curled root hairs gradually rose with each tested stage (Fig. 5A, B, D, E). These results show that process of infection on the root of the control plants proceeded to at least the curling step. On the other hand, in ABA-treated plants, although the proportion of curled root hairs hardly increased during successive stages and curling was down-regulated by ABA, root hair swelling increased gradually and was up-regulated by ABA (Fig. 5A, B, F, G). These results suggest that exogenous ABA inhibited root hair deformation at the stage between root hair swelling and curling, because the infection process did not proceed to curling. Although Phillips (1971) found that the proportion of infection threads increased with the addition of 1.9 µM ABA to pea, a lower concentration of ABA (0.5 µM) suppressed infec-

tion thread formation in white clover in this study (Fig. 5C). In white clover, these results are reasonable, because a stage of infection prior to infection thread formation was blocked by treatment with ABA. In another study of ABA effects, Cho and Harper (1993) reported that isoflavonoid concentrations are decreased in response to exogenous ABA in soybean. Therefore, this possibility can be examined using white clover.

In addition to white clover, we also investigated the effects of ABA on root nodule formation on the roots of *L. japonicus*, which forms determinate-type nodules containing *M. loti*. Unlike what occurred with white clover, the growth of *L. japonicus* plants was not affected by low ABA concentrations (0.01–0.1 µM) (Fig. 2A–C), and root nodule formation was clearly inhibited at all concentrations of ABA tested (Fig. 4). Moreover, the number of lateral roots apparently increased in comparison with the negative control (Fig. 2D). The increase in the number of lateral roots in *L. japonicus* was consistent with the results for white clover (data not shown).

The hypernodulation *har1* mutant of *L. japonicus* shows several interesting phenotypes: one is bushy roots due to increased branching of lateral roots (Wopereis et al. 2000, Kawaguchi et al. 2002). Both root nodule formation and lateral root branching are enhanced in this mutant. It was proposed that the mechanism of root nodule formation partially overlaps that of lateral root branching. It is considered that the gene product responsible for this phenotype may be one of the molecules that function in root nodule autoregulation. There are at least two possible explanations that account for all these findings. One is that ABA is downstream of the part of the pathway where the regulation of root nodule formation overlaps with the control of lateral root branching, and the second is that ABA works through a control mechanism that is different from the previously mentioned pathway, because ABA causes different responses at each differentiation step.

As described above, root growth in white clover is inhibited by exogenous ABA. This result leads to the question of whether the decrease in nodule number is due to the reduced root area available for nodule formation. Although the length of the main root of white clover that was inoculated with H1(pC4S8) was reduced to 60% of the mean control value at an ABA concentration of 5.0 µM, the number of nodules was reduced much more – to only 10% (Fig. 1B, 3B). These data indicate that the relative decrease in nodule number was not in proportion to the decrease in the length of the main root. The same tendency was found in soybean. Although Bano and Harper (2002) have already reported that exogenous ABA (1, 5, 10, and 50 µM) decreases nodule number in both the wild type and a supernodulation mutant of soybean, the growth of roots was reduced to 78% of the control when ABA at a concentration of even 0.1 µM was used (Cho and Harper 1993). On the other hand, in *L. japonicus*, in spite of the drastic reduction in nodule number, plant growth was not affected by the addition of low concentrations of ABA (Fig. 2A–C, 4). Therefore, the
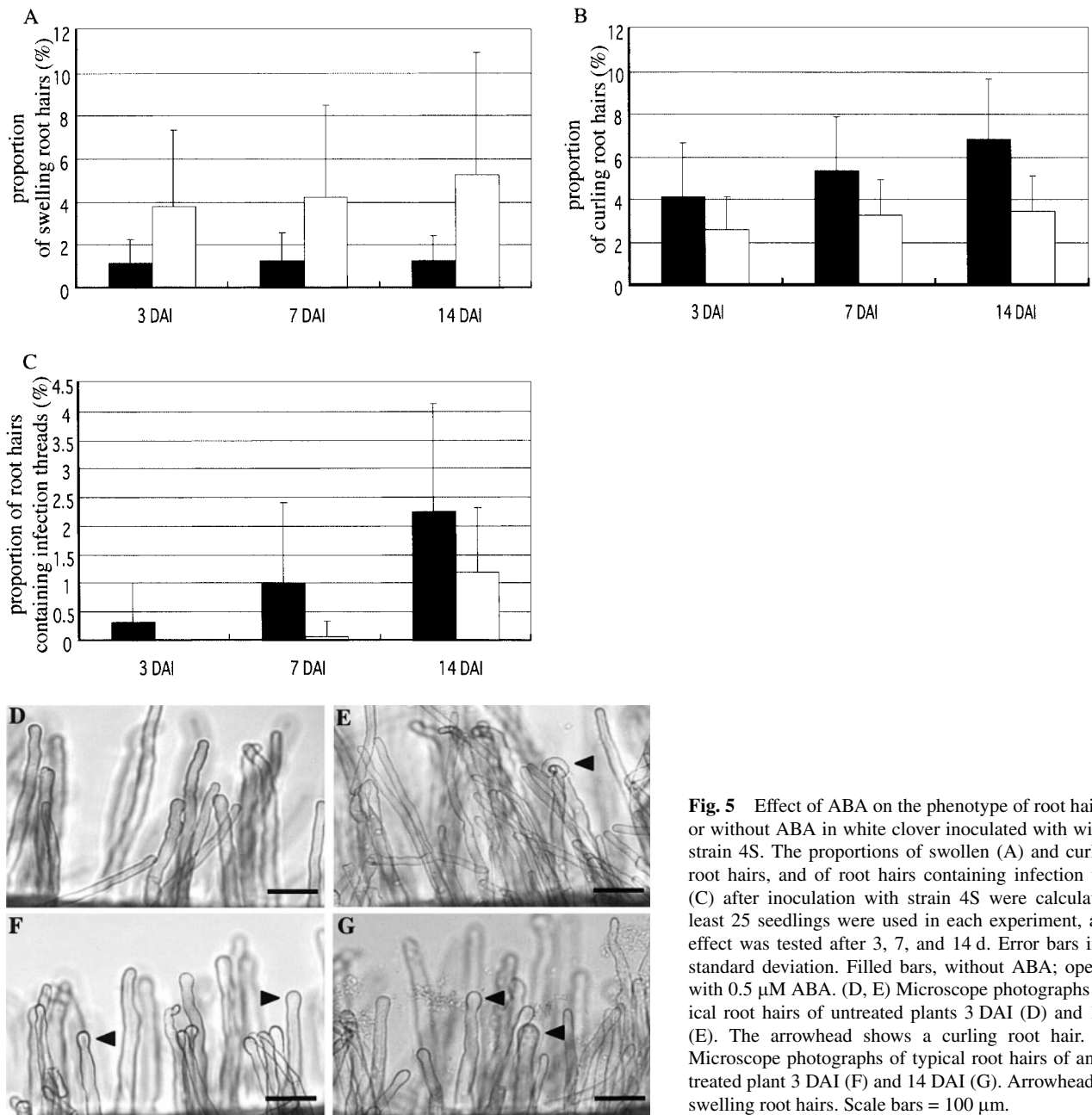
A

B

C

Fig. 5 Effect of ABA on the phenotype of root hairs with or without ABA in white clover inoculated with wild-type strain 4S. The proportions of swollen (A) and curled (B) root hairs, and of root hairs containing infection threads (C) after inoculation with strain 4S were calculated. At least 25 seedlings were used in each experiment, and the effect was tested after 3, 7, and 14 d. Error bars indicate standard deviation. Filled bars, without ABA; open bars, with 0.5 µM ABA. (D, E) Microscope photographs of typical root hairs of untreated plants 3 DAI (D) and 14 DAI (E). The arrowhead shows a curling root hair. (F, G) Microscope photographs of typical root hairs of an ABA-treated plant 3 DAI (F) and 14 DAI (G). Arrowheads show swelling root hairs. Scale bars = 100 µm.

idea that the decrease in nodule number may be due to a reduction in the size of the nodulation zone can be dismissed.

Others have found that endogenous ABA levels in plants were increased by the addition of exogenous ABA. However, ours appears to be the first study of root nodule formation in plants in which the concentration of endogenous ABA was decreased by treatment with an exogenous reagent (abamine). Before using abamine, we tested fluridon, which is an inhibitor of carotenoid synthesis on the indirect pathway of ABA synthesis. But because the effect of fluridon on the growth of plants was very severe (data not shown), we tried abamine instead. Abamine is a specific inhibitor of NCED (Asami et al. 2003)

on the indirect pathway of ABA synthesis and has little effect on plant growth at low concentrations (1 or 10 µM abamine). In *L. japonicus*, although abamine did not have much influence on the growth of plants (Fig. 6C), the endogenous ABA level was decreased (Fig. 6A, B). Twenty-eight DAI with *M. loti*, the nodule number on lateral roots of abamine-treated plants was three times that in control plants (Fig. 6D). Although the effect of abamine on the number of root nodules was dramatically different between main root and lateral root, we could not detect any difference in the endogenous ABA concentration (data not shown). This result suggests that the mechanisms controlling nodule number on main root and on lateral root are
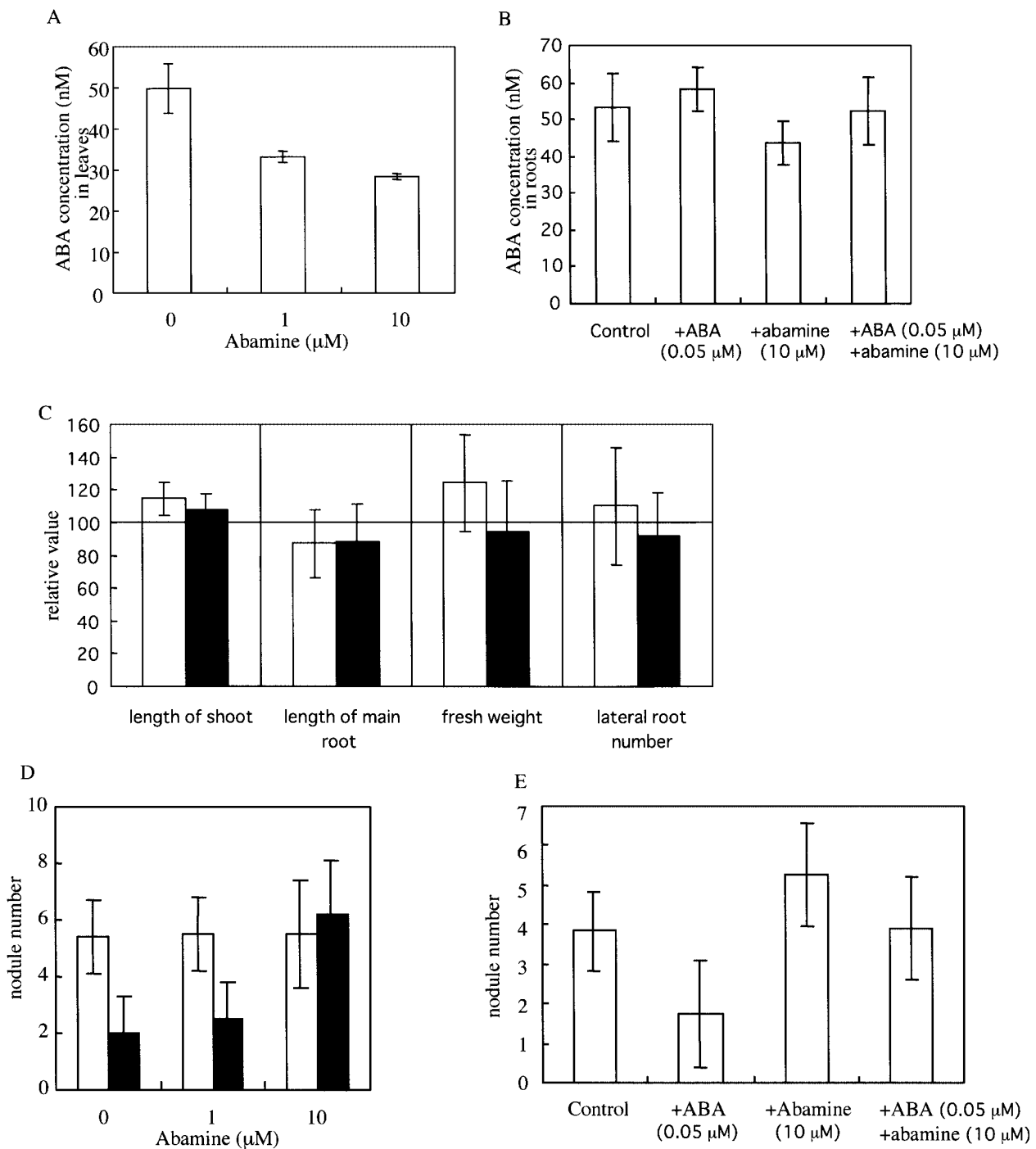
Fig. 6 Effects of exogenous abamine on endogenous ABA concentration and on growth parameters of *L. japonicus* 28 DAI with *M. loti*. At least 45 seedlings were used in each experiment. Error bars indicate standard deviations. (A) ABA concentrations in leaves of *L. japonicus* grown on soil mix. (B) ABA concentration in roots of *L. japonicus* grown on Fåhraeus agar plates. (C) Growth parameters for plants grown at two concentrations of abamine normalized relative to the mean growth parameters for plants grown in the absence of abamine. These plants were grown on soil mix. For shoot lengths, the normalized mean value of 100 is equivalent to 58.4 mm. For lengths of main root, the normalized mean value of 100 is equivalent to 106.3 mm. For fresh weight, the normalized mean value of 100 is equivalent to 77.1 mg. For numbers of lateral roots, the normalized mean value of 100 is equivalent to 11.1. Open bars, 1 μM abamine; filled bars, 10 μM abamine. (D) Effects of exogenous abamine on numbers of nodules formed on roots of *L. japonicus* 28 DAI with *M. loti*. At least 45 seedlings were used in each experiment. Error bars indicate standard deviations. The open bars indicate the number of root nodules on the main roots and the filled bars show the number of nodules on the lateral roots. (E) Effects of exogenous double treatment with abamine and ABA on numbers of nodules formed on roots of *L. japonicus* 28 DAI with *M. loti*. At least 45 seedlings were used in each experiment. Error bars indicate standard deviations.
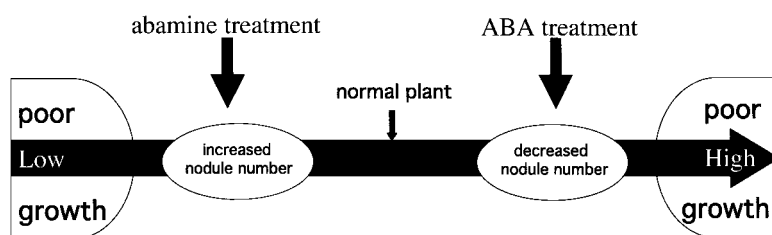
Fig. 7 Schematic representation of the hypothesis suggesting that internal ABA concentration controls the number of root nodules.

not necessarily exactly the same. The endogenous ABA level of double-treated plants (ABA and abamine) was almost the same as that of the untreated plants (control). Therefore, as expected, the nodule number was completely recovered by the double-treatment. These results strongly support the following interpretation.

Fig. 7 is a schematic representation of relations between the level of endogenous ABA and the number of root nodules. The large black arrow represents increasing concentrations of ABA, and the endogenous ABA concentration is that indicated for a "normal" or untreated plant. At higher ABA concentrations, the number of root nodules is decreased and at lower ABA concentrations it is increased. Bano et al. (2002) reported that, in a supernodulation mutant named NOD1-3, ABA concentration in xylem sap increased within 6 h and that in phloem sap decreased within 192 h after inoculation with *Bradyrhizobium japonicum*; this lower concentration of ABA in the phloem sap, compared with that in wild-type soybean (cv. William), caused supernodulation. Our results are also compatible with these results.

Materials and Methods

Measurement of ABA concentration

The ABA concentrations in leaves or roots were measured with a Phytodetek ABA enzyme immunoassay test kit (Agdia Inc.; Elkhart, IN, U.S.A.). Mature leaves or roots of 4-week-old *L. japonicus* grown in medium with or without abamine or ABA were harvested, rinsed in water, frozen in liquid nitrogen, and ground into powder. ABA was extracted by suspending 1 g of tissue in 15 ml of extraction solution (80% methanol, 100 mg liter^{-1} butylated hydroxytoluene, 0.5 g liter^{-1} citric acid monohydrate) and stirring overnight at 4°C. The suspension was centrifuged at 4,000 rpm for 20 min, and the supernatant was transferred to a clean tube and dried under vacuum. The dry residue was dissolved with 1 ml of modified Tris-buffered saline (45 mM Tris-HCl, pH 7.8, 90 μM MgCl$_2$, 0.135 M NaCl, and 10% methanol) and centrifuged at 10,000 rpm for 10 min. The supernatant was filtered with a syringe-driven filter unit (Millex-HV). The ABA concentration in the filtrate was then determined with a Phytodetek ABA enzyme immunoassay test kit (Agdia Inc.).

Plant material and growth conditions

Seeds of white clover (*T. repense* L. cv. Ladino) were surface-sterilized by immersion in disinfectant [0.2% formaldehyde : 0.1% HgCl$_2$: ethanol = 1 : 1 : 1 (vol.)] for 1 min and rinsed several times with sterile distilled water. The seeds were sown on plates of 1% agar; the plates were then placed upside-down and incubated at 25°C in the dark for 2 d. The germinated seedlings were transplanted onto Fåhraeus agar (0.8%) in a tube and inoculated with rhizobia at a con-

centration of 2.5×10^6 cells per plant. The plants were grown at 25°C under 16 h light : 8 h dark conditions.

For surface-sterilization of *L. japonicus* (Gifu) seeds, H$_2$SO$_4$ was used. After incubating the seeds for 3 d at 25°C, germinated seedlings were transferred to an artificial soil mix (vermiculite : perlite = 5 : 1) containing B&D medium or to a Fåhraeus agar plate (0.8%) and inoculated with rhizobia at a concentration of 1.0×10^7 cells per plant. Plant growth and nodulation were assessed on the indicated days. The number of root nodules that developed to a diameter of more than 0.5 mm was counted under a binocular microscope. (±)-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] purchased from Sigma-Aldrich was used in this research.

Observation of root hair deformation in ABA-treated plants

The surface-sterilized seeds of white clover were sown on plates containing 1% agar; the plates were placed upside down and incubated at 25°C in the dark for 2 d. These germinating seedlings were transferred to Fåhraeus liquid medium in the presence or absence of 0.5 μM ABA and inoculated with wild-type strain 4S at a concentration of 2.5×10^6 cells per plant. The plants were grown at 25°C under 16 h light : 8 h dark conditions. Three DAI, root hairs in the first 3 mm of root from the root top were checked under the microscope for swelling, curling, and infection thread formation. The same part of the root of the same tested samples was checked again 7 and 14 DAI.

Bacterial strains and culture conditions

R. leguminosarum bv. *trifolii* strain 4S and strain H1(pC4S8) were used for inoculating white clover. *M. loti* MAFF 303099 was used for inoculating *L. japonicus*. Rhizobia were grown at 28°C in liquid YM medium until the optical density at 600 nm reached 1.0. These rhizobia were washed three times with sterile water and resuspended in Fåhraeus or B&D liquid medium at a concentration of 10^7 cells ml^{-1}.

Abamine treatment

Abamine (1 or 10 μM) was added to Fåhraeus media for the germination and inoculation tests.

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