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Transgenic *Lotus japonicus* with an Ethylene Receptor Gene *Cm-ERS1/H70A* Enhances Formation of Infection Threads and Nodule Primordia

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Ethylene inhibits the establishment of symbiosis between rhizobia and legumes. To examine how and when endogenous ethylene inhibits rhizobial infection and nodulation, we produced transgenic *Lotus japonicus* carrying the mutated melon ethylene receptor gene *Cm-ERS1/H70A* that confers ethylene insensitivity and fixes the transgene in the T_3 generation. The resultant transgenic plants showed reduced ethylene sensitivity because of 1-aminocyclopropane-1-carboxylate resistance and increased flowering duration, probably due to a dominant negative mechanism. When inoculated with *Mesorhizobium loti*, transgenic plants showed markedly higher numbers of infection threads and nodule primordia on their roots than did either wild-type or azygous plants during the early stage of cultivation period as well as during later stages, when the number of mature nodules had reached a steady state. In addition, transcripts of *NIN*, a gene governing infection thread formation, increased in the inoculated transgenic plants as compared with the wild-type plants. The infection responses of transgenic plants were similar to those of wild-type plants treated with ethylene inhibitors. These results imply that the endogenous ethylene in *L. japonicus* roots inhibits rhizobial infection at the primary nodulation, probably via *NIN* gene, and suggest that ethylene perception assists negative feedback regulation of secondary nodule initiation.

Keywords: Ethylene receptor — Infection — Legume — *Lotus japonicus* — *NIN* — Nodulation.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; AVG, L-α-(2-aminoethoxyvinyl) glycine; DAI, days after inoculation; STS, silver thiosulfate; X-Gal, 5-bromo-4-chloro-3-indolyl-β-galactopyranoside.

Introduction

The formation of nitrogen-fixing nodules is the consequence of a series of interactions between *(Brady)rhizobia* and leguminous host plants (Fisher and Long 1992). Although legumes form nodules on their roots in response to lipooligosaccharide signals (Nod factor), the perception of endogenous signals, particularly plant hormones, is important for proper symbiotic development (Caetano-Anolles and Gresshoff 1991).

Ethylene is produced and perceived in response to a wide variety of environmental and developmental cues, including germination, flowering, drought, pathogen attack, and nodulation (Abeles et al. 1992, Spaink 1997). In particular, ethylene inhibits infection and nodulation of most legumes, including *Lotus japonicus* (Nukui et al. 2000). Lee and LaRue (1992) reported that exogenous ethylene application inhibits infection thread elongation into the inner cortex of *Pisum sativum*. In *P. sativum*, endogenous ethylene exerts positional effects on nodule meristem morphogenesis (Heidstra et al. 1997). Ethylene evolution in legume roots increases after application of rhizobial cells (Suganuma et al. 1995, Ligero et al. 1986), Nod factor (van Spronsen et al. 1995), nitrate (Ligero et al. 1987, Caba et al. 1998), and light irradiation (Lee and LaRue 1992), suggesting that these environmental factors control nodulation via levels of ethylene.

The hyper-infected *sickle* mutant of *Medicago truncatula* is insensitive to ethylene (Penmetsa and Cook 1997), although other hyper-nodulated mutants, *sunn* of *M. truncatula* (Penmetsa et al. 2003) and *har1* of *L. japonicus* (Wopereis et al. 2000), were normally sensitive to ethylene. Therefore, ethylene perception is thought to be involved in the control of nodule numbers, as is a well-known systemic feedback regulatory system (autoregulation) (Caetano-Anolles and Gresshoff 1991, Nishimura et al. 2002, Krusell et al. 2002). The relationship between autoregulation and ethylene signaling is still not clear.

Recently, rhizobia have been found to actively produce at least two factors that reduce the level of endogenous ethylene in host legumes and that enhance nodulation by the endosymbionts (Ma et al. 2002). Rhizobitoxine produced by *Bradyrhizobium elkanii* enhances nodulation by lowering the production of endogenous ethylene (Yuhashi et al. 2000, Okazaki et al. 2003). 1-Aminocyclopropane-1-carboxylate (ACC) deaminase, which degrades ACC (an immediate precursor of ethylene), has been found in fast-growing rhizobia, including *Rhizobium leguminosarum* and *M. loti* (Yasuta et al. 2001). Indeed, ACC deaminase in *R. leguminosarum* bv. *viciae* has been confirmed to enhance nodulation of *Pisum sativum* (Ma et al. 2003). Recent findings regarding rhizobial strategies to lower ethylene levels in host legumes emphasize the importance of

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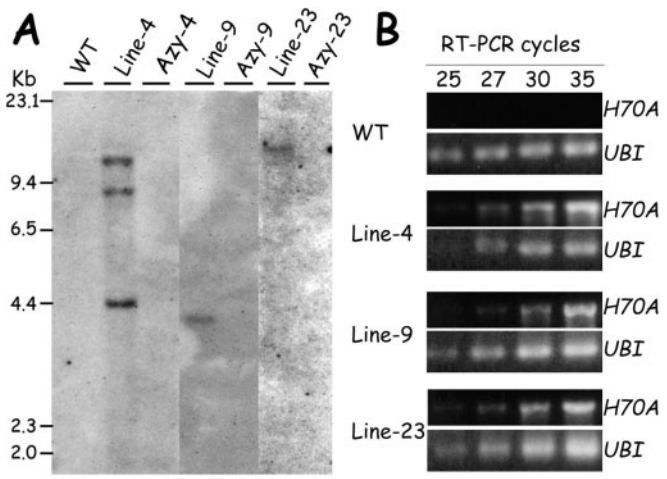

Fig. 1 Genomic Southern hybridization and RT-PCR analysis of transgenic *L. japonicus*. (A) Southern hybridization using *Cm-ERS1* as a probe. Genomic DNA was digested with *Hin*dIII, hybridized at 68°C, and washed at high stringency (0.1× SSC, 1% SDS, 68°C). WT indicates wild type of *L. japonicus*. Line-4, line-9 and line-23 are independent transformants, while Azy-4, Azy-9 and Azy-23 are the corresponding azygous plants. (B) RT-PCR analysis of *Cm-ERS1/H70A* (H70A) gene expression in wild-type and Lj-H70A series plants. Reverse transcription was performed using 2 μg of total RNA. Number of cycles was set at 25, 27, 30 and 35. *Polyubiquitin* (UBI) cDNA fragments were amplified as controls for equal amounts of cDNA template.

ethylene-mediated interactions in the establishment of symbiosis between both partners (Ma et al. 2002).

The expression of ethylene receptors lacking ethylene-binding ability confers reduced sensitivity to ethylene in heterologous plants dominantly (Bleecker 1999). In petunia and tomato, transformation with a mutant *Arabidopsis* ethylene receptor gene (*etr1-1*) conferred reduced sensitivity to ethylene (Wilkinson et al. 1997). A point mutation was introduced into the melon ethylene receptor *Cm-ERS1* (Sato-Nara et al. 1999) by changing the 70th amino acid histidine to alanine, which abolished the ethylene binding ability. This mutant gene was designated *Cm-ERS1/H70A* (Ezura et al. unpublished result).

To address the inhibitory mechanism of ethylene on nodulation, we transformed *L. japonicus* B-129 'Gifu' (Handberg and Stougaard 1992) with *Cm-ERS1/H70A*. Here we report the reduced ethylene sensitivity and altered nodulation phenotypes of the transgenic *L. japonicus*.

Results

Production of transgenic plants

L. japonicus B-129 'Gifu' was transformed with the melon ethylene receptor gene *Cm-ERS1/H70A*, and the transgenic lines were designated as the Lj-H70A series. In the T_0 generation, 24 regenerated and diploid plants were obtained from independent callus samples. Using a *Cm-ERS1*-specific PCR assay, we detected the transgene in 16 of the 24 plants. We then used Southern blotting to confirm the presence of the transgene in nine independent lines (data not shown). Seeds were obtained from these nine lines by self-pollination. Four lines (Lj-H70A-5, -6, -12, and -14) flowered but did not produce fruit. Seed germination depended on the lines: Lj-H70A line-2, 33%; line-3, 33%; line-4, 31%; line-8, 53%; line-9, 56%; line-10, 60%; line-13, 27%; and line-23, 67%. In the $T_{1\text{-}2}$ generation, azygous plants of each line were prepared from segregated inheritances and were used as negative controls.

We performed Southern hybridization of the T_2 generation of the transgenic lines line-4, line-9 and line-23, and of the T_1 and T_2 generations of azygous lines azy-4, azy-9 and azy-23 and wild type (Fig. 1A). Line-4 possessed three copies of the introduced *Cm-ERS1/H70A* gene, whereas line-9 and line-23 each had a single copy (Fig. 1A). Wild-type and azygous lines did not show any bands (Fig. 1A). We then used *Cm-ERS1/H70A*-specific PCR to investigate segregation of the T_3 generation. In line-4, the ratio of *Cm-ERS1/H70A* positive vs. negative genotypes was 3 : 1, suggesting that this line was heterologous (data not shown). Line-9 and line-23 did not segregate at all in the T_3 generation, indicating that both lines were homologous (data not shown). Reverse transcription-PCR (RT-PCR) assays showed that expression of the *Cm-ERS1/H70A* gene occurred in line-4, line-9, and line-23 but not in the wild-type plants (Fig. 1B). However, the control *polyubiquitin* (UBI) cDNA fragment appeared in all plants tested (Fig. 1B). Because the *Cm-ERS1/H70A* gene was driven by the CaMV-35S promoter, the transgene likely was expressed throughout the entire plant, including the root system.

Ethylene sensitivity

To test of ethylene sensitivity of the transgenic plants, we grew wild-type, Lj-H70A series, and azygous plants on Murashige–Skoog medium (Murashige and Skoog 1962) containing 5 μM ACC for 30 d (Fig. 2A). Wild-type and azygous plants (azy-4, azy-9 and azy-23) showed symptoms typical of ethylene treatment: yellowing by chlorophyll degradation, inhibition of shoot elongation, and thick, short, brown roots (Fig. 2A). By contrast, line-4, line-9 and line-23 showed healthy shoots with greenish, long internodes, and healthy, long white roots (Fig. 2A). We monitored senescence and abscission of flowers after pollination as indicators of ethylene sensitivity. In wild-type plants, flower petals remained fresh until 6 d after flowering. They then began to wither, and detached from the pedicel during pod development (Fig. 2B). At 7 d after pollination, wild-type and azygous plants showed senescence and abscission of flowers. However, Lj-H70A series plants retained fresh petals (Fig. 2B), indicating delay of petal senescence and detachment. This phenomenon also occurred in the flowers of the ethylene-insensitive tomato mutant *Nr* (Lanahan et al. 1994). Our results indicate that introduction of the *Cm-ERS1/H70A* gene conferred reduced ethylene sensitivity, as we had expected, according to the so-called dominant negative mecha-

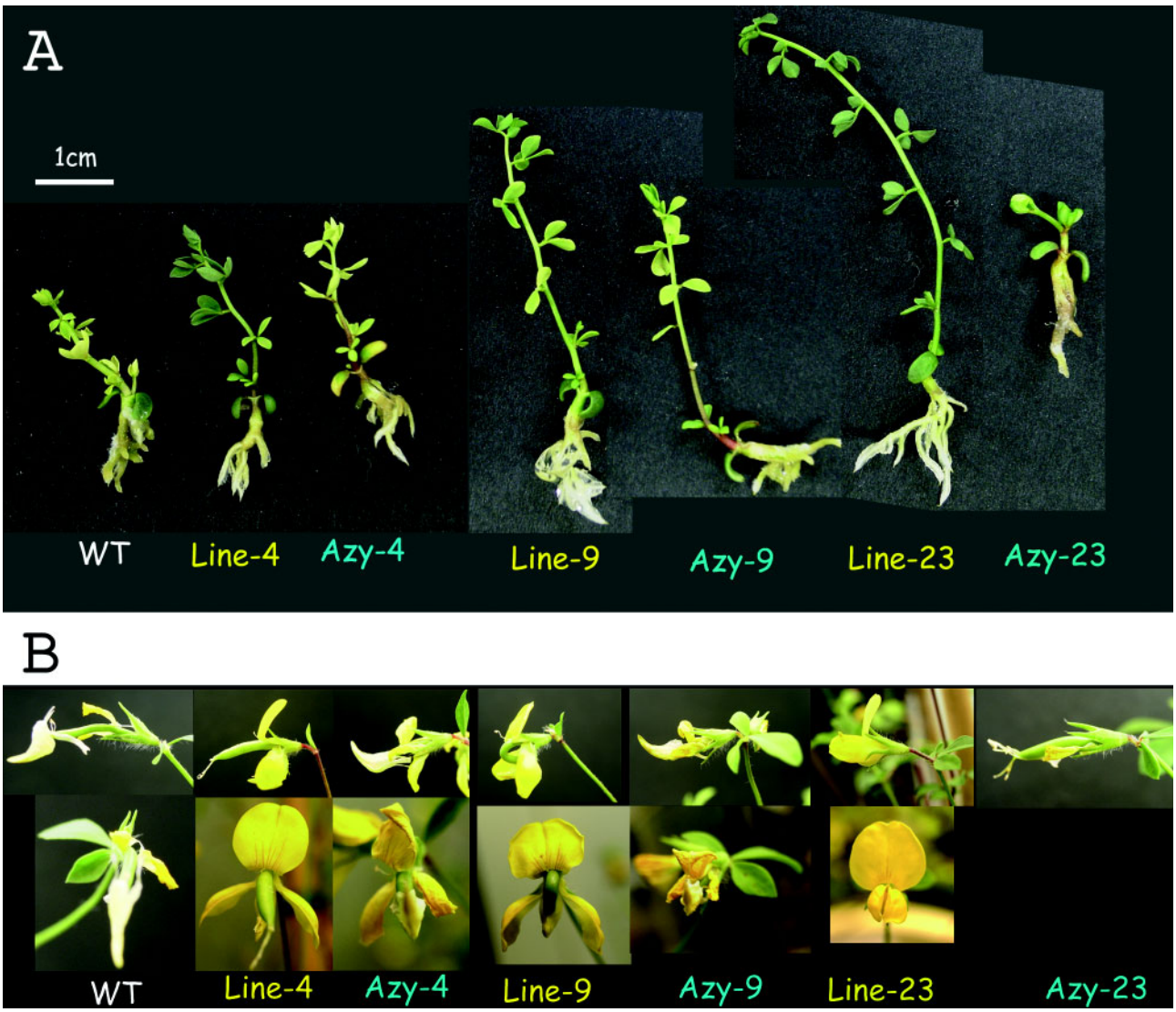

Fig. 2 Reduced ethylene sensitivity caused by transformation with *Cm-ERS1/H70A*. (A) Wild-type (white label), transgenic (yellow labels), and azygous (blue labels) plants were germinated for 30 d on Murashige–Skoog medium containing 5 μM ACC. (B) Flowering phenotypes of Lj-H70A series plants. Wild-type and azygous flowers showed senescence and abscission petals 7 d after flowering, whereas Lj-H70A series plants still had fresh petals at this time, because of reduced-sensitivity to ethylene. WT indicates wild type of *L. japonicus*. Line-4, line-9 and line-23 are independent transformants, while Azy-4, Azy-9 and Azy-23 are the corresponding azygous plants.

nism (Rodriguez et al. 1999). According to results of growth tests in the presence of ACC, line-23 showed the greatest reduction in ethylene sensitivity among plants of the other lines of Lj-H70A series (Fig. 2A).

Quantification of infection threads

To assess whether the transgenic *L. japonicus* with reduced ethylene sensitivity showed altered infection phenotypes, we used *Lac*Z-labeled *M. loti* to visualize the infection threads present throughout the entire root system (Fig. 3A). Because infection threads occurred even in transgenic plants, we compared the data for transgenic and wild-type plants. At 7 d after inoculation (DAI), Lj-H70A lines examined had significantly higher (P <0.05 for line-4, and P <0.01 for line-9 and line-23) numbers of infection threads than did wild-type plants (Table 1). At 14 DAI, the number of infection threads for line-23 was still higher (P <0.05) than that in wild-type plants (Table 1). In comparison, the number of infection threads did not differ significantly between wild type and azygous-23. These results indicate that the reduced-ethylene sensitivity of the transgenic plants enhances infection thread formation.

Time-course study of primordia and nodules

To evaluate the nodulation processes of transgenic plants, we used a stereoscopic microscope to observe nodule primordia (Fig. 3B, C) and mature nodules (Fig. 3D) throughout the

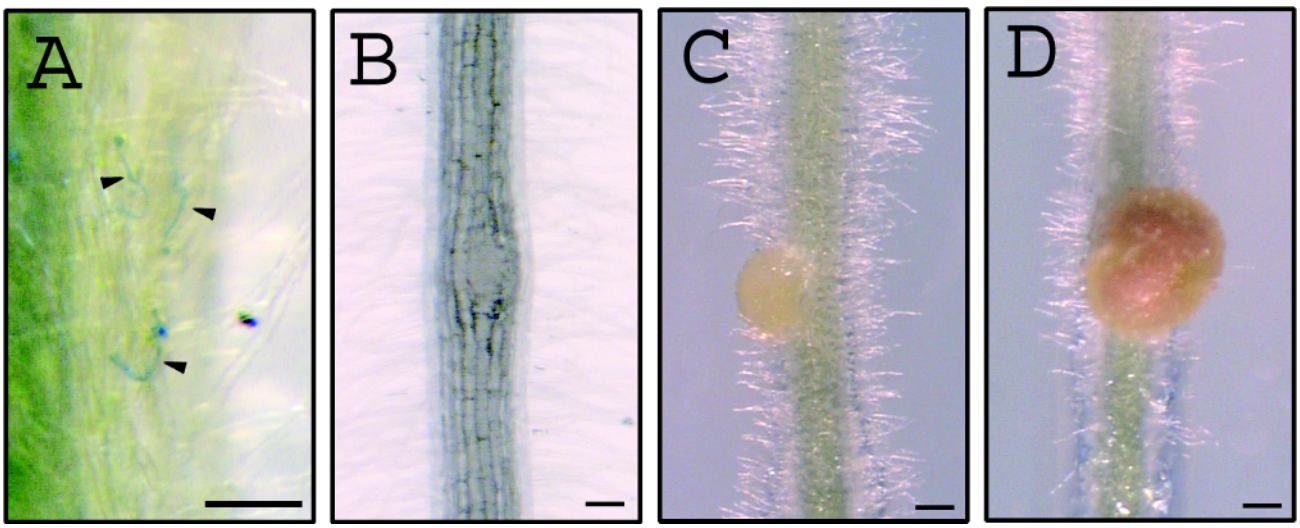

Fig. 3 Root responses of *L. japonicus* to rhizobial inoculation. (A) Infection threads (arrowheads) in root hairs at 7 DAI. Host plants were inoculated with *Lac*Z-labeled *M. loti*, and infection threads were visualized and observed stereoscopic microscopy. Nodulation events were classified as primordium (B and C) or mature nodule (D). Primordium and nodule (B, C, D) were photographed at 28 DAI. Scale bar indicates 100 μm.

entire root system of individual plants. At 3 DAI, we could discern small swellings on the root surface (Fig. 3B) due to cortical cell division triggered by rhizobial infection (Hayashi et al. 2000). Some of the swellings developed into semi-spherical transparent premature nodules (Fig. 3C), followed by the formation of opaque mature nodules (Fig. 3D). Therefore, we counted the number of swellings (Fig. 3B), premature nodules (Fig. 3C), and mature nodules (Fig. 3D) during the time-course of nodulation. We refer to the swellings (Fig. 3B) and premature nodules (Fig. 3C) together as nodule primordia.

In line-23 plants, the number of primordia was significantly higher than in wild-type plants during an early stage of the cultivation period (6–8 DAI, P <0.01) as well as in a later stage (21–28 DAI, P <0.01) (Fig. 4A). This line also showed a significant increase in the rate of mature nodulation compared with wild-type plants (6–14 DAI, P <0.01) (Fig. 4A). The kinetics of primordia and nodule formation of line-4 (Fig. 4B) were similar to those of line-23 (Fig. 4A), although initially the primordia number and nodulation rate increased relatively slowly. The difference likely depends on the degree to which ethylene sensitivity was reduced (Fig. 2A). By contrast, final levels of mature nodule numbers did not differ between the transgenic and wild-type plants (Fig. 4A, B).

Table 1 Number of infection threads in wild-type, Lj-H70A series, and azygous plant

	7 DAI		14 DAI	
	n	No. infection threads/plant	*n*	No. infection threads/plant
Wild type	14	21.0±2.4	13	46.8±3.2
Line-4	11	31.6±4.6 [b]	Not done	Not done
Line-9	14	41.9±4.7 [a]	Not done	Not done
Line-23	10	39.3±6.5 [a]	12	61.0±6.7 [b]
Azygous-23	11	25.1±4.1	13	38.0±4.9

Each value is the mean ± standard error of at least 10 plants.
[a] Significantly different (P <0.01, Student's t-test) from value for wild-type.
[b] Significantly different (P <0.05, Student's t-test) from value for wild-type.

To confirm that the primordia formation and nodulation rate increased by alteration of ethylene perception, we applied ethylene inhibitors to the root systems of inoculated wild-type *L. japonicus*. The ethylene biosynthesis inhibitor L-α-(2-aminoethoxyvinyl) glycine (AVG) inhibits ACC synthase, a key enzyme of ethylene biosynthesis (Yu et al. 1979). Silver thiosulfate (STS) inhibits ethylene perception (Veen 1983). AVG application (0.5 μM) dramatically increased the formation of primordia and nodules, whereas STS application (5 μM) enhanced the formation of primordia and nodules only during later stages of cultivation period (14–28 DAI). The results of these experiments indicate that altering ethylene perception potentially enhances the number of primordia during early and later stages of the cultivation period (Fig. 4C), which is similar to the primordia kinetics of the Lj-H70A line-23 (Fig. 4A) and line-4 (Fig. 4B). Together, these results suggest that ethylene perception in *L. japonicus* simultaneously inhibits primordia formation both the early and later stages of cultivation period.

Spatial distribution of primordia and nodules

To evaluate the location and timing of nodule formation in the entire root system of wild-type plant and Lj-H70A series, we observed spatial distribution of primordia and nodules at 28 DAI. At the time of inoculation, the position of root tip was

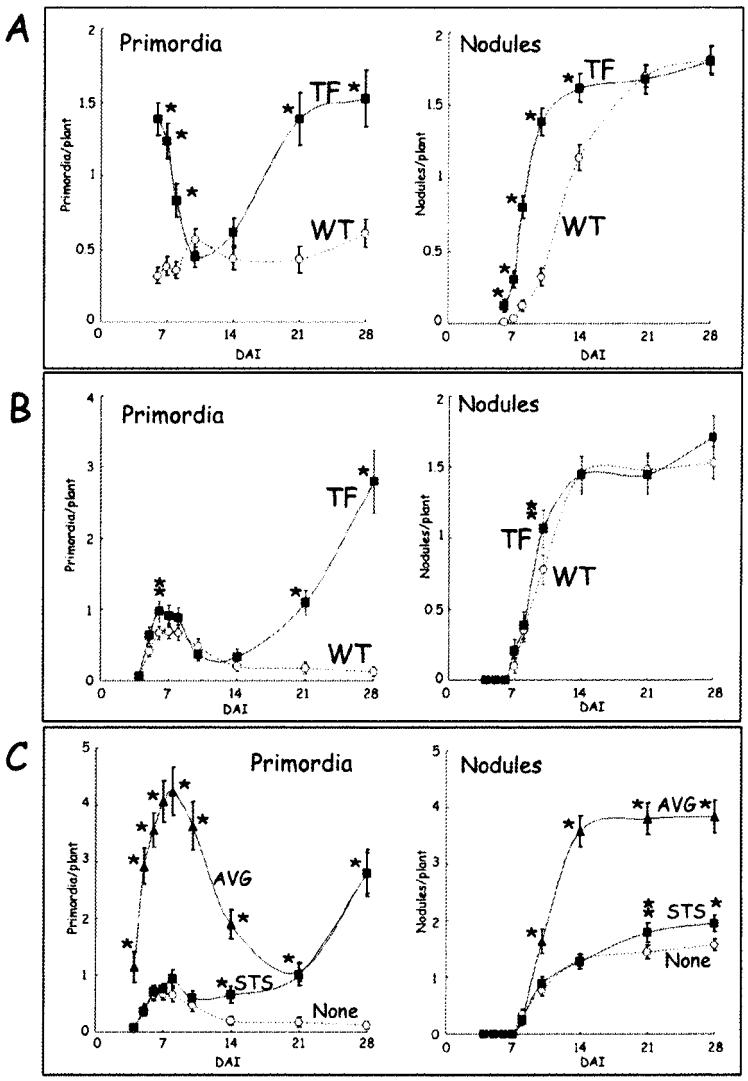

Fig. 4 Effect of a heterologous ethylene receptor gene *Cm-ERS1/H70A* and ethylene inhibitors on nodulation kinetics. (A) Wild type and line-23. (B) Wild type and line-4. (A and B), open circles indicate wild-type plants; closed squares indicate line-23 (A) or line-4 (B). (C) Application of ethylene inhibitors (AVG and STS) to wild type. Open circles indicate untreated wild-type plants; closed squares indicate application of 5 μM STS; closed triangles indicate application of 0.5 μM AVG. Error bars indicate standard errors. *, P <0.01 vs. wild type; **, P <0.05 vs. wild type.

marked (RT mark in Fig. 5). The distribution indicates a distance from the root tip mark along the root growth. Line-23 increased the number of primordia at all positions as compared with wild-type plants (Fig. 5A). Line-4 showed increased primordia formation at +0.5 cm to +4 cm based on the root tip position as compared with wild-type plants (Fig. 5B). Thus, both transgenic plants commonly had increased primordium formation at +0.5 cm to +4 cm, where secondary infection occurred along with the root growth. This increase of primordia number corresponded to the marked enhancement in primordium kinetics at 21 and 28 DAI (Fig. 4A, B). On the other hand, mature nodule distribution did not differ between wild-type and Lj-H70A series (Fig. 5A, B). These results strongly suggest that endogenous ethylene inhibits the primordium formation by secondary infection. However, the enhanced nodule primordia ought to be arrested, probably by feedback regulatory mechanisms, because of similar levels of mature nodules in transgenic and wild-type plants (Fig. 4A, B).

Expression analysis of the early nodulation genes SYMRK and NIN in the root systems of transgenic plants

Transgenic Lj-H70A series plants showed enhanced nodule primordia (Fig. 4), probably because of the increase in infection threads (Table 1). Two *L. japonicus* genes, *SYMRK* and *NIN*, have been verified to be essential for infection thread formation (Schauser et al. 1999, Stracke et al. 2002). To examine the relationship between *SYMRK* and *NIN* expression and ethylene perception, we analyzed the expression of these genes and the transgene *Cm-ERS1/H70A* under various conditions at 2 DAI by RT-PCR analysis (Fig. 6). *Cm-ERS1/H70A* was expressed exclusively in line-4 and line-23 plants (Fig. 6A), indicating the validity of introduction and expression of the transgene. *SYMRK* was consistently expressed in wild-type and transgenic plants when inoculation with rhizobium. However, *NIN* expression in the root system was induced exclusively by inoculation of *M. loti* (data not shown). *NIN* transcripts increased more in line-4 and line-23 than in the wild type or

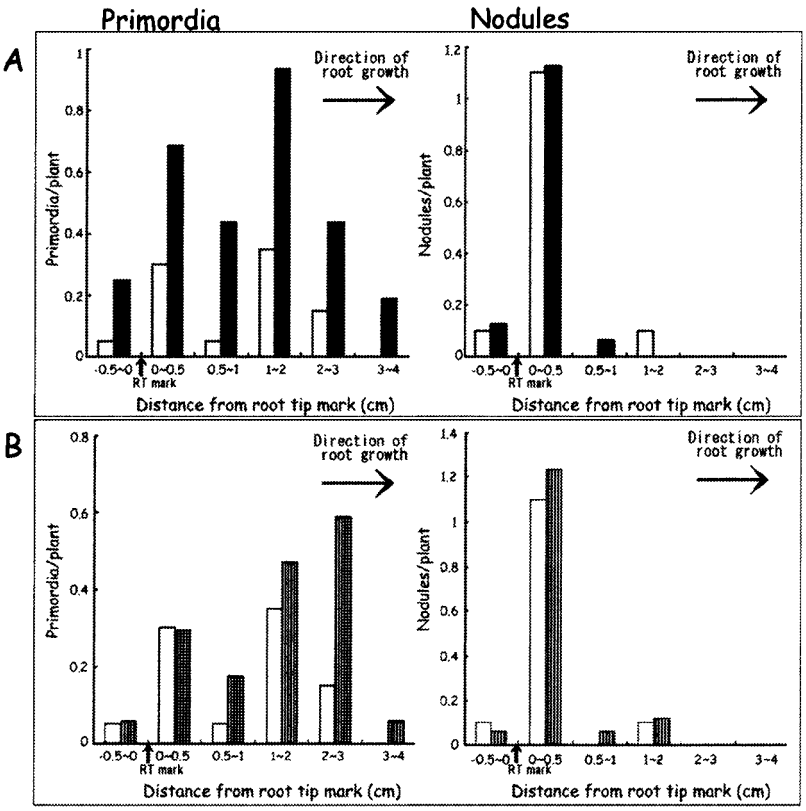

Fig. 5 Spatial distribution of primordia and nodules formed on the entire root system. Distance from the root tip mark was measured at 28 DAI. (A) Wild type (open bar) and line-23 (solid bar). (B) Wild type and line-4 (stripe bar). At the time of inoculation, the root tip was marked (RT-mark). Arrow indicates direction of root growth.

azy-4, azy-23 (Fig. 6A). Moreover, the application of AVG (0.25 and 0.5 μM) and STS (5 and 10 μM), inhibitors of ethylene biosynthesis and perception, enhanced *NIN* expression in inoculated wild-type *L. japonicus* (Fig. 6B). Therefore, it is possible that the enhancement of infection thread formation in transgenic plants (Table 1) occurred through the up-regulation of *NIN* expression.

Discussion

Transgenic *Lotus* plants expressing a modified heterologous ethylene receptor gene, *Cm-ERS1/H70A*, showed reduced ethylene sensitivity. We introduced a mutation into *Cm-ERS1* in light of information regarding *Arabidopsis etr1-1* (Rodriguez et al. 1999), which confers reduced ethylene sensitivity to heterologous plants including petunia, carnation, and tomato (Wilkinson et al. 1997, Bovy et al. 1999). Our results suggest that *Cm-ERS1/H70A* is equally useful for reducing ethylene sensitivity in heterologous *Lotus* plants. We first introduced the mutant ethylene receptor gene into legumes, and examined the role of ethylene in the establishment of symbiosis between rhizobia and legumes. Transgenic legumes with reduced ethylene sensitivity may prove to be a powerful tool for elucidating the roles of ethylene in this symbiosis.

Transgenic *Lotus* plants manifesting reduced ethylene sensitivity showed markedly enhanced infection thread formation and nodule primordial formation when inoculated with *M. loti*. This is consistent with the result reported from the ethylene-insensitive *sickle* mutant of *M. truncatula* (Penmetsa and Cook 1997). In pea (Lee and LaRue 1992), ethylene inhibits invasion of infection threads into cortical cells, but does not inhibit infection thread formation. Together, these results suggest that, in legumes, an early stage of nodule development (including infection thread formation and emergence of nodule primordia) likely is negatively regulated by ethylene signaling.

To maintain the balance of symbiosis between the host plant and rhizobia, the host plant actively controls the number of successful nodulation events on at least two different levels. As our results show, one level is a premature arrest of the rhizobial infection that involves the ethylene signaling system (Cook and Penmetsa 1997). The second is the negative feedback regulatory system by which nodules systemically inhibit subsequent nodule development in other parts of the root (autoregulation) (Caetano-Anolles and Gresshoff 1991). Ethylene signaling is considered to be deeply involved in both rhizobial infection and systemic feedback regulation. Our transgenic *Lotus* plants with reduced ethylene sensitivity showed enhanced infection thread and primary nodule formation; but, the number of mature nodules was the same as for wild-type plants, indicating that mature nodule development from nodule primordia was arrested by other (probably ethylene-independent) factors. The hypernodulation mutant *har1* shows a normal phenotype in response to ethylene application (Wopereis et al. 2000), strongly supporting the hypothesis

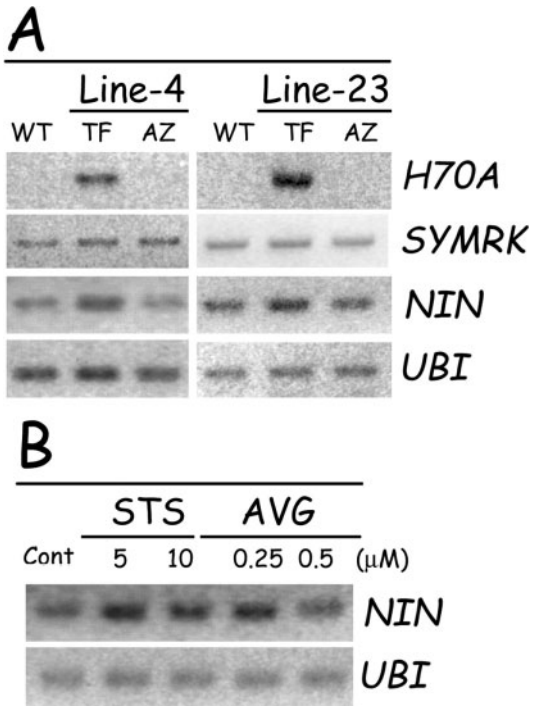

Fig. 6 RNA expression analysis of *Cm-ERS1/H70A*, *SYMRK*, and *NIN* in roots of wild-type, Lj-H70A series, and azygous plants. (A) Wild type (WT), line-4 or line-23 (TF), and azy-4 or azy-23 (AZ). (B) Wild-type plant that is untreated (Cont), treated with 5 μM STS, and treated with 0.5 μM AVG. All samples were harvested at 2 DAI with *M. loti* MAFF303099. *Polyubiquitin* (*UBI*) cDNA fragments were amplified as controls for equal amounts of cDNA template.

that mature nodule formation is regulated by an ethylene-independent mechanism. Nodule development in *L. japonicus* can be divided into two steps: an early step from infection thread formation to nodule primordia formation and a later step of nodule maturation from nodule primordia (Fig. 3). Our data show that ethylene signaling mainly accounts for primordia formation of secondary nodule formation at the later stages of cultivation period, and suggest a link between the ethylene-dependent negative regulatory system and autoregulation.

Ethylene inhibition begins at infection thread formation during nodulation (Table 1). To clarify the relationship between known nodulation genes and ethylene inhibition, we investigated the expression of *SYMRK* and *NIN* in the root system. RNA expression analysis suggested that ethylene signaling regulates *NIN* gene transcription and then affects the inception of infection thread formation. NIN is required for the formation of infection threads and initiation of primordia (Schauser et al. 1999).

In conclusion, we propose the following model for nodulation inhibition controlled by ethylene perception. When ethylene binds its receptors, the receptors cease phosphorylation to downstream in the signaling pathway, thereby initiating the ethylene response (Bleecker 1999). This signaling affects nodulation signal transduction, perhaps through its effect on *NIN* transcription (Fig. 6). Ethylene perception regulates transcription of *NIN* but not *SYMRK*; therefore, the point at which ethylene inhibits the process of nodulation may lie between root hair curling and infection thread formation (Schauser et al. 1999, Stracke et al. 2002). By controlling *NIN* transcription, ethylene perception causes inhibition of infection thread formation. Ethylene might negatively regulate independently primary nodulation and secondary nodulation, which is associated with the autoregulation system.

Materials and Methods

Plant material

L. japonicus B-129 'Gifu' was grown in an air-conditioned room as reported previously (Nukui et al. 2000). For seed collection, confirmation of germination rate, and DNA preparation, plants were grown in pots filled with a mixture of equal volumes of commercial soil (Kureha Chemical Industry, Tokyo, Japan) and vermiculite.

Bacterial strain

Plasmid pGD499 (including a *Lac*Z gene) was introduced into *M. loti* strain MAFF303099 by triparental mating (Ditta et al. 1985). The resultant strain MAFF303099 (pGD499) was cultured at 30°C in TY medium (Beringer 1974) containing 15 μg ml^{-1} of tetracycline.

Production of transgenic plants and ploidy analysis

A point mutation was introduced into melon ethylene receptor *Cm-ERS1* (Sato-Nara et al. 1999) by changing the 70th amino acid histidine to alanine, which abolishes ethylene binding, and the mutated gene was designated *Cm-ERS1/H70A* (Ezura et al. unpublished results). Transgenic *Lotus* plants were produced by using *Agrobacterium*-mediated transformation methods (Handberg and Stougaard 1992) with the neomycin phosphotransferase gene (*NPT*II) for kanamycin/G-418 resistance as a selectable marker; transgenic plants were designated as the Lj-H70A series. Putative transgenic plants were selected according to the results of specific PCR assays using primers for the selectable marker gene and *Cm-ERS/H70A* gene (described later). *L. japonicus* nuclei were isolated from the young leaves, and the nuclear DNA content was measured by using Ploidy Analyzer PA (Partec GmbH, Münster, Germany) according to the manufacturer's instructions. Diploid plants were used to confirm the presence of the transgene.

Confirmation of transgene

To confirm the presence of the transgene in Lj-H70A series plants, we performed specific PCR assays and genomic Southern hybridization. Genomic DNA was extracted from young leaves of wild-type and Lj-H70A series plants by using SDS-phenol methods followed by sodium acetate precipitation (Liu et al. 1995). Inheritance of the transgene in Lj-H70A series plants also was confirmed by segregation analysis. To amplify *Cm-ERS1/H70A* and *NPT*II gene sequences, we used the following primer sets: for *Cm-ERS1/H70A*, CaMV35S promoter –20 (5′-CGGGGGACTCTAGAGGATCC-3′) as the forward primer and Cm ERS1+588 rev (5′-GTAGACTTAGTCCATTCCGT-3′) as the reverse primer (Ezura et al. unpublished data); and for *NPT*II, NEO1 (5′-ATGATTGAACAAGATGGATTGCACGCAGGT-3′) as the forward primer and NEO2 (5′-TCAGAAGAACTCGTCAAGAAGGCGATAGAA-3′) as the reverse primer. Reactions were carried out in a GeneAmp PCR System 9700 (Perkin-Elmer, CT, U.S.A.) with ExTaq (Takara, Tokyo, Japan), starting with denaturation

at 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 7 min, according to the supplier's instructions (Takara, Tokyo, Japan). Amplified DNA fragments were subjected to electrophoresis on 1% agarose gels at 100 V for 20 min, stained with ethidium bromide, and observed under UV illumination. For Southern hybridization, genomic DNAs (50 μg each) were digested with *Hin*dIII. The restricted fragments were separated on 1% agarose gels at 20 V for 17 h and transferred onto positively charged nylon membranes (Roche Diagnostics, Mannheim, Germany). The *Cm-ERS1* gene was used as a hybridization probe and was labeled by DIG DNA labeling kit (Roche Diagnostics, Mannheim, Germany). Hybridization and washing procedures were as recommended by the manufacturer.

RNA isolation for expression analysis

At 2 DAI, total RNA was extracted from inoculated and uninoculated roots of wild-type, Lj-H70A series, and azygous plants by using the SV Total RNA Isolation System (Promega, WI, U.S.A.) according to the manufacturer's instructions. The RNA was treated with DNase I to degrade contaminating genomic DNA and was purified according to the manufacturer's instructions (Promega).

RT-PCR

Reverse transcription reactions were performed by using Omniscript reverse transcriptase (Qiagen, CA, U.S.A.) according to the manufacturer's instructions. For normalization of the concentration of the RT product for its use as a template for specific PCR, product volumes were adjusted to yield bands of equal intensity by using a DNA competitor for the *polyubiquitin* gene as an internal standard. DNA competitors were generated and target DNA (*polyubiquitin*) concentrations were confirmed by using a competitive DNA construction kit (Takara). For amplification of *Cm-H70A*, *SYMRK*, *NIN*, and *polyubiquitin* cDNA, the appropriately adjusted volume of each RT product was used as a template. Amplification was carried out using *Taq* DNA polymerase (Promega) and GeneAmp System 9700 (Perkin-Elmer) for the appropriate number of cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 50 s, according to the supplier's instructions (Takara, Tokyo, Japan). Amplification of *Cm-H70A*, *SYMRK*, and *NIN* required 24 cycles, and *polyubiquitin* needed 22 cycles. The primer sets for *Cm-ERS1/H70A* were the same as those used for amplification of genomic DNA. The primer sets FWD P60243 (5′-TGCAGTGAGATCATCCAGGCTC-3′) and REV P60244 (5′-TCTAAGTTGGTCATCTCAGCAATGCTGG-3′) were used for *SYMRK* (Stracke et al. 2002), NIN Fwd (5′-AGGGGAAAGGTGTGTCTGTG-3′) and NIN Rev (5′-CTCGCCTGACTTTCTTCCAC-3′) for *NIN*, and UBI-F (5′-CTACAACATTCAGAAGGAGTCCA-3′) and UBI-R (5′-CACAGGCCAGAAGAGGCCACAACA-3′) for *polyubiquitin* (GenBank accession number AW719307) (Stracke et al. 2002). PCR products were separated on 2% agarose S gels (Nippon Gene, Tokyo, Japan) for 30 min at 100 V and visualized with ethidium bromide.

Ethylene sensitivity test

We performed two types of ethylene sensitivity tests. First, plants were tested for resistance to the ethylene precursor ACC (Sigma-Aldrich Japan, Tokyo). Plants were grown in vitro on 0.4% Gellan gum (Wako, Osaka, Japan) plates containing Murashige–Skoog medium, 3% sucrose, and 5 μM ACC for 30 d with a 16 : 8-h day : night cycle at 25°C. Thirty d after sowing, plants were evaluated for ethylene sensitivity by observing the morphology and amount of chlorophyll degradation. Second, senescence and abscission of flowers after pollination were monitored as an indication of ethylene sensitivity. Flowers were tagged on a pedicle when anthesis initiated. Seven d after flowering, flower features were observed.

Plant culture conditions for nodulation

Conditions for surface sterilization of seed and culture were the same as in our previous report (Nukui et al. 2000). The surface-sterilized seeds were immersed in sterilized distilled water overnight at room temperature on a shaker at 100 rpm, transferred onto square plate (No. 2 square scale, Eiken Kizai, Tokyo, Japan) with B & D nitrogen-free medium (Broughton and Dilworth 1971) containing 1% agar, and germinated in the dark at 25°C for 2 d. The plates were moved to an air-conditioned container (NK System Biotron LH-300, Nippon Medical & Chemical Instruments, Osaka, Japan) with a 16 : 8-h day : night cycle, and kept there for 5 d at 25°C. The lower part of the square plate was shielded from light by using a shade to protect the root system. Seven d after sowing, plants were inoculated at 10^7 cells per plate with *M. loti* MAFF303099 or the *Lac*Z-labeled strain.

Quantification of infection threads

The number of infection threads was evaluated by bright-field stereomicroscopy, using *M. loti* MAFF303099 harboring pGD499, which includes a constitutively expressing *Lac*Z gene. At 7 and 14 DAI, the inoculated plant roots were visualized by using X-Gal (Wako) (Keating et al. 2002). The total number of visible blue infection threads was counted under a stereomicroscope. Student's *t*-test was used to assess the statistical significance of differences in nodule number from wild type. Each value is presented as the mean ± standard error (SE).

Nodulation test

Nodulation foci, primordia, and nodules were observed at 6, 7, 8, 10, 14, 21, and 28 DAI. Nodulation was counted under a stereomicroscope, and values were assessed for statistical significance, as described in the previous section. Each value is presented as the mean ± SE; $n = 81$ for wild type and 65 for line-23 (Fig. 4A), and $n = 40$ for wild type and $n = 34$ for line-4 (Fig. 4B).

Effects of STS and AVG application on nodulation and expression of mRNA

Wild-type seeds were sown onto a square plate with B & D agar media (Broughton and Dilworth 1971) containing STS (5 and 10 μM) or AVG (0.25 and 0.5 μM). Preparation of STS and AVG was as previously reported (Nukui et al. 2000). Conditions for growth, nodulation, and RNA expression analysis were as described earlier. Nodulation was observed at 3, 4, 5, 6, 7, 8, 10, 14, 21, and 28 DAI. Student's *t*-tests were done as described earlier. Each value is presented as a mean ± SE; $n = 39$ for untreated controls, 36 for STS-treated plants, and 35 for AVG-treated plants.

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