LjnsRING, a Novel RING Finger Protein, is Required for Symbiotic Interactions Between Mesorhizobium loti and Lotus japonicus

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Nodule-specific (nodulin) genes are thought to play crucial roles during establishment of the nitrogen-fixing symbiosis between legume plants and Rhizobium bacteria. On the basis of a gene expression database for early stages of the nodulation process of Lotus japonicus, previously constructed by a cDNA macroarray analysis, we identified a novel nodulin gene, LjnsRING, which encodes a protein with a typical RING-H2 finger domain that is well conserved in a number of plant E3 ubiquitin ligases. LjnsRING transcripts were almost exclusively expressed in nodules, and very low expression was detected in roots and shoots. RNA interference (RNAi) knockdown of LjnsRING by hairy root formation caused impaired root growth together with abortion of nodule formation. Examination with lacZ-labeled Mesorhizobium loti indicated that infection thread formation in the RNAi transgenic hairy roots was significantly inhibited. Analysis using a chimeric gene of LjnsRING promoter and β-glucuronidase (GUS) coding region demonstrated that LjnsRING transcription in nodules was restricted to the infected cells. These results suggest the requirement for LjnsRING in rhizobial infection and the subsequent nodule formation process.

Keywords: Lotus japonicus — Mesorhizobium loti — Nodulation — RNAi — Agrobacterium rhizogenes.

Abbreviations: d.p.i., days post-inoculation; EST, expressed sequence tag; GFP, green fluorescent protein; GUS, β-glucuronidase; ihpRNA, intron-containing hairpin RNA; IT, infection thread; ORF, open reading frame; RNAi, RNA interference; RT–PCR, reverse transcription–PCR; siRNA, small interfering RNA.

The nucleotide sequence of the LjnsRING cDNA reported in this paper has been submitted to DDBJ/EMBL/GENEBANK databases under the accession number, AB272096.

Introduction

Legume plants are able to form nitrogen-fixing nodules by symbiotic interactions with soil-borne bacteria of the genera Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium and Azorhizobium (referred to as Rhizobium hereafter). Infection and nodulation processes are triggered by specific lipochitin-oligosaccharide signal molecules, Nod factors, secreted by rhizobia in response to plant-derived flavonoid compounds. In general, the interactions start from the colonization of rhizobia on legume root hairs, followed by invasion of rhizobia into root epidermis and then cortical cells through infection threads (ITs). Concomitantly with the bacterial infection process, cell division is induced in the root cortex to form a nodule primordium, which develops into a highly organized symbiotic organ, the root nodule, in which rhizobia differentiate to bacteroids and fixation of atmospheric nitrogen takes place.

Recent progress in molecular genetic studies using two model legumes, Lotus japonicus and Medicago truncatula, has revealed a number of host plant genes that are essential for establishing symbiosis with Rhizobium bacteria. These isolated genes are mostly involved in the very early steps of symbiotic interactions that precede the initiation of bacterial infection and nodule organogenesis; they are, for instance, putative Nod factor receptors (Limpens et al. 2003, Madsen et al. 2003, Radutoiu et al. 2003) and the components of the immediate downstream signaling pathways common for symbiosis with both Rhizobium bacteria and mycorrhizal fungi (Stracke et al. 2002, Imaizumi-Anraku et al. 2005, Trichine et al. 2006). However, identification of the genes involved in bacterial infection and/or nodule organogenesis is still very limited.

Besides the identification of the host plant genes required for nitrogen-fixing symbiosis from symbiotic mutants of the model legumes by a map-based cloning strategy, functional analyses of nodule-specific genes also provide essential clues to understanding the molecular mechanisms underlying the nodulation process. It has been well documented that formation of nitrogen-fixing nodules is accompanied by transcriptional activation of a unique set of host plant genes that are almost exclusively expressed in the nodulation process. Those genes are termed ‘nodulin genes’ and have been identified from many legume species (Legocki and Verma 1980, van Kammen 1984, Kouchi and Hata 1993). Although their exact functions are mostly unknown at present, recent transgenic studies

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for an early nodulin, ENOD40, and leghemoglobin demonstrated that they play essential roles in nodule formation and/or symbiotic nitrogen fixation (Charon et al. 1999, Ott et al. 2005, Kumagai et al. 2006). In particular, an RNA interference (RNAi) technique using hairy root transformation and/or an Agrobacterium-mediated stable transformation systems for L. japonicus has been shown to be a powerful tool in the analysis of the functions of nodule-specific genes (Kumagai and Kouchi 2003, Ott et al. 2005, Kumagai et al. 2006).

Accumulation of expressed sequence tags (ESTs) from model legumes has enabled comprehensive analysis of gene expression profiles during the nodulation process (Colebatch et al. 2002, Fedorova et al. 2002, Colebatch et al. 2004). We have already performed such an analysis by means of a cDNA array composed of 18,144 non-redundant ESTs collected from various organs of L. japonicus, and identified a large number of nodule-specific genes (Kouchi et al. 2004). Using the gene expression database constructed (http://www.kazusa.or.jp/en/plant/lotus/EST/cDNA.html), we have initiated systematic analyses of functions of nodule-specific genes by means of the RNAi technique with hairy root transformation. Among them, we describe here the characterization of a novel nodulin gene, LjnsRING, which encodes a RING-H2 finger protein and is required for bacterial infection and the subsequent nodulation process.

Results

Isolation, structure and expression of LjnsRING

LjnsRING was originally identified as an EST clone, GENL061g01 (accession No. BP065629), that showed obvious nodule-specific expression by our previous cDNA macroarray analysis during early stages of nodulation (Kouchi et al. 2004, see also http://www.kazusa.or.jp/en/plant/lotus/EST/cDNA.html). Since the GENL061g01 clone did not appear to represent the entire mRNA sequence, we performed 5'- and 3'-RACE (rapid amplification of cDNA ends) procedures with RNA isolated from root nodules of L. japonicus. As a consequence, we isolated the full-length cDNA for LjnsRING of which the putative open reading frame (ORF) was 3,708 bp encoding a polypeptide of 1,236 amino acids. A database search of Lotus Genome Project (http://www.kazusa.or.jp/lotus/index.html) allowed us to identify the LjnsRING gene in TM0073 (accession No. AP004915) on chromosome 4. The LjnsRING gene comprises 14 exons in approximately 11 kb nucleotide length in the Lotus genome (data not shown).

The predicted amino acid sequence of LjnsRING protein is shown in Fig. 1. From the N-terminus, it has five HHE domains with unknown function, a CHY zinc-finger motif and a RING-H2 finger motif, both of which are presumably involved in protein–protein interactions. A public database search revealed the presence of homologs of the same domain structure with high amino acid sequence similarity in M. truncatula, Arabidopsis thaliana and Oryza sativa (Fig. 2A, B), although these putative orthologs have not yet been assigned to specific biochemical functions. The complete alignment of LjnsRING with these orthologs is given in the Supplementary figure. The RING (really interesting new gene) motif has recently been implicated in specific ubiquitination events (Freemont 2000). The RING-H2 finger motif of LjnsRING showed significant similarity with those conserved in a number of well-characterized E3 ubiquitin ligases, in particular with that of rice EL5 that is potentially involved in defense responses against pathogen attack (Takai et al. 2002) (Fig. 2C).

Genomic Southern blot analysis together with searching databases of the Lotus Genome Project and EST Index strongly suggested that LjnsRING is present in the Lotus genome as a single copy (data not shown). RNA gel blot analysis with RNAs prepared from various
tissues of *L. japonicus* revealed that the accumulation of the *LjnsRING* transcripts only occurs in nodules (Fig. 3A). Real-time reverse transcription–PCR (RT–PCR) analysis, however, indicated that the transcripts were also present in both roots and shoots at very low levels (Fig. 3B). The expression of the *LjnsRING* gene in the roots appeared to be up-regulated around 4–7 d after rhizobial inoculation.

Nodulation phenotypes of RNAi transgenic roots

To explore the possible functions of *LjnsRING* in the nodulation process, we generated *Agrobacterium rhizogenes*-mediated hairy roots transformed with RNAi constructs that form double-stranded hairpin RNAs (Wesley et al. 2001, Kumagai and Kouchi 2003). Three distinct regions about 350–480 nucleotides in length each near to the 3′ end of the *LjnsRING* mRNA, as indicated in Fig. 1 as the corresponding amino acid sequences, were used to make the constructs of intron-containing hairpin RNAs (ihpRNAs) under the control of the cauliflower mosaic virus 35S promoter. These constructs were each transferred into *Lotus* hairy roots induced by *A. rhizogenes* LBA1334. Fig. 4 shows typical nodulation phenotypes of the plants with RNAi transgenic hairy roots. Transgenic roots showed impaired growth with many short lateral roots and/or those aborted at the stage of lateral root primordia (Fig. 4B, C). The shoot growth of plants with transgenic hairy roots was also strongly retarded. Nodule formation...
on the transgenic roots was severely inhibited, although some transgenic roots formed small bumps on rare occasions. These phenotypes were common among the hairy roots transformed with the constructs of RNAi-1 to -3 (Table 1). Average numbers of fully developed nodules per root at 18 days post-inoculation (d.p.i.) were <1 for all transformants with three different RNAi constructs, whereas the control hairy roots formed >8 nodules at the same stage.

Expression of LjnsRING in transgenic hairy roots was examined by real-time RT-PCR (Fig. 5A). The level of the LjnsRING transcripts in the transgenic roots without inoculation of M. loti was 35-50% of that of the control roots, although the basal level of the expression in uninfected roots was very low (Fig. 5A-a). In contrast, the difference in the transcript levels between RNAi transgenic roots and the control hairy roots appeared to be much more significant after inoculation of M. loti (Fig. 5A-b), reflecting that LjnsRING expression is strongly dependent on nodulation. Since accumulation of small interfering RNA (siRNA) is a hallmark of RNA silencing, we performed gel blot hybridization analysis of low molecular weight RNA prepared from transgenic hairy roots using in vitro transcribed 32P-labeled cRNA probes. As shown in Fig. 5B, the presence of siRNA (21–23 nucleotides) could be confirmed in the hairy roots transformed with RNAi-2 and -3 constructs, thus showing ihpRNA-mediated silencing of LjnsRING.

Notably, supplement of exogenous nitrogen (1 mM nitrate) into the rooting medium did not completely restore the impaired growth of the plants with RNAi-transgenic hairy roots (data not shown), indicating that retarded growth of the roots and shoots was not solely due to nitrogen deficiency by poor nodulation on the transgenic hairy roots. Therefore, it is likely that LjnsRING plays roles in plant development other than those in symbiotic nodule formation, even though its expression in non-symbiotic tissues is quite low.

LjnsRING is related to the bacterial infection process

Symbiotic phenotypes of the transgenic hairy roots were further analyzed in respect to the rhizobial infection process, by using lacZ-labeled M. loti to visualize IT formation (Fig. 6A) (Tansengco et al. 2003, Kumagai et al. 2006). Although the temporal pattern of infection on hairy roots induced by A. rhizogenes fluctuated greatly as compared with normal roots, the results clearly indicate that IT formation was significantly inhibited in LjnsRING RNAi roots (Fig. 6B). Average numbers of ITs per root were 5.55 ± 1.07 (SE) for the control roots, whereas they were 0.20 ± 0.14, 1.05 ± 0.43 and 0.65 ± 0.29 for RNAi-1, -2 and -3, respectively.

To localize LjnsRING expression in the root nodules, we performed hairy root transformation with a chimeric gene of the LjnsRING promoter (approximately 4 kb upstream from the ORF) and the GUS coding sequence. Histochemical GUS staining revealed, in uninfected roots, very low GUS activity in central vascular tissues near the root tip and the vascular junction at the base of lateral

Table 1  Growth and nodulation of the hairy roots transformed with RNAi constructs for LjnsRING 18 days post-inoculation of M. loti

<table>
<thead>
<tr>
<th></th>
<th>No. of nodules</th>
<th>Root length (cm)</th>
<th>Shoot length (cm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.22 ± 0.09</td>
<td>4.80 ± 1.06</td>
<td>5.28 ± 1.18</td>
<td>9</td>
</tr>
<tr>
<td>RNAi-1</td>
<td>0.11 ± 0.333</td>
<td>2.556 ± 1.27</td>
<td>3.08 ± 0.60</td>
<td>9</td>
</tr>
<tr>
<td>RNAi-2</td>
<td>0.56 ± 1.10</td>
<td>2.056 ± 1.00</td>
<td>3.47 ± 0.50</td>
<td>9</td>
</tr>
<tr>
<td>RNAi-3</td>
<td>0.09 ± 0.30</td>
<td>2.427 ± 0.76</td>
<td>3.16 ± 1.00</td>
<td>12</td>
</tr>
</tbody>
</table>

Number of nodules and root length were measured only for GFP-positive roots.
roots (Fig. 7A, B). In developing (Fig. 7C) and mature nodules (Fig. 7D, E), strong GUS activity appeared only in the central infected zone. An image with higher magnification of the central zone demonstrates that the abundant expression of *LjnsRING* is restricted to nodule-infected cells (Fig. 7F).

The relationship of bacterial invasion and *LjnsRING* transcription was further examined by using a *nifH* disruption (Δ*nifH*) mutant of *M. loti* which completely lacks nitrogenase activity. Nitrogen fixing activity of the nodules formed by the wild-type *M. loti* Tono appeared at around 8–9 d.p.i. During early stages of nodule development (up to 11 d.p.i.), the levels of *LjnsRING* transcripts in nodules formed by the Δ*nifH* mutant were almost comparable with those in nodules formed by the wild-type *M. loti* strain, while they were reduced significantly at a later stage (16 d.p.i.) possibly by premature senescence of the ineffective nodules formed by the Δ*nifH* mutant (Fig. 8). Thus, *LjnsRING* expression appears to be tightly associated with bacterial invasion into nodule cells, and does not correlate with the onset of nitrogen fixation.

**Discussion**

Here, we report the identification, expression and functional characterization of a novel nodule-specific (nodulin) gene, *LjnsRING*, from a model legume, *L. japonicus*. RNAi knockdown of *LjnsRING* by hairy root transformation led to severely impaired root growth.
with formation of many short lateral roots and lateral root primordia together with inhibition of nodulation (Fig. 4 and Table 1). Expression of *LjnsRING* was highly nodule-specific, and the basal transcript levels in non-symbiotic tissues were very low (Fig. 3). Nevertheless, impaired root growth appears to be the direct effect of *LjnsRING* RNA silencing rather than nitrogen deficiency due to poor nodulation, because supply of exogenous nitrogen failed to restore the root growth. Although the reduction of the basal levels of *LjnsRING* transcripts in uninfected roots by RNA silencing was not so large (Fig. 5A), it is clear that impaired root growth is due to RNAi knockdown of *LjnsRING* because RNAi transformations with three distinct regions of the *LjnsRING* mRNA sequence resulted in exactly the same phenotype.

The shoot growth of the plants with transgenic hairy roots was significantly retarded (Fig. 4A), and this impaired shoot growth could not be restored by supplemental nitrogen, despite the fact that plants with transgenic hairy roots also formed a number of non-transgenic hairy roots and many healthy adventitious roots. This might be interpreted as possible transmission of siRNA formed in the hairy roots to the shoot to induce RNA silencing in the shoot (Kumagai and Kouchi 2003). However, this is unlikely because we detected neither accumulation of the siRNAs nor significant reduction of *LjnsRING* transcripts in the shoots of the plants with transgenic hairy roots (data not shown). It is likely rather that RNAi knockdown of *LjnsRING* causes serious imbalance of certain phytohormone(s) in transgenic hairy roots that causes the inhibition of shoot growth. In addition, our effort to generate stable transformants with the *LjnsRING* RNAi constructs by conventional *A. tumefaciens*-mediated hypocotyl transformation was unsuccessful because the callus formation and/or regeneration processes were strongly inhibited by the RNAi transformation, also suggesting the possible involvement of *LjnsRING* in phytohormone-related signaling.
With regard to nodulation phenotypes, RNAi knockdown of \textit{LjnsRING} results in significant inhibition of IT formation (Fig. 6). This observation suggests that poor nodulation of RNAi transgenic roots is primarily due to defects in the rhizobial infection process. Nodule formation in the transgenic hairy roots was strongly inhibited, and/or aborted at the stage of small bumps. These phenotypes resemble a number of legume symbiotic mutants with defects in IT formation and/or its growth, which show formation of small bumps instead of developed nodule structures (Kuppusamy et al. 2004, Yano et al. 2006). \textit{LjnsRING} is most abundantly expressed in mature nodules (Fig. 3A) and, therefore, must play an essential role(s) in nodule functions. However, abortion of the rhizobial infection and subsequent nodule formation processes by RNAi knockdown of \textit{LjnsRING} makes it difficult to evaluate further its role in nodule functioning. Since \textit{LjnsRING} is expressed most abundantly in nodule-infected cells (Fig. 7E, F) and is essential in the early infection process, it is tempting to hypothesize that in developing and mature nodules, \textit{LjnsRING} functions in respect to perception and/or persistence of endosymbiotic bacteria in the host cell cytoplasm. Besides the abundant expression of \textit{LjnsRING} in nodule-infected cells, it is expressed weakly in central vascular bundles near the root tip as well as in the vascular junction at the base of lateral roots, as indicated by the promoter::GUS experiments (Fig. 7A, B). The region of \textit{LjnsRING} expression near the root tip is just the zone of growing root hairs where rhizobial infection mostly occurs. This may also be interpreted as indicating possible involvement of \textit{LjnsRING} in the infection process. \textit{LjnsRING} protein has a RING-H2 zinc finger motif (Figs. 1, 2C). The RING finger domain occurs in a wide variety of proteins with diverse biological functions, but its involvement in ubiquitin-mediated proteolysis has been most intensely studied in recent years. In particular, a number of E3 ubiquitin ligases have been shown to have a RING-H2 finger motif as the domain for specific interaction with the E2 ubiquitin-conjugating enzyme (ubiquitin carrier protein) (Freedman 2000). E3 ubiquitin ligase transfers the activated ubiquitin from E2 to the specific target protein and then the polyubiquitinated target protein is destined for degradation by a protease complex, proteasome. Targeted proteolysis mediated by the ubiquitin/proteasome system is a common mechanism to regulate protein functions in eukaryotic cells, and has been shown to play crucial roles in many vital biological processes in plants, such as regulation of cell cycle and organ development, phytohormone-related signaling, defense responses and photomorphogenesis. E3 ubiquitin ligase plays a central role to determine the specificity to target proteins. The structure of the RING-H2 domain in \textit{LjnsRING} is highly homologous to those found in E3 ubiquitin ligases from plants, and consensus cysteine and histidine residues are perfectly conserved (Fig. 2C). Therefore, it is most likely that \textit{LjnsRING} is a nodule-specific E3 ubiquitin ligase. A CHY zinc finger domain located just upstream of the RING-H2 motif may serve to interact with substrate protein(s). It has been shown that in rice EL5, a tryptophan residue (indicated by blue color in Fig. 2C) located after the third metal-chelating motif is essential for binding to the corresponding E2 protein, OsUBC5b, thus being crucial for the E3 ubiquitin ligase activity of EL5 (Katoh et al. 2005). The residue at this position is substituted by tyrosine in \textit{LjnsRING} as well as in its rice and Arabidopsis orthologs, BAD82554 and ABE78891, respectively. Therefore, \textit{LjnsRING} family proteins may interact with E2 protein(s) other than UBC5b. There have been two reports so far about possible involvement of RING proteins in the legume–\textit{Rhizobium} symbiosis (Nishimura et al. 2002, Karlowski and Hirsch 2003). These previously identified genes, however, do not show nodule-specific expression, and it is not likely that they are directly involved in the nodulation process. It is noteworthy that the critical importance of protein degradation by the ubiquitin/proteasome system has recently been proposed in respect to cell cycle arrest and endoreduplication in nodule-infected cells (Kondorosi et al. 2004). Our attempt to detect polyubiquitination activity in vitro by a translational fusion with maltose-binding protein (MBP) in combination with wheat germ lyase as a source of E1 and E2 proteins (Takai et al. 2003) has been unsuccessful so far. However, it will be intriguing to confirm E3 ubiquitin ligase activity of \textit{LjnsRING} and to find proteins that interact with the RING-H2 and CHY Zn-finger domains.

**Materials and Methods**

**Plasmid construction**

The gene constructs were made according to standard DNA manipulation protocols (Sambrook and Russell 2001). Binary vectors for hairy root transformation, pC1300GFP and pC1301GFP, were made from pCAMBIA1300 and pCAMBIA1301 (CAMBIA, Canberra, Australia), respectively, by replacing the hygromycin-resistant gene with green fluorescent protein [gGFP (S65T)] (Niwa et al. 1999, Kumagai and Kouchi, 2003). Three distinct regions of the \textit{LjnsRING} coding sequence were amplified by PCR from an EST clone GENL1061g10 (accession No. BP665629) with the primer sets shown in Table 2. The amplified cDNA fragments were 484, 387 and 343 bp in length for RNAi-1, -2 and -3, respectively. The amplification products were digested with \textit{XhoI} /\textit{KpnI} and with \textit{BamHI} /\textit{ClaI}, and ligated into pHANNIBAL plasmid vector (Wesley et al. 2001), in which the sense and antisense RNA sequences were located in tandem with a pyruvate dehydrogenase kinase (pdh) intron between them, and this ihpRNA construct was placed behind the cauliflower mosaic virus 35S promoter. Then the entire RNAi
Hairy root transformation

Hairy root transformation of *L. japonicus* ‘Gifu’ using *A. rhizogenes* LBA1334 was performed according to a procedure described previously (Kumagai and Kouchi 2003). In brief, the seedlings (5–6 d old) were placed in a Petri dish and then cut at the base of the hypocotyl. The seedlings were transferred onto the HRE agar medium containing 150 μM cefotaxime and grown for 10 more days. Hairy roots that emerged from the base of the hypocotyls were tested for GFP fluorescence. The resultant binary vector plasmids were transferred into *A. rhizogenes* LBA1334 by direct transfer (Kumagai and Kouchi 2003).

**Table 2** Primer sets used in this study

<table>
<thead>
<tr>
<th>Transformation</th>
<th>RNAi-1</th>
<th>ATGGATCCTCGAGCAGAGTATGTC</th>
<th>Forward</th>
<th>ATGGATCCTCGAGCAGAGTATGTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAi-2</td>
<td>ATGGATCCTCGAGCATCTGTA</td>
<td>GTCTGTAG</td>
<td>Reverse</td>
<td>CTGAT</td>
</tr>
<tr>
<td>RNAi-3</td>
<td>ATGGATCCTCGAGCAAGCCCTTG</td>
<td>GTACAGAC</td>
<td></td>
<td>ATATCGATGGTACCTTGCCACAACA</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>Promoter assay</td>
<td>TGGTGATTTTGTGGCTTATT</td>
<td></td>
<td>GCGTCGCCATGGGCTTTAT</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>TTACCTTTGTGCTCCTTCCTTC</td>
<td>ACAAACAGCACACACACACAGCCAATCC</td>
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<td></td>
</tr>
<tr>
<td>LjnsRING</td>
<td>TCTTGGGACTGTCCCTTCTTGC</td>
<td>TGGTCTGGTTCCTCACGTC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Detection of small interfering (si) RNA**

Procedures for detection of siRNA were exactly the same as described previously (Kumagai et al. 2006). In brief, total RNAs were isolated from transgenic hairy roots using Trizol (Invitrogen), and low-molecular weight RNAs were enriched by eliminating high-molecular weight RNAs by PEG (polyethylene glycol) precipitation. After electrophoresis of low-molecular weight RNAs on a 15% denaturing polyacrylamide gel with 7 M urea, they were electro-blotted onto a nylon membrane (Hybond NX, Amersham). The membranes were hybridized with partially hydrolyzed 52P-labeled antisense RNA probes in vitro transcribed from the *LjnsRING* cDNA fragments which were used for construction of the RNAi plasmids. Hybridization and washing conditions were as described previously (Kumagai et al. 2006).

**DNA sequencing**

Isolated cDNA and PCR amplification products were sequenced by the dideoxy chain termination method using an automated DNA sequencer (Model 3700; Applied Biosystems, CA, USA). Both strands were sequenced entirely.

**Histochemical GUS staining**

Hairy roots transformed with the *LjnsRING* promoter::GUS construct were immersed in a staining solution (2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 100 mM sodium phosphate, pH 7.0) and were put under a vacuum for a few minutes, followed by incubation overnight at 25°C in the dark. Root nodules were excised from transgenic hairy roots, embedded in 5% agar and sectioned at 80–100 μm thickness using a microlicer (DTK-1000, Dohan EM, Osaka, Japan). The sections were placed on a slide and stained for 10–16 h in the staining solution. The stained materials were observed with a dissection binocular or ordinary light microscope.
Analysis of infection thread formation

Hairy roots were inoculated with *M. loti* strain MAFF303099 that harbored the β-galactosidase (*lacZ*) gene as a constitutive marker (Tansengco et al. 2003), and grown for 7 d in a vermiculite pot. Transgenic hairy roots were selected by GFP fluorescence, fixed in 2.0% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.2) for 2 h at room temperature, and then stained in 0.8 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and 2.5 mM each of potassium ferrocyanide and potassium ferricyanide in the same buffer for 16 h at 37°C. After washing in 0.1M Tris–HCl buffer, the root tissue was clarified in chloral hydrate (8 g) dissolved in 1 ml of glycerol and 2 ml of water, and observed under a light microscope.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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