A Positive Regulatory Role for *LjERF1* in the Nodulation Process Is Revealed by Systematic Analysis of Nodule-Associated Transcription Factors of *Lotus japonicus*[1][W]

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We have used reverse genetics to identify genes involved in legume-rhizobium symbiosis in *Lotus japonicus*. We obtained the sequences of 20 putative transcription factors from previously reported large-scale transcriptome data. The transcription factors were classified according to their DNA binding domains and patterns of expression during the nodulation process. We identified two homologues of *Medicago truncatula MtHAP2-1*, which encodes a CCAAT-binding protein and has been shown to play a role in nodulation. The functions of the remaining genes in the nodulation process have not been reported. Seven genes were found to encode proteins with AP2-EREBP domains, six of which were similar to proteins that have been implicated in ethylene and/or jasmonic acid signal transduction and defense gene regulation in Arabidopsis (*Arabidopsis thaliana*). We identified a gene, *LjERF1*, that is most similar to Arabidopsis *ERF1*, which is up-regulated by ethylene and jasmonic acid and activates downstream defense genes. *LjERF1* showed the same pattern of up-regulation in roots as Arabidopsis *ERF1*. The nodulation phenotype of roots that overexpressed *LjERF1* or inhibited *LjERF1* expression using an RNA interference construct indicated that this gene functions as a positive regulator of nodulation. We propose that *LjERF1* functions as a key regulator of successful infection of *L. japonicus* by *Mesorhizobium loti*.

Plants are sessile organisms and cannot respond to environmental insults, such as heat, drought, flooding, or lack of light, by moving their location. They are also constantly exposed to potentially damaging organisms, including bacteria, fungi, and insects. Plants have evolved highly developed systems to adjust to their surrounding conditions. These so-called stress responses and resistance programs are initially regulated at the level of gene transcription by a host of specific transcription factors.

The sequencing of the genome of the model legume *Lotus japonicus* is nearly complete (Sato et al., 2008). Primary annotation has assigned 30,000 genes in the draft genomic sequence, including approximately 1,500 genes that encode putative transcription factors. This number represents approximately 5% of all annotated genes in the *L. japonicus* genome.

Leguminous plants have developed a unique mechanism of nitrogen fixation that involves a symbiotic relationship with soil bacteria known as rhizobia. The ability to sustain this symbiotic relationship is restricted to leguminous species, with the exception of the nonlegume *Parasponia* genus. Successful bacterial infection is established between host plant and rhizobia through a complex set of signals, resulting in the formation of symbiotic nodules in the root of the host plant in which nitrogen fixation is carried out by the rhizobium.

To date, a small number of host transcription factors have been characterized in terms of their function in the nodulation process. *NIN* was originally identified in an analysis of a transposon-tagged mutant of *L. japonicus* that is unable to form nodules (Schauser et al., 1999). The expression of *NIN* is strictly dependent on infection by *Mesorhizobium loti* or exogenous purified nodulation (Nod) factors, and it has been postulated that *NIN* is required for rhizobial invasion through root hairs. GRAS family proteins that are essential for Nod factor-mediated signal transduction have been isolated from both *Medicago truncatula* and *L. japonicus* (Kaló et al., 2005; Murakami et al., 2006). *M. truncatula MtHAP2-1* is a CCAAT-binding transcription factor whose expression is regulated by microRNA169. It was recently identified as a key regulator of symbiotic nodule development (Combier et al.,

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2006). Recently, an AP2-EREBP transcription factor, *MtERN1* (for ERF required for nodulation), was found to be essential for Nod factor-mediated signal transduction (Middleton et al., 2007). Two additional AP2-EREBP genes, *MtERN2* and *MtERN3*, were identified as trans-acting factors that regulate the expression of an early nodulin gene, *ENOD11*, through a Nod factor-responsive cis-element in the promoter region (Andriankaja et al., 2007).

Transcriptome analysis of *M. truncatula* using cDNA microarrays has identified a number of transcription factors whose expression changes early in the nodulation process (El Yahyaoui et al., 2004; Lohar et al., 2006). Previously, we used two approaches, cDNA macroarray analysis and serial analysis of gene expression, to identify nodulation-related genes in *L. japonicus* (Kouchi et al., 2004; Asamizu et al., 2005). We identified 20 transcription factors whose expression is up-regulated during nodulation. In this study, we have characterized the majority of these putative nodulation-associated transcription factors, with particular emphasis on members of the AP2-EREBP family of transcription factors, which appear to be the most abundant proteins in this group. We also carried out a functional analysis of one of the transcription factors, *LjERF1*, to determine its role in the nodulation process in more detail.

RESULTS

Classification of Transcription Factor Genes

Twenty putative nodulation-associated transcription factor genes (Kouchi et al., 2004; Asamizu et al., 2005) were classified according to their DNA-binding domains (Davuluri et al., 2003; Table I). Seven of the genes encoded proteins that are members of the AP2-EREBP family of transcription factors. The remainder encoded members of the CCAAT (two genes), bZIP (two genes), C2H2 (two genes), Homeobox (one gene), NAC (one gene), WRKY (one gene), C3H (one gene), MADS (one gene), C2C2-Dof (one gene), and CPP (one gene) families of transcription factors. These results were consistent with previous transcriptome analyses of early symbiotic events in *M. truncatula* and *Sinorhizobium meliloti*, which also identified transcription factors of the AP2-EREBP, CCAAT, NAC, WRKY, and MADS families of proteins (El Yahyaoui et al., 2004; Lohar et al., 2006). *M. truncatula MtHAP2-1*, which encodes a CCAAT-binding transcription factor and is regulated by microRNA169, has been shown to be essential for the differentiation of nodule cells (Combier et al., 2006). The two CCAAT-binding transcription factors of *L. japonicus* that emerged from our analysis, *CBF-A01* and *CBF-A22*, exhibited 76% and 70% amino acid sequence identity, respectively, with *MtHAP2-1*.

Table I. *Relative expression levels compared with uninfected root after M. loti infection*

The expression of each gene was normalized using an internal control (AV772463; Supplemental Table S1). ND, Not determined.

Gene Name	Accession No.	Transcription Factor Family	3 h	24 h	2N	4N	7N	12N
Group I (initial response)								
LjERF1	AB378626	AP2-EREBP	1.8	1.7	1.2	0.1	0.4	0.5
HDZ-M48	AB378627	Homeobox	1.8	1	0.7	0.1	0.1	0.1
CBF-A22	AB378628	CCAAT	1.6	1.3	1.1	0.3	0.5	0.2
bZIP-R91	AB378629	bZIP	1.9	1.1	0.9	0.2	0.3	0.9
LjRAP2.4	AB378630	AP2-EREBP	1.9	1.1	1.4	0.6	1.6	0.5
ZF-M39	AB378631	C2H2	1.8	1.1	0.8	1	1.2	0.8
LjERF2	AB378632	AP2-EREBP	1.7	1.3	0.6	0.8	1.4	1.6
Group II (induced at 2N)								
CBF-A01	AB378633	CCAAT	ND	ND	14.3	6.2	14.7	12.2
LjERF16	AB378634	AP2-EREBP	ND	ND	5.1	5.8	3.7	2.9
ZF-G96	AB378635	C2H2	ND	ND	2.7	3.1	1.5	0.7
LjERF18	AB378636	AP2-EREBP	ND	ND	1.6	15.4	25.2	11
LjWRKY30	AB378640	WRKY	ND	ND	1.8	1	2.7	1.8
Group III (induced at 4N)								
LjERF17	AB378637	AP2-EREBP	ND	ND	0.4	9.4	11.5	4.9
LjERFI9	AB378638	AP2-EREBP	ND	ND	1.2	1.7	3.1	4.7
NAM-A43	AB378639	NAC	ND	ND	1.3	1.8	2	2.3
Group IV (induced at 12N)								
RING-G83	AB378641	C3H	ND	ND	0.4	0.2	0.9	9.9
MADS-A18	AB378642	MADS	ND	ND	0.4	0.1	0.2	8
bZ1P-M43	AB378643	bZIP	ND	ND	1.1	0.2	0.5	2.1
Not clear								
Dof-M153	AB378644	C2C2-Dof	1.1	0.8	0.4	0.1	0.1	0.3
CPP-L56	AB378645	CPP	1.1	0.7	0.6	0.3	0.4	0.4

Expression Analysis of Transcription Factor Genes in Response to Inoculation with *M. loti*

To determine the expression profiles of transcription factor genes during the nodulation process, we used quantitative-reverse transcription (RT-Q)-PCR to examine uninfected roots, infected roots at 2 and 4 d after inoculation (DAI) with *M. loti*, nodule primordia collected at 7 DAI, and mature nodules at 12 DAI. Genes for which induced expression was not clearly detected were examined by RT-Q-PCR at 3 and 24 h after *M. loti* inoculation. The transcription factor genes were classified according to a time course of induction (Table I). Seven transcription factor genes (*LjERF1*, *HDZ-M48*, *CBF-A22*, *bZIP-R91*, *LjRAP2.4*, *ZF-M39*, and *LjERF2*) were classified as group I and were induced as early as 3 h after inoculation. The expression of four of the early induced genes of group I was repressed at a later stage of nodulation (4 DAI). Group II consisted of five transcription factor genes (*CBF-A01*, *LjERF16*, *ZF-G96*, *LjERF18*, and *LjWRKY30*) that were induced at 2 DAI, although their expression pattern in earlier stages was not examined. Persistently induced expression throughout the nodulation process (2–12 DAI) was observed for *CBF-A01*, *LjERF16*, and *LjERF18*. Group III included three transcription factor genes (*LjERF17*, *LjERF19*, and *NAM-A43*) that were induced at 4 DAI. Group IV included three transcription factor genes (*RING-G83*, *MADS-A18*, and *bZIP-M43*) whose expression was induced at 12 DAI. We were unable to detect the induced expression of two genes, *Dof-M154* and *CPP-L56*, at any time point examined.

Phylogenetic Analysis of Nodulation-Induced AP2-EREBP Family Genes

Members of the AP2-EREBP family of transcription factors were the most abundant in our analysis, and their expression was induced at various time points during nodulation (Table I). These results suggested that they may play important roles in the nodulation process. To date, three AP2-EREBP genes have been shown to be essential in the nodulation process in *M. truncatula*. *ERN1* was isolated as the causative gene in a nodulation mutant and has been identified as a component of the Nod factor signal transduction pathway downstream of *DMI3* (Middleton et al., 2007). Recently, two additional genes, *ERN2* and *ERN3*, were identified as trans-acting factors that interact with the Nod factor-responsive cis-element in the promoter region of *ENOD11* (Andriankaja et al., 2007). *ERN1* to *ERN3* encode AP2-EREBP domain proteins and are highly homologous to Arabidopsis (*Arabidopsis thaliana*) *RAP2.11*, a gene of unknown function that was classified as a group V gene in a detailed genome-wide classification of Arabidopsis and *Oryza sativa* AP2-EREBP domain proteins (Nakano et al., 2006).

To determine the relationship of the AP2-EREBP genes of *L. japonicus* to their homologues in Arabidopsis, we carried out a phylogenetic analysis using the ClustalW server of the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/; Fig. 1). *LjRAP2.4* showed the highest level of similarity to Arabidopsis *RAP2.4*, which belongs to group I in the classification scheme of Nakano et al. (2006). The remaining six *L. japonicus*

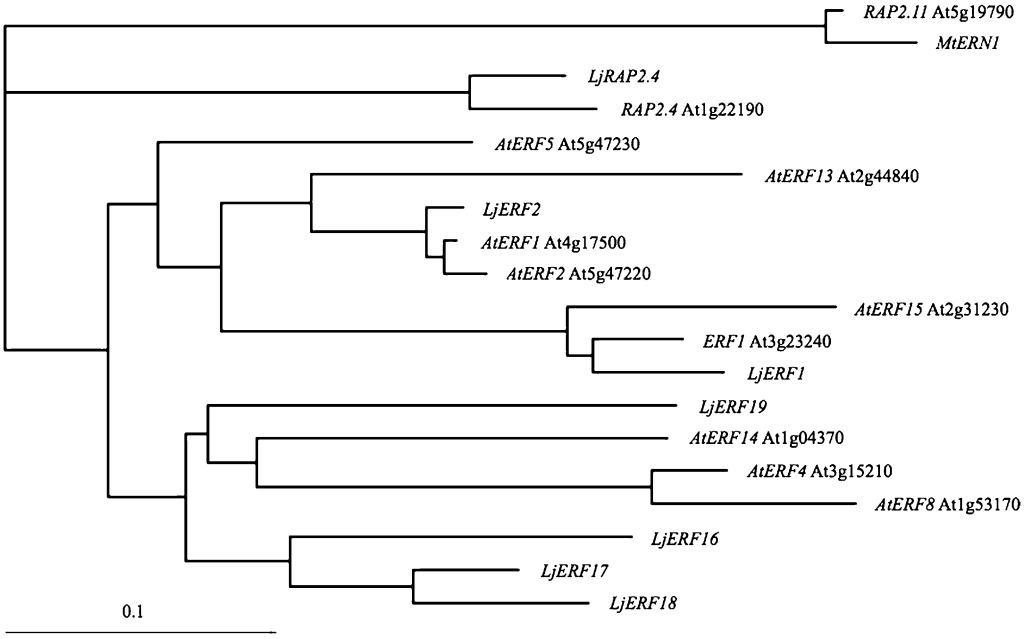

Figure 1. Phylogenetic tree of nodulation-associated *L. japonicus* AP2-EREBP genes. Phylogenetic analysis was carried out using the sequences of the AP2-EREBP domains of the *L. japonicus* (Lj) genes and their Arabidopsis (At) homologues. Locus identifiers (such as At1g22190) are shown for the Arabidopsis genes. Arabidopsis *RAP2.11* and *M. truncatula MtERN1* were included as an outgroup.

genes were classified into groups VIII and IX. *LjERF1* was closest to Arabidopsis *ERF1*. *LjERF2* was classified in a different clade that included *AtERF1* and *AtERF2*. The remaining four genes were relatively divergent in the legume lineage. *LjERF16*, *LjERF17*, and *LjERF18* were similar to each other and classified in a clade that included *AtERF4*, *AtERF8*, and *AtERF14* as well as *LjERF19*. These results indicated that the analyzed *L. japonicus* AP2-EREBP transcription factors are phylogenetically distinct from *M. truncatula ERN1* to *ERN3*.

Response to Ethylene and Jasmonic Acid of AP2-EREBP Family Genes

Previous studies have shown that ethylene and jasmonic acid (JA) are involved in the nodulation process (Penmetsa and Cook, 1997; Oldroyd et al., 2001; Nakagawa and Kawaguchi, 2006; Sun et al., 2006). Furthermore, Arabidopsis AP2-EREBP genes in groups VIII and IX have often been implicated in ethylene and/or JA signaling (Nakano et al., 2006). When we examined the effect of ethylene and JA on the expression of *L. japonicus* AP2-EREBP genes, we found that they fell into one of two groups: ubiquitously induced genes (induced in both shoots and roots) and root-specific genes (Fig. 2). One exception was *LjERF16*, which showed a complex pattern of down-regulation in response to the plant growth regulators. *LjERF2*, *LjRAP2.4*, and *LjERF18* were induced in both shoots and root. The induction of *LjERF2* and *LjERF18* was observed in response to both ethylene and JA, and *LjERF18* induction was higher in shoots than in root. The induction of *LjRAP2.4* by ethylene and JA was observed in the root, and a synergistic effect was observed in shoots and root. A significant level of induction of *LjERF1*, *LjERF19*, and *LjERF17* was observed only in the root. *LjERF17* was preferentially induced by JA. A synergistic effect of ethylene and JA was observed for *LjERF1* and *LjERF19*. *LjERF1* exhibited synergistic up-regulation in response to ethylene and JA in a root-specific manner.

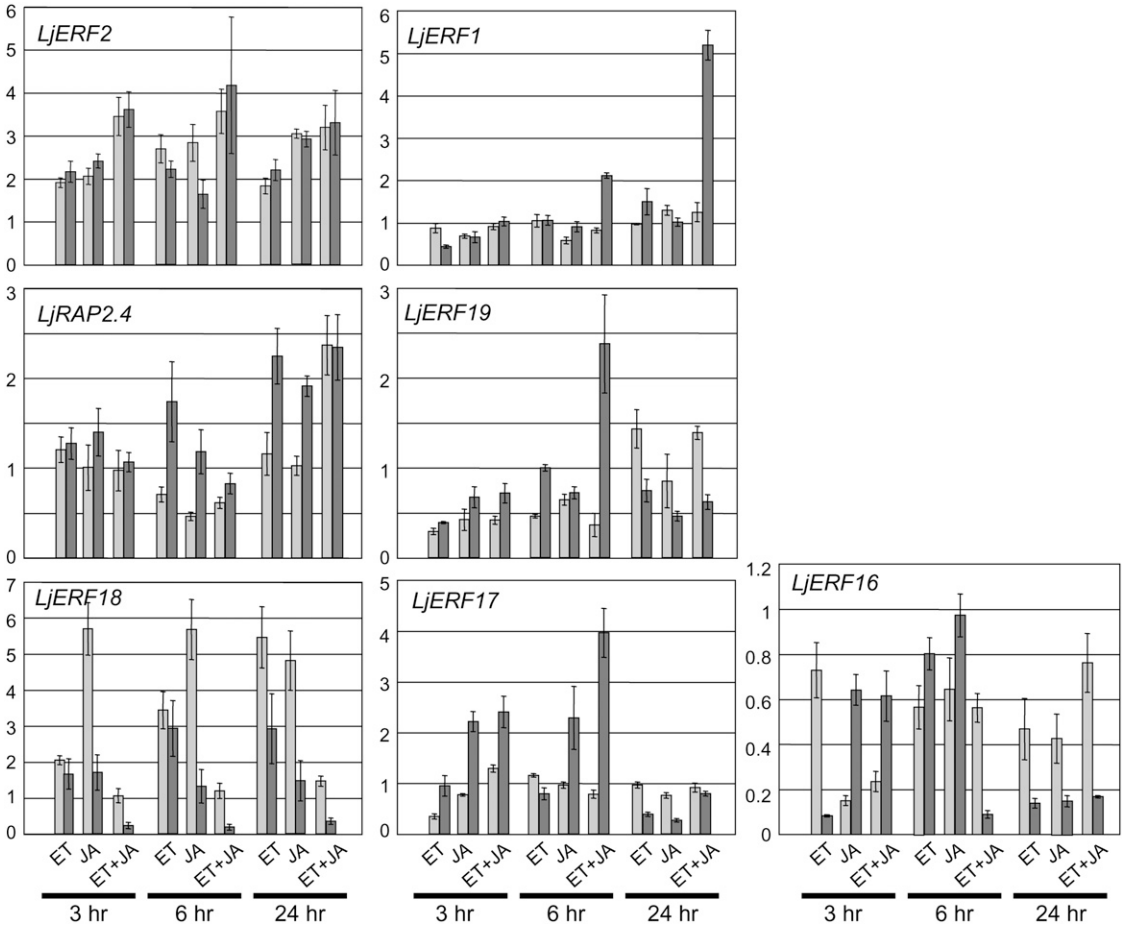

Figure 2. Effects of ethylene (ET) and/or JA on the expression of AP2-EREBP genes in shoots and root. The expression levels of the indicated *L. japonicus* AP2-EREBP genes in shoots (light gray) and root (dark gray) are expressed relative to controls. Controls are either nontreated shoots or root. Data represent averages ± SE of three independent experiments. The *y* axis in each bar graph indicates the relative transcript level.

Effect of the *LjERF1* Overexpression on Nodulation

We chose to analyze the function of *LjERF1* in early nodulation in more detail for several reasons: phylogenetic analysis indicated that *LjERF1* is highly similar to Arabidopsis *ERF1* (Lorenzo et al., 2003); the expression of *LjERF1* was up-regulated very early (3–24 h) after *M. loti* inoculation (Table I); and *LjERF1* exhibited synergistic induction by ethylene and JA. We first examined the effect of the overexpression of *LjERF1* on the nodulation process. We transformed *L. japonicus* hairy roots with an *LjERF1*-overexpressing vector, as described in "Materials and Methods." *LjERF1* expression in transformed hairy roots was 8.9-fold higher than in control roots that were transformed with an empty vector. Roots were then inoculated with *M. loti* MAFF303099, and the number of nodules formed on the roots was counted at 3 weeks after infection. The average number of nodules formed on transformed, *LjERF1*-overexpressing hairy roots was 11.1, while that on control roots was 6.5. When we analyzed the data using Student's *t* test, we found that this difference was statistically significant ($P < 0.01$; Table II).

Effect of Suppression of *LjERF1* Expression on Nodulation

We also examined the effect of suppression of *LjERF1* expression on nodulation using RNA interference (RNAi). We transformed *L. japonicus* hairy roots with two *LjERF1*-specific RNAi constructs (Fig. 3). Suppression of *LjERF1* expression by RNAi was confirmed using real-time RT-PCR by amplifying a region of the *LjERF1* mRNA that was not used in the RNAi constructs. *LjERF1* mRNA was down-regulated approximately 60% in transformed hairy roots compared with control roots. The phenotype of transformed hairy roots was observed at 3 weeks after inoculation with *M. loti* MAFF303099. As shown in Figure 3, the growth of transformed plants was severely retarded compared with that of control plants. The average number of nodules formed on control hairy roots was 3.3, while that on the two RNAi-transformed plants, RNAi-1 and RNAi-2, was 0.8 and 0.9, respectively, and this difference was statistically significant ($P < 0.01$ and $P < 0.02$, respectively; Table II). To determine whether the growth suppression phenotype of RNAi-transformed plants was due to the availability of nitrogen, plants were grown in the presence of 1 mM nitrate (Fig. 3). RNAi-transformed plants grew normally when exogenous nitrogen was supplied, which indicated that the growth retardation of RNAi plants is due to nitrogen deficiency resulting from poor nodulation.

Table II. *Results of t test of mean numbers of nodules formed on control and LjERF1-overexpressed or RNAi hairy roots*

Plant	Control	*LjERF1* Overexpression/ RNAi	Statistical Significance[a]
Overexpressor	6.5 ± 0.7	11.1 ± 1.3	0.01
RNAi-1	3.3 ± 0.8	0.8 ± 0.5	0.01
RNAi-2	3.3 ± 0.8	0.9 ± 0.6	0.02

[a]Statistical significance level of observed difference in nodule number calculated by Student's *t* test.

Spatial Expression of *LjERF1*

To investigate the spatial response of *LjERF1* to *M. loti* infection, we transformed hairy roots with a chimeric gene composed of the *LjERF1* promoter (1,000 bp upstream from the open reading frame) and the GUS coding sequence. Histochemical GUS staining at 3 h after the *M. loti* inoculation revealed GUS activity in an epidermal region 2 to 3 mm above the root tip, which corresponded to the infection zone (Fig. 4). This time point was consistent with the results of the RT-Q-PCR analysis, in which highest induction of *LjERF1* was observed (Table I). No GUS-stained roots were observed at 24 h after inoculation or at later time points. We also performed a GUS assay using the entire hairy roots at 10 d after *M. loti* inoculation. No GUS staining was observed in the nodule primordia or mature nodules of these roots (Fig. 4), which is also consistent with the RT-Q-PCR results. GUS staining was detected in epidermal and cortical cells, where no visible morphological changes for nodule formation were observed (Fig. 4).

Expression of *PR10-1* during Normal Nodulation and in *LjERF1*-Overexpressing or *LjERF1* RNAi Plants

Overexpression of *LjERF1* increased the number of nodules formed on hairy roots, and RNAi resulted in the inhibition of nodulation. These results were somewhat surprising, because it was previously reported that Arabidopsis *ERF1* is involved in the activation of ethylene/JA-dependent pathogen defense genes (Lorenzo et al., 2003). It is generally believed that in order for legume-*Rhizobium* symbiosis to be established, plant resistance mechanisms must be temporarily or locally suppressed (Mithöfer, 2002). The precise mechanisms of suppression of defense responses remain to be elucidated. To determine whether *LjERF1* was involved in the regulation of plant defense mechanisms, we examined the expression of *LjPR10-1* in wild-type, *LjERF1*-overexpressing, and *LjERF1* RNAi plants. *M. truncatula MtPR10-1* expression is induced by pathogens (Gamas et al., 1998), and *LjPR10-1* was shown to be induced during the establishment of *L. japonicus*-microorganism symbiosis (Kouchi et al., 2004; Deguchi et al., 2007). We first examined the expression of *LjPR10-1* in response to *M. loti* infection in wild-type plants by RT-Q-PCR (Fig. 5). The expression of *LjPR10-1* was induced at 3 h after *M. loti* infection and decreased after 24 h. To confirm the specificity of the response, we infected plants with two symbiosis-defective *M. loti* mutant strains, *nodAC*$^-$ and *nodD*$^-$. The specificity of *LjPR10-1* induction in response to a compatible rhizobium was also confirmed by examining

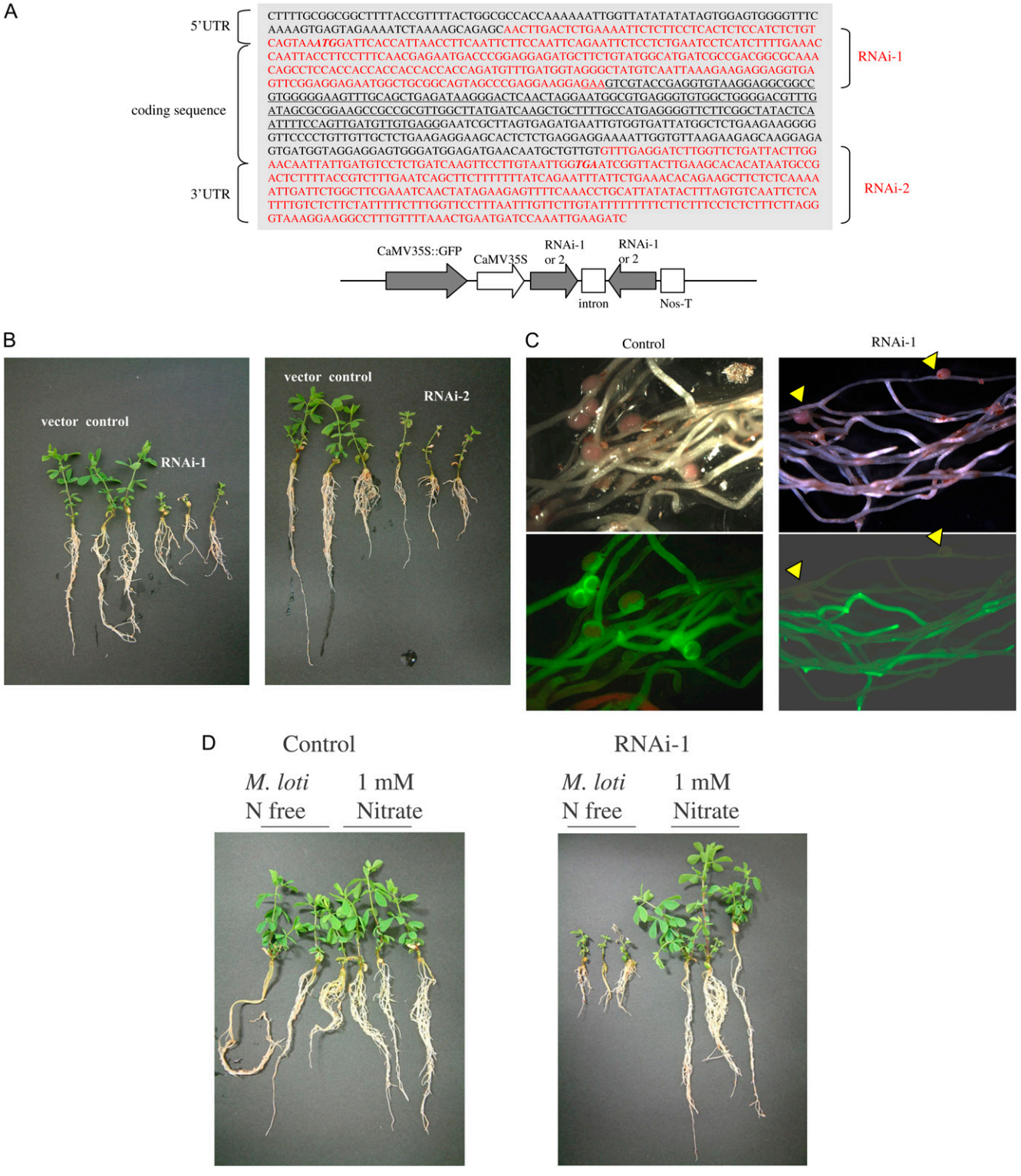

Figure 3. Nodulation phenotype of *LjERF1*-specific RNAi-transformed hairy roots. A, Two constructs targeting different regions of *LjERF1* (RNAi-1 and RNAi-2) were used to transform plants. B, Plants were inoculated with *M. loti* and grown in the absence of nitrogen. Photographs were taken 3 weeks after infection and show the growth phenotype of plants that were transformed with an empty vector (control), RNAi-1, or RNAi-2. C, Fluorescence microscopy of transformed hairy roots. The formation of nodules on control hairy roots was evident, but nodules were observed only in nontransformed hairy roots in the RNAi plants, as distinguished by GFP fluorescence. D, The growth of plants in which the expression of *LjERF1* was suppressed by RNAi was similar to that of control plants in the presence of a nitrogen source.

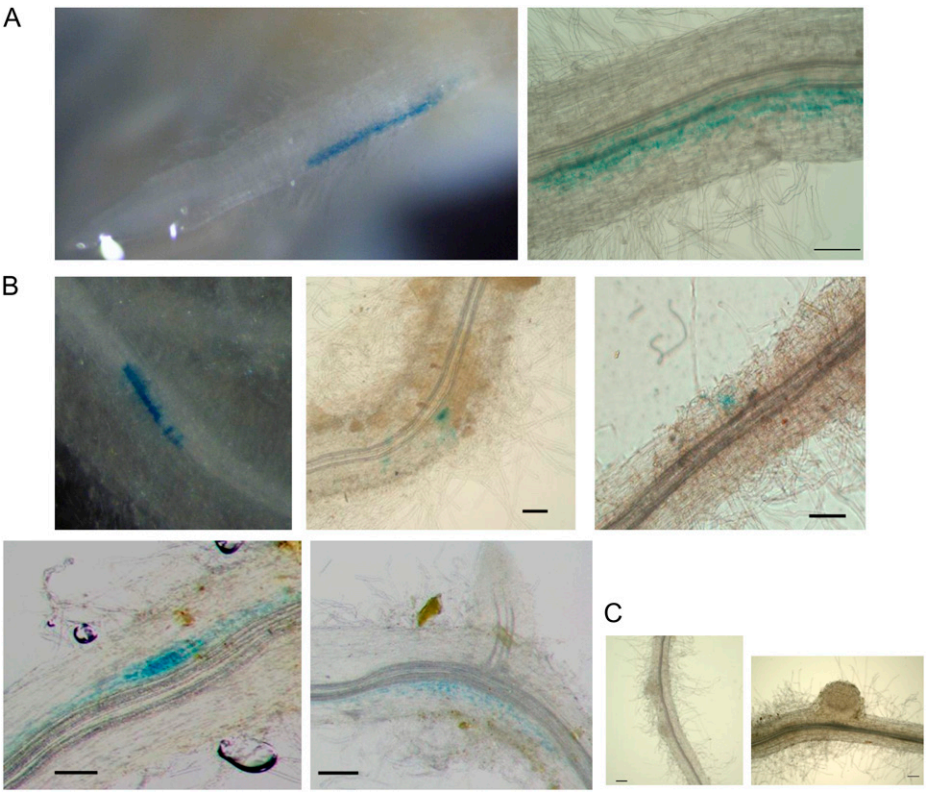

Figure 4. *LjERF1* promoter::GUS assay after *M. loti* inoculation. A, Histochemical GUS staining was observed in an epidermal region of the *LjERF1* promoter::GUS transgenic hairy root at 3 h after *M. loti* inoculation. B and C, GUS staining at 10 d after *M. loti* inoculation indicated that *LjERF1* is induced in epidermal and cortical regions where no morphological changes for nodulation were observed (B) but not in the nodule primordia or mature nodules (C). Bars = 0.1 mm.

the expression pattern of *LjERF1*, which does not respond to symbiosis-defective *M. loti* strains. These results indicated that the expression of *LjPR10-1* is temporally induced only upon infection by wild-type *M. loti*. We then examined the expression of *LjPR10-1* in nodulating hairy roots that overexpressed *LjERF1* or in which *LjERF1* expression was suppressed by RNAi (Fig. 5). Overexpression of *LjERF1* did not affect the expression level of *LjPR10-1*, whereas suppression of *LjERF1* expression by RNAi resulted in up to a 2-fold induction of *LjPR10-1* expression.

DISCUSSION

We used a reverse genetics approach to characterize 20 transcription factor genes of *L. japonicus* that were identified in previous transcriptome studies as genes whose expression is induced during nodulation (Kouchi et al., 2004; Asamizu et al., 2005). One of the transcription factor genes identified in *M. truncatula* transcriptome studies (El Yahyaoui et al., 2004; Lohar et al., 2006), *M. truncatula MtHAP2-1*, encodes a CCAAT-binding transcription factor and is a key regulator of nodule development (Combier et al., 2006). In this study, we identified two *L. japonicus* CCAAT-binding proteins. The proteins shared 84% sequence identity with each other and exhibited distinct patterns of induction during the nodulation process (Table I). In contrast to *M. truncatula*, *L. japonicus* forms a determinate type of nodule. We speculate that the two CCAAT-binding proteins identified in this study may play a coordinated role in meristem development in the determinate type of nodule. Future studies in our laboratory will focus on the involvement of these proteins in *L. japonicus* nodule organogenesis.

We also identified a MADS box gene, *MADS-A18*, that may play a role in nodule organogenesis. The gene was induced at 12 DAI in mature nodules, in which symbiotic nitrogen fixation by resident *M. loti* is established. It has been suggested that nodule MADS box proteins in *M. sativa* are involved in defining or maintaining the differentiated state of the nodule organ (Heard et al., 1997; Zucchero et al., 2001). In situ hybridization demonstrated that the genes encoding these proteins are expressed in infected cells, and it has been suggested that two MADS box proteins, Nmh7 and Ngl9, form heterodimers, since homo/heterodimerization of MADS box proteins is a prerequisite for DNA binding (Zucchero et al., 2001). In this study, we identified one MADS box gene that was induced during the *L. japonicus* nodulation process. It will be interesting to identify potential binding partners of this MADS box protein in *L. japonicus* using protein-protein interaction screens and to determine whether it forms homodimers or heterodimers.

We identified several transcription factor genes that may be involved in the pathogen infection response, including genes that encoded WRKY and AP2-EREBP family proteins. In the establishment of legume-rhizobium symbiosis, the resistance mechanisms of the plant must be suppressed at the site of infection (Mithöfer,

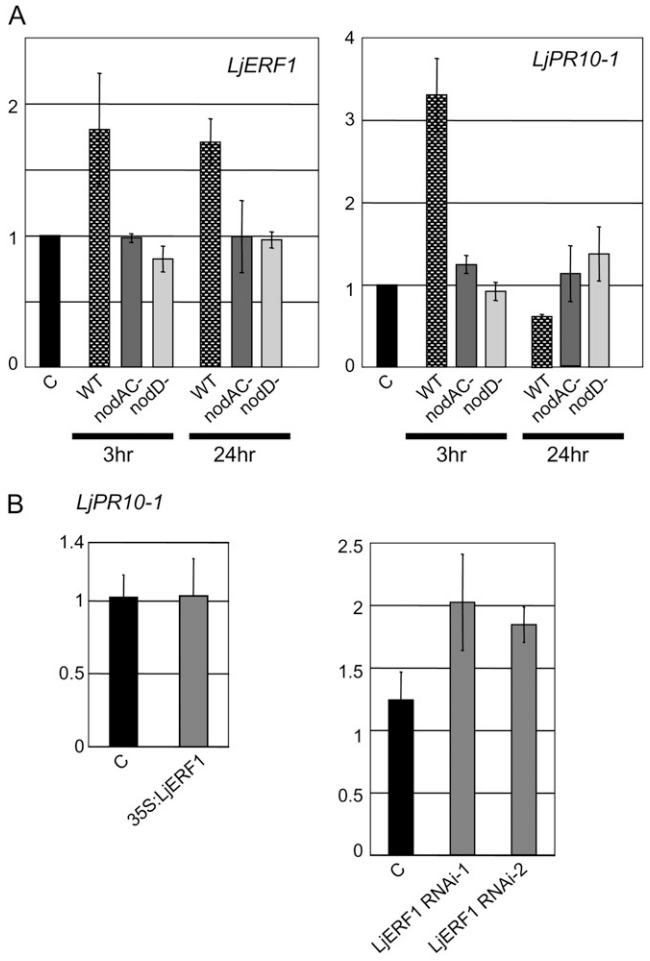

Figure 5. Expression of *LjPR10-1* examined by RT-Q-PCR. A, Expression of *LjERF1* and *LjPR10-1* in response to infection by wild-type and noninfectious mutant strains of *M. loti*. B, Expression of *LjPR10-1* in nodule-containing roots that overexpress *LjERF1* (overexpressor) or nonnodulating roots in which the expression of *LjERF1* was suppressed by RNAi at 3 weeks after *M. loti* inoculation. The *y* axes indicate relative expression levels compared with those in control plants.

2002). In the later stages of nodulation, when the supply of nitrogen is sufficient, the defense system must be switched on to prevent excessive nodule formation, which can result in the loss of photoassimilates that are required to maintain the nitrogen fixation system. None of the genes that regulate either system have been characterized to date. We identified a WRKY transcription factor, *LjWRKY30*, whose induction level was highest at 7 DAI, when the nodule primordium is developed. WRKY proteins are known to have regulatory functions in response to pathogen infection (Eulgem and Somssich, 2007). Previously, cDNA array analysis demonstrated that defense-related genes are up-regulated at 7 DAI, including genes for an elicitor-inducible Dof protein and an elicitor-inducible β-1,3-glucanase (Kouchi et al., 2004). The WRKY protein identified in our study may be involved in the regulation of downstream defense genes directly or indirectly to block excess rhizobium infection under nitrogen-sufficient conditions.

We identified seven AP2-EREBP genes that were induced during nodulation in *L. japonicus*. This is a relatively large number, as a previous transcriptome analysis of *M. truncatula* described only one (El Yahyaoui et al., 2004). This result is most likely due to the increased number of time points examined in our study.

L. japonicus AP2-EREBP genes found in this study may be involved in elevated ethylene and JA sensing during *M. loti* infection and may function by regulating downstream genes, including defense genes. A hyperinfected mutant, *skl*, of *M. truncatula* is insensitive to ethylene, which suggests that ethylene is involved in the nodulation process (Penmetsa and Cook, 1997). It has also been suggested that ethylene functions upstream or at the point of calcium spiking to inhibit the Nod factor signal transduction pathway (Oldroyd et al., 2001). The effect of JA on nodulation also has been examined. It has been suggested that JA functions as a signaling molecule in the systemic suppression of nodulation (Nakagawa and Kawaguchi, 2006) and in the antagonistic cross talk between the pathways of ethylene signaling and calcium spiking (Sun et al., 2006).

We examined the expression of seven *L. japonicus* AP2-EREBP genes in response to ethylene and JA (Fig. 2). Most of the genes could be grouped according to two patterns of expression: ubiquitous expression (induced in both shoots and root) and root-specific expression. *LjERF16* was not activated by ethylene or JA, which suggests that it may be regulated by other inducers, such as salicylic acid or abscisic acid, as is the case for *AtERF4* (McGrath et al., 2005). The expression patterns of the *L. japonicus* AP2-EREBP genes in response to ethylene and JA did not indicate a clear correlation between their phylogenetic relationship and expression during the nodulation process. These results indicate that *L. japonicus* AP2-EREBP genes are involved in various stages of nodulation and that the regulation of their expression is complex and likely involves a balance in the ethylene and JA levels within the cell.

We found that *LjERF1*, the closest homologue of Arabidopsis *ERF1*, is a positive regulator of the early process of nodulation. The overexpression of *LjERF1* resulted in a statistically significant increase in nodule number (Table II), and RNAi resulted in a severe inhibition of nodulation (Fig. 3). *LjERF1* expression was induced at 3 h after *M. loti* infection (Table I) and was specifically induced by compatible wild-type *M. loti* (Fig. 5). The expression was localized to an epidermal region at the infection zone (Fig. 4). GUS staining observed in hairy roots at 10 DAI may indicate that new infection events accompanied by *LjERF1* expression are occurring continuously.

To determine whether *LjERF1* affected the expression of defense genes, we examined the expression of *LjPR10-1*, an ortholog of *M. truncatula MtPR10-1*. Previous transcriptome studies of *L. japonicus* nodulation and arbuscular mycorrhizal fungi colonization have

reported that the expression of several defense genes, including *PR10-1* (*LjPR10-1*), is initially induced, then repressed (Kouchi et al., 2004; Deguchi et al., 2007). In this study, we showed that the expression of *LjPR10-1* is initially induced, then suppressed in response to infection by compatible *M. loti* (Fig. 5). Taken together with previous results, our results indicate that *LjPR10-1* is involved in pathogen defense in nodulating roots.

Unlike in Arabidopsis, in which the expression of *ERF1*, driven by the cauliflower mosaic virus (CAMV) 35S promoter, resulted in the activation of defense genes (Lorenzo et al., 2003), *LjERF1* overexpression did not induce the expression of *LjPR10-1* (Fig. 5B). However, we did not observe suppression of *LjPR10-1*, as expected based on the down-regulation of *LjPR10-1* at 24 h after infection with wild-type *M. loti* (Fig. 5A). This is likely due to the fact that we used nodulating roots at 3 weeks after *M. loti* inoculation to examine the effect of *LjERF1* overexpression. In these samples, the basal level of *LjPR10-1* may have offset the effect of constitutive *LjERF1* expression, even if it suppressed the expression of *LjPR10-1* locally. We also observed a slight induction of *LjPR10-1* expression in *LjERF1* RNAi plants. The observed induction of *LjPR10-1* was relatively small, and it seems likely that *LjERF1* does not activate *LjPR10-1* in nodulating roots. Since *LjERF1* was induced in the early stages of infection (3–24 h; Table I), it is possible that it is involved in the suppression of defense genes for the establishment of rhizobium infection.

The mechanism by which the host plant recognizes a compatible rhizobium as ''self'' is an unsolved question. Our results provide evidence that *LjERF1* functions as a key regulator of this process. However, additional studies, aimed at identifying the direct targets of this transcription factor, are needed to clarify its role in plant defense systems.

MATERIALS AND METHODS

RT-Q-PCR

RNA was extracted using the RNeasy Kit (QIAGEN) from the uninfected roots of soil-grown *Lotus japonicus* 'Gifu B-129' and *Mesorhizobium loti* Tono-infected samples at 3 and 24 h after inoculation and 2, 4, 7, and 12 DAI (N2, N4, N7, and N12, respectively). In the course of RNA extraction, samples were treated with RNase-Free DNase Set (QIAGEN) for genomic DNA removal. The entire root was collected for the 3- and 24-h samples, the infection zone (5 mm in length, 3 mm above the root tip) was collected for the N2 and N4 samples, and visible nodule primordia and mature nodules were collected for the N7 and N12 samples (Kouchi et al., 2004). RT-Q-PCR was performed using 1 μg of total RNA as a template and the DyNAmo HS SYBR Green qPCR Kit (Finnzymes). Results were quantitated using the DNA Engine Opticon2 system (Bio-Rad). Each experiment was performed in triplicate with two biological repeats. Transcript levels were normalized to a *PERK1* homolog (AV772463), since its stable expression was suggested in a previous serial analysis of gene expression (Asamizu et al., 2005). Primers were designed for each gene using the Primer3 program (Rozen and Skaletsky, 2000). The sequences of the primers used in this study are available in Supplemental Table S1.

Treatment with Ethylene and JA

The seeds of Gifu B-129 were sterilized and then germinated on half-strength Broughton and Dilworth (B&D) medium containing 1 mM nitric acid. Fourteen-day-old seedlings were transferred to the same medium containing 10^{-5} M 1-aminocyclopropane-l-carboxylic acid (Wako), 10^{-6} M methyl JA (Wako), or both. The shoots and roots of hormone-treated seedlings were collected at 3, 6, and 24 h after treatment, separated, and then frozen in liquid nitrogen.

Overexpression of *LjERF1* and *LjERF1*-Specific RNAi

The vector for the overexpression of *LjERF1* was constructed as follows. The full-length cDNA of MWM012d12 was obtained by RT-PCR using primers specific for the 5′ and 3′ untranslated regions (5′-CTTTTGCGG-CGGCTTTTACC-3′ and 5′-CAAAGACGGTAAAAGAGTCG-3′). The amplified fragment was cloned into pENTR (Invitrogen) and then transferred by Gateway reaction to the binary vector pHBR (a kind gift from Dr. Y. Murakami, RIKEN). pHBR encodes GFP [sGFP (S65T); Niwa et al., 1999] driven by the CAMV 35S promoter for the selection of transformed hairy roots. The overexpression of *LjERF1* was mediated by tandem duplicated CAMV 35S promoters. The expression of *LjERF1* was confirmed by triplicate RT-Q-PCR analysis that resulted in a mean overexpression value of 8.9- ± 1.5-fold.

The vectors for RNAi were constructed as follows. A 300-bp fragment of *LjERF1*, including 46 bp of the 5′ untranslated region and 254 bp of coding sequence that did not contain the AP2 domain (RNAi-1), and a 374-bp region of the 3′ untranslated region (RNAi-2) were amplified by RT-PCR using the following primers: 5′-CTTGACTCTGAAAATTCTC-3′, containing an *Xho*I or *Bam*HI site at the 5′ end, and 5′-TTCTCCTTCCTCGGGCTAC-3′, containing a *Kpn*I or *Cla*I site at the 5′ end, for RNAi-1; and 5′-TGAATCGGTTACTTG-AAG-3′, containing an *Xho*I or *Bam*HI site at the 5′ end, and 5′-GATCTTCAA-TTTGGATCATTA-3′, containing a *Kpn*I or *Cla*I site at the 5′ end, for RNAi-2. Amplified fragments were digested with *Xho*I/*Kpn*I or *Bam*HI/*Cla*I and ligated into the corresponding sites of pKANNIBAL (Wesley et al., 2001) in inverse orientations with an intron between them. The construct, consisting of the CAMV 35S promoter and the intron-containing hairpin RNA, was transferred to the binary vector pHKN29 using *Not*I. pHKN29 is a modified version of pCAMBIA1300 in which the kanamycin resistance gene is replaced by sGFP (S65T) (Kumagai and Kouchi, 2003). The suppression of *LjERF1* was confirmed by triplicate RT-Q-PCR analysis that resulted in a mean down-regulation value of 60% ± 4.2%.

The overexpression and RNAi binary vectors were transferred into *Agrobacterium rhizogenes* LBA1334 (Offringa et al., 1986) by electroporation.

Hairy Root Transformation

Induction and transformation of *L. japonicus* hairy root using *A. rhizogenes* LBA1334 were performed as described previously (Kumagai and Kouchi, 2003). Briefly, seeds were sterilized and germinated on filter paper submerged in water for 4 d in the dark at 23°C to obtain elongated hypocotyls, followed by 2 d in a photocycle of 16 h of light/8 h of dark in a growth chamber. The seedlings were cut just above the base of the hypocotyls and put into a suspension of *A. rhizogenes* in a petri dish for a several minutes. The seedlings were transferred onto agar plates containing Jensen (N^+) medium (Díaz et al., 1989) and cocultivated for 5 d in a growth chamber. Plants were then transferred onto Schenk and Hildebrandt medium containing 100 μg mL^{-1} cefotaxime and grown for 10 d until the hairy roots were developed from the section of hypocotyls. Emerged hairy roots were assayed for GFP using a fluorescence microscope. Plants harboring transformed hairy roots were transferred to pots filled with vermiculite and supplied with half-strength B&D medium containing 1 mM nitric acid and grown in a growth chamber in a 16-h photocycle at 23°C. After 5 to 7 d, plants were inoculated with *M. loti* MAFF303099 and allowed to continue growing with the same medium without the nitrogen source.

LjERF1 Promoter::GUS Construction

The 1,000-bp upstream promoter region of *LjERF1* was PCR amplified by the primers 5′-CACCGTATAGGGTTCGAACCCTAGGGAGCAC-3′ and 5′-CATTTACTGACAGAGATGGAGAGTGAGGAAGAG-3′. The amplified fragment was cloned into the pENTR D-TOPO vector (Invitrogen) and then transferred to the modified binary vector pCAMBIA1381Z (AF234306; Hajdukiewicz et al., 1994). pCAMBIA1381Z was converted to a Gateway destination vector by ligating a bunt-ended Gateway cassette into the *Sma*I site in the multicloning site. In addition, for the selection of transformed hairy roots, the CaMV 35S promoter and hygromycin resistance gene of pCAMBIA1381Z were replaced by the GFP gene sGFP (S65T) (Niwa et al., 1999) driven by the

nopaline synthase promoter. The *LjERF1* promoter fragment was placed in front of the GUS gene by the LR recombinase reaction.

Histochemical GUS Staining

Hairy roots transformed with the *LjERF1* promoter::GUS construct were placed on nitrogen-free half-strength B&D medium for 5 d after GFP selection (see "Hairy Root Transformation" above), and the hairy roots were inoculated with *M. loti* MAFF303099 by placing the roots between two sheets of filter paper that were immersed in a *M. loti* cell suspension. After 3 and 24 h, hairy roots were immersed in a GUS 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 placed under vacuum for a few minutes, followed by incubation overnight at 37°C in the dark. Otherwise, plants were transferred to pots filled with vermiculite and supplied with nitrogen-free half-strength B&D medium and grown in a growth chamber with a 16-h photocycle at 23°C. Ten days after inoculation with *M. loti*, hairy roots were immersed in a GUS staining solution. The stained materials were observed with a light microscope.

Noninfectious Mutant Strains of *M. loti*

Nod gene disruptants of *M. loti* MAFF303099 were generated by replacing the coding regions of each gene with a gentamicin resistance cassette (*aacC1*) from pMS246 (Becker et al., 1995). *M. loti* M1101 has a deletion of *nodA* and *nodC* genes and thus cannot produce Nod factors (Takeda et al., 2005). This strain is therefore designated *nod AC*$^{-}$. Another strain has a deletion of the *nodD* gene, and this strain is also infection defective (Y. Shimoda, unpublished data). This strain is therefore called *nodD*$^{-}$.

Sequence data from this article can be found in the GenBank/EMBL/DDBJ data libraries under accession numbers AB378626 to AB378645.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. List of primers used in RT-Q-PCR.

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LITERATURE CITED

Andriankaja A, Boisson-Dernier A, Frances L, Sauviac L, Jauneau A, Barker DG, de Carvalho-Niebel F (2007) AP2-ERF transcription factors mediate nod factor dependent Mt *ENOD11* activation in root hairs via a novel cis-regulatory motif. Plant Cell **19:** 2866–2885

Asamizu E, Nakamura Y, Sato S, Tabata S (2005) Comparison of the transcript profiles from the root and the nodulating root of the model legume *Lotus japonicus* by serial analysis of gene expression. Mol Plant Microbe Interact **18:** 487–498

Becker A, Schmidt M, Jager W, Puhler A (1995) New gentamicin-resistance and lacZ promoter-probe cassettes suitable for insertion mutagenesis and generation of transcriptional fusions. Gene **162:** 37–39

Combier JP, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, Moreau S, Vernié T, Ott T, Gamas P, Crespi M, et al (2006) *MtHAP2-1* is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. Genes Dev **20:** 3084–3088

Davuluri RV, Sun H, Palaniswamy SK, Matthews N, Molina C, Kurtz M, Grotewold E (2003) AGRIS: Arabidopsis Gene Regulatory Information Server, an information resource of Arabidopsis *cis*-regulatory elements and transcription factors. BMC Bioinformatics **4:** 25

Deguchi Y, Banba M, Shimoda Y, Chechetka SA, Suzuri R, Okusako Y, Ooki Y, Toyokura K, Suzuki A, Uchiumi T, et al (2007) Transcriptome profiling of *Lotus japonicus* roots during arbuscular mycorrhiza development and comparison with that of nodulation. DNA Res **14:** 117–133

Díaz CL, Melchers LS, Hooykaas PJJ, Lugtenberg BJJ, Kijne JW (1989) Root lectin as a determinant of host-plant specificity in the Rhizobium-legume symbiosis. Nature **338:** 579–581

El Yahyaoui F, Küster H, Ben Amor B, Hohnjec N, Pühler A, Becker A, Gouzy J, Vernié T, Gough C, Niebel A, et al (2004) Expression profiling in *Medicago truncatula* identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program. Plant Physiol **136:** 3159–3176

Eulgem T, Somssich IE (2007) Networks of WRKY transcription factors in defense signaling. Curr Opin Plant Biol **10:** 366–371

Gamas P, de Billy F, Truchet G (1998) Symbiosis-specific expression of two *Medicago truncatula* nodulin genes, *MtN1* and *MtN13*, encoding products homologous to plant defense proteins. Mol Plant Microbe Interact **11:** 393–403

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol **25:** 989–994

Heard J, Caspi M, Dunn K (1997) Evolutionary diversity of symbiotically induced nodule MADS box genes: characterization of *nmhC5*, a member of a novel subfamily. Mol Plant Microbe Interact **10:** 665–676

Kaló P, Gleason C, Edwards A, Marsh J, Mitra RM, Hirsch S, Jakab J, Sims S, Long SR, Rogers J, et al (2005) Nodulation signaling in legumes requires *NSP2*, a member of the GRAS family of transcriptional regulators. Science **308:** 1786–1789

Kouchi H, Shimomura K, Hata S, Hirota A, Wu GJ, Kumagai H, Tajima S, Suganuma N, Suzuki A, Aoki T, et al (2004) Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, *Lotus japonicus*. DNA Res **11:** 263–274

Kumagai H, Kouchi H (2003) Gene silencing by expression of hairpin RNA in *Lotus japonicus* roots and root nodules. Mol Plant Microbe Interact **16:** 663–668

Lohar DP, Sharopova N, Endre G, Peñuela S, Samac D, Town C, Silverstein KA, VandenBosch KA (2006) Transcript analysis of early nodulation events in *Medicago truncatula*. Plant Physiol **140:** 221–234

Lorenzo O, Piqueras R, Sánchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15:** 165–178

McGrath KC, Dombrecht B, Manners JM, Schenk PM, Edgar CI, Maclean DJ, Scheible WR, Udvardi MK, Kazan K (2005) Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. Plant Physiol **139:** 949–959

Middleton PH, Jakab J, Penmetsa RV, Starker CG, Doll J, Kaló P, Prabhu R, Marsh JF, Mitra RM, Kereszt A, et al (2007) An ERF transcription factor in *Medicago truncatula* that is essential for Nod factor signal transduction. Plant Cell **19:** 1221–1234

Mithöfer A (2002) Suppression of plant defence in rhizobia-legume symbiosis. Trends Plant Sci **7:** 440–444

Murakami Y, Miwa H, Imaizumi-Anraku H, Kouchi H, Downie JA, Kawaguchi M, Kawasaki S (2006) Positional cloning identifies *Lotus japonicus NSP2*, a putative transcription factor of the GRAS family, required for *NIN* and *ENOD40* gene expression in nodule initiation. DNA Res **13:** 255–265

Nakagawa T, Kawaguchi M (2006) Shoot-applied MeJA suppresses root nodulation in *Lotus japonicus*. Plant Cell Physiol **47:** 176–180

Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol **140:** 411–432

Niwa Y, Hirano T, Yoshimoto K, Shimizu M, Kobayashi H (1999) Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. Plant J **18:** 455–463

Offringa IA, Melchers LS, Regensburg-Tuink AJ, Costantino P, Schilperoort RA, Hooykaas PJ (1986) Complementation of *Agrobacterium tumefaciens* tumor-inducing aux mutants by genes from the T(R)-region of the Ri plasmid of *Agrobacterium rhizogenes*. Proc Natl Acad Sci USA **83:** 6935–6939

Oldroyd GE, Engstrom EM, Long SR (2001) Ethylene inhibits the Nod factor signal transduction pathway of *Medicago truncatula*. Plant Cell **13:** 1835–1849

Penmetsa RV, Cook DR (1997) A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. Science **275:** 527–530

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. *In* S Krawetz, S Misener, eds, Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365–386

Sato S, Nakamura Y, Kaneko T, Asamizu E, Kato T, Nakao M, Sasamoto S, Watanabe A, Ono A, Kawashima K, et al (2008) Genome structure of the legume, *Lotus japonicus*. DNA Res (in press)

Schauser L, Roussis A, Stiller J, Stougaard J (1999) A plant regulator controlling development of symbiotic root nodules. Nature **402:** 191–195

Sun J, Cardoza V, Mitchell DM, Bright L, Oldroyd G, Harris JM (2006) Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation. Plant J **46:** 961–970

Takeda N, Okamoto S, Hayashi M, Murooka Y (2005) Expression of *LjENOD40* genes in response to symbiotic and non-symbiotic signals: *LjENOD40-1* and *LjENOD40-2* are differentially regulated in *Lotus japonicus*. Plant Cell Physiol **46:** 1291–1298

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, et al (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J **27:** 581–590

Zucchero JC, Caspi M, Dunn K (2001) *ngl9*: a third MADS box gene expressed in alfalfa root nodules. Mol Plant Microbe Interact **14:** 1463–1467