Host legumes control root nodule numbers by sensing external and internal cues. A major external cue is soil nitrate, whereas a feedback regulatory system in which earlier formed nodules suppress further nodulation through shoot–root communication is an important internal cue. The latter is known as autoregulation of nodulation (AUT), and is believed to consist of two long-distance signals: a root-derived signal that is generated in infected roots and transmitted to the shoot; and a shoot-derived signal that systemically inhibits nodulation. In "Lotus japonicus", the leucine-rich repeat receptor-like kinase, HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1), mediates AUT and nitrate inhibition of nodulation, and is hypothesized to recognize the root-derived signal. Here we identify "L. japonicus" CLE-Root Signal 1 (LjCLE-RS1) and LjCLE-RS2 as strong candidates for the root-derived signal. A hairy root transformation study shows that overexpressing LjCLE-RS1 and -RS2 inhibits nodulation systemically and, furthermore, that the systemic suppression depends on HAR1. Moreover, LjCLE-RS2 expression is strongly up-regulated in roots by nitrate addition. Based on these findings, we propose a simple model for AUT and nitrate inhibition of nodulation mediated by LjCLE-RS1, -RS2 peptides and the HAR1 receptor-like kinase.

Keywords: CLE genes • Lotus japonicus • Nitrate • Nod factor signaling • Nodulation • Systemic regulation.

Abbreviations: AUT, autoregulation of nodulation; CLE, CLAVATA3/ESR-related; CLV, CLAVATA; DAI, days after inoculation; ENOD, early nodulin; FCP1, FON2-LIKE CLE PROTEIN 1; FON, FLORAL ORGAN NUMBER; GFP, green fluorescent protein; GUS, β-glucuronidase; HAR1, HYPERNODULATION ABERRANT ROOT FORMATION 1; LjCCaMK, L. japonicus CCaMK; LjCLE-RS, L. japonicus CLE-Root Signal; LjNSP2, L. japonicus NSP2; LM, laser microdissection; LRR, leucine-rich repeat; MALDI TOF-MS, matrix-assisted laser desorption ionization time-of-flight-mass spectrometry; NARK, NODULE AUTOREGULATION RECEPTOR KINASE; NT51, NITROGEN-TOLERANT SYMBIOSIS 1; ORF, open reading frame; RLK, receptor-like kinase; RT–PCR, reverse transcription–PCR; SUNN, SUPER NUMERIC NODULES; SYM29, SYMBIOSIS 29; TDF, trechearry element differentiation inhibitory factor.

The nucleotide sequences reported in this paper have been submitted to DDBJ under accession numbers: LjCLE-RS1, AP010912; LjCLE-RS2, AP010911; LjCLE3, DF093345; LjCLE4, DF093252; LjCLE5, AP009981; LjCLE6, DF093444; LjCLE7, DF093291; LjCLE8, DF093487; LjCLE9, DF093494; LjCLE10, DF093195; LjCLE11, DF093446; LjCLE12, DF093346; LjCLE13, BABK01020001; LjCLE14, BABK01015111; LjCLE15, BABK01005343; LjCLE16, BABK01007606; LjCLE17, DF093443; LjCLE18, BABK01015679; LjCLE19, DF093363; LjCLE20, DF093363; LjCLE21, AP009928; LjCLE22, BABK01008074; LjCLE23, BABK01009817; LjCLE24, AP009913; LjCLE25, BABK01008074; LjCLE26, DF093353; LjCLE27, BABK01010624; LjCLE28, AP010217; LjCLE29, BABK01007274; LjCLE30, BABK01023262; LjCLE31, BABK01039053; LjCLE32, DF093533; LjCLE33, BABK01016033; LjCLE34, DF093335; LjCLE35, DF093323; LjCLE36, BABK01068922; LjCLE37, BABK01043991; LjCLE38, BABK0104370; LjCLV3, AP009713.

Introduction

Symbiotic nitrogen fixation in nodules is required for legume growth in the absence of soil nitrogen, but excessive nodulation can inhibit plant growth. To maintain symbiotic...
balance, legumes have a systemic regulatory system called autoregulation of nodulation (AUT), which involves shoot and root communication via two presumptive long-distance signals, one root-derived and the other shoot-derived. The root-derived signal is generated in response to rhizobia and is translocated to the shoot, whereas the shoot-derived signal is translocated to the root to restrict further nodulation (Caetano-Anollés and Gresshoff 1991, Oka-Kira and Kawaguchi 2006).

Legume mutants defective in AUT, such as Glycine max nitrogen-tolerant symbiosis 1 (nts1)/nodule autoregulation receptor kinase (nark) (Carroll et al. 1985, Delves et al. 1986, Olsson et al. 1989) and Lotus japonicus har1 (Schauser et al. 1998, Szczygowski et al. 1998, Wopereis et al. 2000, Kawaguchi et al. 2002), display a hypernodulating phenotype. Grafting experiments revealed that these mutants are deficient in the shoot-derived signal. Recently, genes that mediate AUT have been identified from L. japonicus, Psium sativum, G. max and Medicago truncatula, HAR1 (Krusell et al. 2002, Nishimura et al. 2002), SYMBIOSIS 29 (SYM29) (Krusell et al. 2002), NTS1/NARK (Nishimura et al. 2002, Searle et al. 2003) and SUPER NUMERIC NODULES (SUNN) (Schnabel et al. 2005), respectively. Each gene encodes a receptor-like kinase containing 21 leucine-rich repeats (LRRs) in the extracellular domain. Therefore, they are thought to be receptors for the root-derived signal in the shoot.

HAR1 and orthologs are highly similar to the Arabidopsis receptor-like kinase CLAVATA1 (CLV1) (Clark et al. 1997), which maintains shoot and floral meristems via short-distance signaling (Clark 2001). Recently CLV1 has been shown to bind a modified CLE peptide encoded by CLV3 (Fletcher et al. 1999, Ogawa et al. 2008). In rice, the FLORAL ORGAN NUMBER 1 (FON1) receptor-like kinase (Suzuki et al. 2004), which is closely related to HAR1, is thought to recognize a FON2/FON4 peptide belonging to the CLAVATA3/ESR-related (CLE) family of peptides (Chu et al. 2006, Suzuki et al. 2006). These findings led us to speculate that HAR1 might recognize a Lotus CLE peptide induced by rhizobial infection as the root-derived AUT signal.

Here we describe L. japonicus CLE peptide genes, LjCLE-RS1 and -RS2, as strong candidates for the root-derived signal. Furthermore, we report that LjCLE-RS2 is strongly induced by nitrate addition.

### Results

LjCLE genes are identified from the Lotus genome database

To date, 91.3% of the gene space is determined in the L. japonicus genome (Sato et al. 2008). An in silico search of the L. japonicus genome sequence database for genes with a CLE domain led us to identify 39 LjCLE genes (Supplementary Table S1), including a gene that shows high similarity to Arabidopsis CLV3 (LjCLV3) and a gene having multiple CLE domains in a single open reading frame (ORF; LjCLE32), which is similar to Oryza sativa CLE (Kinoshita et al. 2007), M. truncatula CLE and Triticum aestivum CLE proteins (Oelkers et al. 2008). In most of the LjCLE proteins, secretion signals were predicted at the N-termini by SignalP (http://www.cbs.dtu.dk/services/SignalP/), but not in LjCLE5, LjCLE14, LjCLE26 and LjCLE33.

Three LjCLE genes are up-regulated by rhizobial inoculation

The root-derived signal is believed to be induced by rhizobial infection. To search for a CLE gene with a similar induction pattern, we analyzed the expression of the 39 LjCLE genes at 6 days after inoculation (DAI) with Mesorhizobium loti MAFF303099. No significant change in LjCLE gene expression was observed in the shoots (Fig. 1A), but three LjCLE genes (LjCLE-RS1, LjCLE-RS2 and LjCLE3) were significantly up-regulated in inoculated roots (Fig. 1B). The level of LjCLE-RS1 expression in inoculated roots was >100 times higher than in uninoculated roots. LjCLE-RS1 and -RS2 expression was not detected in shoots, whereas LjCLE3 was constitutively expressed in shoots, but not in uninoculated roots.

LjCLE-RS1 and -RS2 inhibited nodulation systemically in a HAR1-dependent manner

To explore their function, LjCLE-RS1, -RS2 and LjCLE3, controlled by the cauliflower mosaic virus 35S promoter, were introduced into Lotus hairy roots via Agrobacterium transformation. To distinguish between transformed and untransformed roots, a green fluorescent protein (GFP) gene was also integrated along with the LjCLE genes and introduced into the T-DNA on the binary vector pH7WG2D1 (Karimi et al. 2002). After Agrobacterium transformation, we inoculated the roots with M. loti and scored on both the transgenic root system induced by Agrobacterium and on the normal untransformed root system. In the GFP-positive transgenic roots, nodule development was significantly inhibited when LjCLE-RS1 was overexpressed (Fig. 2A, C). Almost the same effect was observed for overexpression of the LjCLE-RS2 gene. Notably, nodule suppression by overexpression of LjCLE-RS1 and -RS2 was also observed in GFP-negative roots (Fig. 2C). These findings indicate that the suppressive effects of nodulation by LjCLE-RS1 and -RS2 are transmitted systemically from transformed to untransformed roots. In contrast, overexpression of LjCLE3 did not affect total nodule number. To consolidate the systemic effect of LjCLE-RS1 and -RS2 on nodulation, we attempted another hairy root transformation by stubbing the hypocotyls with a fine needle. In this system, the normal untransformed root system can be separated from the hairy roots where LjCLE-RS1 and -RS2 are introduced. After induction of
the hairy roots, we inoculated *M. loti* only into the normal untransformed root system and scored on nodules 2 weeks later. As a result, overexpression of *LjCLE-RS1* and -RS2 systemically suppressed nodulation, whereas overexpression of *LjCLE3* did not repress nodulation (Fig. 2G–M).

To test whether *LjCLE-RS1*- and -RS2-induced nodule suppression requires the HAR1 receptor-like kinase, we overexpressed both genes in transgenic hairy roots of har1-4 hypernodulating mutants (Nishimura et al. 2002). Transformed and untransformed roots of har1-4 exhibited a typical hypernodulating phenotype (Fig. 2D, F), indicating that har1 is epistatic to *LjCLE-RS1*- and -RS2-mediated nodule suppression.

**LjCLE-RS1** and -RS2 rapidly respond to rhizobial inoculation

Normally, a regulatory response in *L. japonicus* is detectable 3 DAI with *M. loti* using a split root system (Suzuki et al. 2008). A time course analysis for gene expression revealed that the induction of *LjCLE-RS1* and -RS2 was up-regulated 3h after *M. loti* inoculation (Fig. 3A, B). The timing for induction was almost the same as that for *NIN* (Schauser et al. 1999), one of the earliest *nodulin* genes expressed during nodule initiation (Fig. 3C). The transcript level of *LjCLE-RS1* was substantially increased, up to approximately 200-fold, and reached a maximum 24 h after inoculation. These results satisfy requirements for the presumptive root-derived signal with regard to the timing of induction.

**Nod factor and several components of the signaling pathway are required for the induction of LjCLE-RS1 and -RS2**

Nod factor production by rhizobia is crucial for the induction of systemic regulation of nodulation (van Brussel et al. 2002, Suzuki et al. 2008). Furthermore, *snf1* har1 double mutant analysis indicated that the HAR1-mediated regulatory system may act downstream of *LjCCaMK*, a component of...
Fig. 2 The phenotypes of *LjCLE-RS1*, *LjCLE-RS2*, *LjCLE3* and *GUS* overexpressing roots. (A–F) Hairy root transformation by excising primary roots. Total nodule number per wild type plant is shown in (A) (n = 7–13). GFP fluorescence of overexpression of *GUS* (B) and of *LjCLE-RS1* (C) on wild-type roots. Note the absence of nodules (C compared with B). Total nodule number per *har1-4* mutant is shown in (D) (n = 16–22). GFP fluorescence of overexpression of *GUS* (E) and *LjCLE-RS1* (F) on *har1-4* roots. (G–M) Hairy root transformation by stabbing of the hypocotyls. The number of nodules on untransformed roots per plant on the wild type is shown in (G) (n = 5–9). Wild-type plants possessing hairy roots overexpressing *LjCLE3* (H) and overexpressing *LjCLE-RS1* (K). (I and J) and (L and M) are details of (H) and (K), respectively. Blue triangles indicate normal untransformed root system. Red triangles indicate the initiation site of the hairy root system. Error bars represent standard deviations. Bars = 1 cm.

The Nod factor signaling pathway (Tirichine et al. 2006). To address the question of whether early activation of *LjCLE-RS1* and *-RS2* depends on Nod factor perception and triggering of the signaling pathway, we inoculated plants with *M. loti* MLI875S, which is mutated in *nodA* and thus unable to synthesize Nod factor. In the inoculated roots, *LjCLE-RS1* and *-RS2* were not up-regulated (Fig. 3D, E). We then analyzed *LjCLE-RS1* and *-RS2* gene expression in castor, *Ljccamk* and...
Ljnsp2 mutants in response to wild-type *M. loti*. The CASTOR ([Imaizumi-Anraku et al. 2005](#)) and *LjCCaMK* ([Tirichine et al. 2006](#)) mutations clearly inhibited *LjCLE-RS1* and -RS2 expression whereas the *Ljnsp2* ([Heckmann et al. 2006; Murakami et al. 2006](#)) mutation resulted in a reduction of *LjCLE-RS1* up-regulation (Fig. 3D, E). These results indicate that Nod factor and several components of the signaling pathway are crucial for *LjCLE-RS1* and -RS2 expression.

**LjCLE-RS2 is strongly up-regulated in response to nitrate**

Nitrogen depletion in the soil is a prerequisite for nodule development and function, and high concentrations of nitrogen as nitrate or ammonia abolish nodulation ([Rautenberg and Kühn 1864; Carroll et al. 1985; Barbulova et al. 2007](#)). Mutations in *HAR1* and *NTS1/NARK* exhibit a nitrate-tolerant symbiotic phenotype ([Carroll et al. 1985; Wopereis et al. 2000; Oka-kira et al. 2005; Barbulova et al. 2007; Magori et al. 2009](#)). To examine whether nitrogen sources could influence *CLE* gene induction, we exposed seedlings to 10 mM KNO₃ for 5 d and analyzed the expression of the 39 *LjCLE* genes. We found that *LjCLE-RS2* was strongly up-regulated in the root in response to nitrate (Fig. 1C). In contrast, *LjCLE-RS1* was suppressed by nitrate (data not shown). Exposure of seedlings to different nitrate concentrations showed that the level of *LjCLE-RS2* transcript accumulation increased by adding 1 mM KNO₃ and reached a maximum at 30 mM KNO₃, a concentration that interferes with *L. japonicus* nodulation (Fig. 4A). We also analyzed the profile of *LjCLE-RS2* expression in *castor, Ljccamk* and *Ljnsp2* mutants, the non-nodulating mutants impaired in the Nod factor signaling pathway. We found that *LjCLE-RS2* was induced at almost the same level in response to nitrate (Fig. 4B), indicating that these signaling pathway members are not required for *LjCLE-RS2* induction via nitrate signaling.

To investigate where *LjCLE-RS1* and -RS2 are expressed, we performed tissue selective expression analysis by using laser microdissection (LM). We used root segments at a distance of 5–10 mm from the root tip for the expression analysis, because significant high levels of expression of *LjCLE-RS1* and -RS2 were detected in these segments (data not shown). Root stele and the tissues outside of the endodermis (cortex...
and epidermis) were dissected by LM (Fig. 5A), and total RNA was extracted from each sample. Reverse transcription–PCR (RT–PCR) analysis showed that *LjCLE-RS1* and *-RS2* were induced in not only the stele but also the outer tissues in response to *M. loti* infection and nitrate treatment (Fig. 5B).

**Discussion**

Through a functional analysis as well as a genome-wide search of *L. japonicus*, we identified three LjCLE genes that were significantly induced by rhizobial inoculation (Fig. 1B). Nod factor and components of the signaling pathway such as CASTOR and LjCCaMK are required for LjCLE gene induction (Fig. 3D, E). So far, a number of early nodulin (*ENOD*) genes are known to be expressed at an early stage of nodulation (Kouchi et al. 2004, Mitra et al. 2004), and some of these promote nodule development and rhizobial infection. In contrast, *LjCLE-RS1* and *-RS2*, suppress nodulation, and thus represent novel *ENOD* genes with an autoregulatory function.

In plants, the CLE genes are members of a well-characterized family that encodes small polypeptides. CLV3, FON2/FON4 and FCP1 (*FON2-LIKE CLE PROTEIN 1*) function in stem cell maintenance of the shoot apical meristem (Fletcher et al. 1999, Chu et al. 2006, Suzaki et al. 2006, Suzaki et al. 2008), and TDIF, CLE41, CLE42 and CLD44 are assumed to function in procambial cell homeostasis (Ito et al. 2006, Hirakawa et al. 2008). In contrast to the constitutive expression of these CLE genes, *LjCLE-RS1* and *-RS2* are induced by external stimuli (Fig. 1B, C). Although microarray analysis showed that CLE2 was induced by nitrate (Scheible et al. 2004), the details were not described. Thus, this is the first report...
unequivocally demonstrating that CLE genes respond to environmental stimuli.

Identification of TDIF, which negatively regulates tra- cheary element differentiation (Ito et al. 2006), and in situ MALDI TOF-MS (matrix-assisted laser desorption ionization time-of-flight-mass spectrometry) analysis of CLV3 (Kondo et al. 2006) in Zinnia and Arabidopsis cells, respectively, revealed that a dodecapeptide with hydroxyproline residues is the product of CLE gene expression. In LjCLE-RS1 and -RS2, such a high similarity is not so obvious, but the 12 amino acids (RLSPGGPDPQHN) constituting the CLE domain are identical to one another, suggesting the evolutionary and functional significance of this domain. However, applying identical to one another, suggesting the evolutionary and functional significance of this domain. However, applying this synthetic peptide to L. japonicus roots and leaves did not suppress nodulation (data not shown). A similar situation was observed in Arabidopsis upon addition of CLE1–CLE7 peptides, which are closely related to LjCLE-RS1 and -RS2 (Fig. 6A). Synthetic CLE peptide treatment had no effect on shoot and root development (Ito et al. 2006, Kinoshita et al. 2007), although overexpression led to the shrinkage and loss of the shoot apical meristem (Strabala et al. 2006). Thus, LjCLE-RS1 and -RS2 may be modified in a manner that differs from the typical modifications of CLV3 and TDIF. Besides the CLE domain, LjCLE-RS1 and -RS2 have conserved motifs consisting of five amino acids (TLQAR) adjacent to the signal sequence and four amino acids (PPSN) close to the C-termini (Fig. 6B). These motifs seem to be specific to legumes because they are not observed in CLE premature proteins of Arabidopsis or rice. The function of these motifs in legumes should be clarified in relation to AUT.

Overexpression analysis in a hairy root system revealed that LjCLE-RS1 and -RS2 strongly suppress nodulation (Fig. 2A, C). Interestingly, the effects traveled systemically from LjCLE-RS1- and -RS2-transformed roots to untransformed roots (Fig. 2G, K–M). Furthermore, the systemic suppression of nodulation was not observed in the har1 hypernodulating mutant (Fig. 2D, F), suggesting that HAR1 functions downstream of LjCLE-RS1 and -RS2. Because HAR1 has been shown by grafting experiments to function in the shoot (Krusell et al. 2002, Nishimura et al. 2002), LjCLE-RS1 and -RS2 gene products specifically expressed in roots are expected to be translocated to the shoot to interact with the HAR1 receptor-like kinase. However, quantitative RT–PCR did not detect LjCLE-RS1 and -RS2 mRNAs in the shoot after rhizobial infection (Fig. 1A), suggesting that the long-distance transport of LjCLE-RS1 and -RS2 gene products would be in a form other than as mRNAs.

In 1864, Rautenberg and Kühn found that nitrogen sources suppressed nodulation. Since then, an explanation for nitrate suppression of nodulation has been elusive. In the mid-1980s, soybean supernodulating mutants were isolated as nitrate-tolerant symbiotic mutants (nts) (Carroll et al. 1985), demonstrating a correlation between increased nodulation and nitrate tolerance. Later, hypernodulating mutants were isolated from a number of legumes, for example Ljhar1, Msun and Psym29. However, the reasons for how hypernodulating mutants exhibit nitrate tolerance are still unknown. Our findings provide a simple model to answer this question based on the finding that nitrate-induced LjCLE-RS2 suppresses nodulation via a HAR1 receptor-like kinase (Fig. 7). According to the model, nitrate tolerance of the hypernodulating mutants is explained because the mutation in HAR1 leads to defective LjCLE-RS2 peptide recognition in the presence of nitrate. Thus, LjCLE-RS2 is a key gene to elucidate nitrogen signaling and developmental plasticity of nodulation that constantly adapts to environmental nitrogen conditions.

Materials and Methods

Biological materials

Lotus japonicus Gifu B-129, non-nodulating mutants: castor-5 (sym71-1), Ljccamk-3 (sym72) and Ljnsp2-1 (sym70) and the hypernodulating mutant: har1-4 (sym78-1) were used in this study. Lotus japonicus was grown with or without M. loti MAFF 303099 or M. loti strain ML8755, which carries a mutation in nodA. An Agrobacterium tumefaciens derivative of CS8 carrying an Ri plasmid was used for hairy root transformation of L. japonicus.

In silico analysis of CLE genes in the L. japonicus genome

The L. japonicus genome sequences in the Kazusa database were scanned for putative CLE homologs by tblastn search using 12 amino acid residues corresponding to functional CLE peptide sequences of Arabidopsis as queries. The candidates with TBLASTN hits were examined for ORFs in the genome with TBLASTN hits were examined for ORFs in the genome of approximately 100 bp upstream of the conserved region. The tblastn search was repeated using putative dodecapeptide sequences identified in the CLE candidates found in the L. japonicus genome as queries until no additional candidate was identified. A signal sequence was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/).

Real-time RT–PCR

Total RNA was isolated with the RNeasy Mini kit (QIAGEN, Hilden, Germany) and the concentration of total RNA was quantified at OD260. Using equal amounts of total RNA (1 µg), cDNA was synthesized with a Quantitect Reverse transcription kit (QIAGEN). Real-time PCR (95°C for 15 min, 45 cycles at 94°C for 15 s, 60°C for 1 min) was performed with ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA) using a Quantitect SYBR Green PCR kit (QIAGEN). The relative expression amount was calculated by the ΔΔCt method according to the manufacturer’s protocol. The Ct value...
Fig. 6 Comparison of the CLE domains of LjCLE-RS1, LjCLE-RS2, LjCLE3 and AtCLE proteins, and amino acid sequence alignments of LjCLE-RS1 and LjCLE-RS2. (A) The amino acid residues of the CLE domain that are identical to LjCLE-RS1 are highlighted in blue. CLE domains that are closely related to LjCLE-RS1 are shaded. (B) Identities and similarities are marked with asterisks and dots, respectively. The positions of signal sequence cleavage sites as predicted by SignalP are shown as triangles. The CLE domain is boxed. The specifically conserved motifs in LjCLE-RS1 and LjCLE-RS2 are shaded.

**CLE domain sequences**

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**Fig. 6** Comparison of the CLE domains of LjCLE-RS1, LjCLE-RS2, LjCLE3 and AtCLE proteins, and amino acid sequence alignments of LjCLE-RS1 and LjCLE-RS2. (A) The amino acid residues of the CLE domain that are identical to LjCLE-RS1 are highlighted in blue. CLE domains that are closely related to LjCLE-RS1 are shaded. (B) Identities and similarities are marked with asterisks and dots, respectively. The positions of signal sequence cleavage sites as predicted by SignalP are shown as triangles. The CLE domain is boxed. The specifically conserved motifs in LjCLE-RS1 and LjCLE-RS2 are shaded.

**Hairy root transformation by exciting primary roots**

For overexpression constructs, ORFs of LjCLE-RS1, -RS2, -RS3 and GUS (β-glucuronidase) were amplified and cloned into pDONR221 (Invitrogen, Carlsbad, CA, USA) using the Gateway BP reaction, and transformed into *Escherichia coli*.
strain DH5α. Inserts were transferred into pH7WG2D, 1 (Karimi et al. 2002) via the Gateway LR reaction. The LR reaction products were transformed into E. coli DH5α. Positive transformants were sequenced and transformed into an A. tumefaciens derivative of C58 carrying Ri plasmid. Primer pairs used were F1/R1 for LjCLE-RS1, F2/R2 for LjCLE-RS2, F3/R3 for LjCLE3 and F4/R4 for GUS (Supplementary Table S2).

Hairy root transformation by stabbing of the hypocotyls

Cultured Agrobacterium cells were suspended in about 0.5 ml of dH2O to make a bacterial paste. Subsequently, the bacterial paste was picked up with a fine needle (φ0.35 mm) and the middle site of hypocotyls of juvenile plants was stabbed two or three times with it. Then plants were placed on the plate and grown in a growth cabinet at 22°C. The numbers of nodules on hairy roots were counted 1 month after inoculation. Transformed hairy roots were distinguished by observing GFP fluorescence using epifluorescent stereomicroscopy.

Sample preparation for laser microdissection (LM)

The pieces of roots at a distance of 5–10 mm from the root tip were fixed in Farmer’s fixative (ethanol:acetate = 3:1) overnight at 4°C. Dehydration and paraffin embedding were performed as described by Inada and Wildermuth (2005) by using a microwave processor. Paraffin-embedded sections were cut to a thickness of 14 μm and mounted on PEN membrane glass slides (Molecular Devices, Sunnyvale, CA, USA) for LM. To remove paraffin, slides were immersed twice in HistoClear II (National Diagnostics, Atlanta, Georgia, USA) for 5 min and then air-dried completely at room temperature. Two or three individual pieces of roots were used for each LM experiment. LM was performed using the Veritas Laser Microdissection System LCC1704 (Molecular Devices). Selected areas were captured by an infrared laser onto CapSure Macro LCM Caps (Molecular Devices), and were subsequently cut by a UV laser. The target cells that fused to the LCM cap were collected by removing the cap from the tissue section. Stele and the rest of root tissues were dissected from 150–200 transverse sections of each sample.

RNA extraction and amplification for tissue-selective RT–PCR

Total RNA was extracted from laser-microdissected cells by using a PicoPure™ RNA isolation kit (Molecular Devices). The Quant-iT™ RiboGreen RNA reagent and kit (Invitrogen) were used for RNA quantification. A 1 ng aliquot of total RNA was amplified by using a WT-Ovation™ RNA amplification system (NuGEN Technologies, San Carlos, CA, USA). Normalized samples were diluted and subjected to RT–PCR. PCR products were loaded on 2% agarose gels and visualized by ethidium bromide. Primer pairs used were F5/R5 for LjCLE-RS1, F6/R6 for LjCLE-RS2 and F7/R7 for Lj18S rRNA (LjSGA_079901) (Supplementary Table S2).

Supplementary data

Supplementary data are available at PCP online.

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The Ministry of Education, Culture, Sports, Science and Technology Grant-in-Aid for Scientific Research for Priority Areas (No. 17018041); the Bio-oriented Technology Research Advancement Institution (BRAIN) Program of Basic Research Activities for Innovative Bioscience; Japan Science and Technology Agency Core Research for Evolutional Science and Technology (CREST); Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, Global COE Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms).

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Fig. 7 A nodule regulation model mediated by LjCLE-RS1 and LjCLE-RS2.
References


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