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Expression of the *CLE-RS3* gene suppresses root nodulation in *Lotus japonicus*

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Abstract

Cell-to-cell communication, principally mediated by short- or long-range mobile signals, is involved in many plant developmental processes. In root nodule symbiosis, a mutual relationship between leguminous plants and nitrogen-fixing rhizobia, the mechanism for the autoregulation of nodulation (AON) plays a key role in preventing the production of an excess number of nodules. AON is based on long-distance cell-to-cell communication between roots and shoots. In *Lotus japonicus*, two CLAVATA3/ESR-related (CLE) peptides, encoded by *CLE-ROOT SIGNAL 1* (*CLE-RS1*) and -*RS2*, act as putative root-derived signals that transmit signals inhibiting further nodule development through interaction with a shoot-acting receptor-like kinase HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1). Here, an *in silico* search and subsequent expression analyses enabled us to identify two new *L. japonicus CLE* genes that are potentially involved in nodulation, designated as *CLE-RS3* and *LjCLE40*. Time-course expression patterns showed that *CLE-RS1/2/3* and *LjCLE40* expression is induced during nodulation with different activation patterns. Furthermore, constitutive expression of *CLE-RS3* significantly suppressed nodule formation in a *HAR1*-dependent manner. TOO MUCH LOVE, a root-acting regulator of AON, is also required for the CLE-RS3 action. These results suggest that CLE-RS3 is a new component of AON in *L. japonicus* that may act as a potential root-derived signal through interaction with HAR1. Because *CLE-RS2*, *CLE-RS3* and *LjCLE40* are located in tandem in the genome and their expression is induced not only by rhizobial infection but also by nitrate, these genes may have duplicated from a common gene.

Key words: autoregulation of nodulation, CLE, legume, *Lotus japonicus*, nodulation, root nodule symbiosis

Introduction

In plants, cell-to-cell communication has important roles not only for development but also for responses to environmental stimuli. There are diverse kinds of mobile signals that may include phytohormones, small RNAs, transcription factors, or small peptides. Among these signaling molecules, recent genetic and biochemical studies have focused on the roles of small peptides (Djordjevic et al. 2015; Endo et al. 2014). The CLAVATA3 (CLV3)/ESR-related (CLE) family is one of the best characterized small peptide family in plants, and in most cases leucine-rich repeat (LRR) receptor-like kinases (RLKs) function as the receptors that transmit signals to the downstream pathway (Cock and McCormick 2001; Miyawaki et al. 2013). In plant development, the currently available data indicate that a significant feature of the signal transduction events mediated by CLE-LRR-RLK modules are associated with controlling the balance between cell proliferation and differentiation in stem cells. First, Arabidopsis *CLV3* is expressed in the stem cell region located at the tip of the shoot apical meristem (SAM). CLV3 non-cell autonomously represses the expression of *WUSCHEL* (*WUS*), which encodes a WUS-related homeobox (WOX) transcription factor (Brand et al. 2000; Fletcher et al. 1999; Haecker et al. 2004; Mayer et al. 1998; Schoof et al. 2000). CLV3 physically interacts with an LRR-RLK, CLV1, that is located in cells beneath the stem cell region that overlaps with *WUS*-expressing cells (Clark et al. 1997; Ogawa et al. 2008). WUS also acts as a mobile signal and can move to the stem cell region, thereby directly activating *CLV3* expression (Daum et al. 2014; Yadav et al. 2011). The CLV-WUS negative feedback loop is crucial for the maintenance of stem cell homeostasis in the SAM. Second, in the Arabidopsis root apical meristem (RAM), *CLE40* is expressed in differentiated columella cells, and the encoded peptide is transferred to the columella stem cell region, where it controls stem cell fate (Stahl et al. 2009). Genetic data suggest that ARABIDOPSIS CRINKLY 4 (ACR4), which is not an LRR-RLK but another type of RLK, is required for CLE40 action. In addition, CLV1 is expressed in the RAM and physically interacts with ACR4 (Stahl et al. 2013). The CLE40/ACR4-CLV1 signaling pathway appears to be involved in the down-regulation of *WOX5*, a key regulator of quiescent center specification. Finally, in Arabidopsis vascular stem cells, proliferation is controlled by an interaction between a CLE peptide, TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), and an LRR-RLK, TDIF RECEPTOR (TDR)/PHLOEM INTERCALATED WITH XYLEM (PXY) (Fisher and Turner 2007; Hirakawa et al. 2008; Ito et al. 2006). WOX4 is identified as a key target of the TDIF-TDR/PXY signaling pathway (Hirakawa et al. 2010).

The signaling pathways mediated by the above-mentioned CLE peptides can be characterized as types of short-range cell-to-cell communication, in which the peptides move within a local region such

as between adjacent cells or in the most distant cases across several cell layers. In contrast, long-distance action of Arabidopsis *CLE6* has been shown; the root-specific expression of *CLE6* rescues the shoot phenotype caused by gibberellin deficiency (Bidadi et al. 2014). Recently, the production of several small peptides that belong to the C-terminally encoded peptide (CEP) family were reported to be induced in the root in response to nitrogen deficiency (Tabata et al. 2014). Subsequently, these CEP family peptides are translocated to the shoot and recognized by two LRR-LRKs (Tabata et al. 2014). In addition, using soybean xylem sap, several small peptides were identified as long-distance mobile signals that belong to the CLE and CEP families (Okamoto et al. 2015). Although the emerging roles for CLE peptides indicate their use as long-distance mobile signals and other key peptides have been identified, there is little known of the molecular function of long-distance signals in comparison with short-range signals.

Autoregulation of nodulation (AON) is a conserved mechanism observed among diverse leguminous species by which plants restrict the number of root nodules, the symbiotic organs containing nitrogen-fixing rhizobia, to conserve energy related to nodulation (Caetano-Anolles and Gresshoff 1991; Oka-Kira and Kawaguchi 2006; Suzaki et al. 2015). The basic concept of AON is as follows. Rhizobial infection not only initiates a signaling pathway resulting in nodule formation but also induces the production of mobile negative factors for nodulation called root-derived signals that are translocated to the shoot through the xylem. When the signal is perceived in the shoot, the second signals, referred to as shoot-derived inhibitors (SDIs), are generated. The SDIs are then transferred to the root through the phloem and block further nodule development. Among the 39 *LjCLE* genes identified from *Lotus japonicus*, the expression of two *CLE* genes, *CLE-ROOT SIGNAL 1* (*CLE-RS1*) and -*RS2,* is induced immediately in response to rhizobial inoculation after direct activation by an RWP-RK type transcription factor NODULE INCEPTION (NIN) (Okamoto et al. 2009; Schauser et al. 1999; Soyano et al. 2014). In addition, in *Medicago truncatula*, exogenous application of cytokinin to roots induces the expression of *MtCLE13*, a functional counterpart of the *CLE-RS1/2* genes (Mortier et al. 2010; Mortier et al. 2012), suggesting that activation of the nodulation-related *CLE* genes occur at the downstream part of cytokinin signaling in roots, of which finding was recently confirmed in *L. japonicus* (Soyano et al. 2014). There is direct evidence that CLE-RS2 meets at least one criterion for a root-derived signal because mature CLE glycopeptides derived from the CLE domain of CLE-RS2 are detected in xylem sap of plants that express *CLE-RS2* (Okamoto et al. 2013). The mature CLE-RS2 peptide can physically interact with HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1), an LRR-RLK that is orthologous to CLV1 (Krusell et al. 2002; Nishimura et al. 2002; Okamoto et al. 2013). The constitutive expression of either *CLE-RS1* or *CLE-RS2* almost completely abolishes nodulation, and functional *HAR1* is required for CLE-RS1/2 action (Okamoto et al. 2009). Moreover, a loss-of function mutation in the

HAR1 gene significantly increases nodule numbers, and reciprocal grafting experiments between roots and shoots indicate that shoot-acting *HAR1* is involved in the control of nodule number (Krusell et al. 2002; Nishimura et al. 2002; Wopereis et al. 2000). Hence, the CLE-RS1/2-HAR1 module is hypothesized to play a pivotal role in the negative regulation of nodulation in AON. KLAVIER (KLV), another shoot-acting LRR-RLK, seems to be involved in CLE-RS1/2-mediated negative regulation of nodulation (Miyazawa et al. 2010; Oka-Kira et al. 2005). Recently cytokinin production was reported to be induced in the shoot by the downstream part of the CLE-RS1/2-HAR1 signaling pathway (Sasaki et al. 2014). In addition, shoot-applied cytokinin is able to move to roots and inhibit nodulation. These results suggest that shoot-derived cytokinin may be an SDI candidate. There might be a proteasome-mediated degradation process for an unidentified protein in the most downstream part of AON in roots because the negative effect of shoot-applied cytokinin is masked by a mutation in the F-box protein TOO MUCH LOVE (TML) (Magori et al. 2009; Sasaki et al. 2014; Takahara et al. 2013). In soybean, microRNA (miR) 172c appears to control nodule number by repressing its target gene, *NODULE NUMBER CONTROL 1* encoding an AP2-type transcription factor, and a mutation in the *NODULE AUTOREGULATION RECEPTOR KINASE* gene, which encodes an LRR-RLK that is orthologous to HAR1, increases the expression level of *miR172c* in roots (Searle et al. 2003; Wang et al. 2014). These results suggest that in soybean SDIs may play a role in the miRNA-mediated transcriptional control of genes involved in the regulation of nodulation. Although our knowledge of AON has been furthered, identification of additional components of AON will be undoubtedly essential for a deeper understanding of the mechanism.

In this study, an *in silico* search enabled us to identify five new *CLE* genes from the *L. japonicus* genome. Expression analyses of the *LjCLE* genes suggested that it is likely that two of them are involved in nodulation. In addition, our data revealed that nodulation-related *CLE* genes had diverse expression patterns. Constitutive expression of *CLE-RS3* in the root significantly suppressed nodulation possibly through long-distance communication between roots and shoots. Functional *HAR1* was required for *CLE-RS3* action. These results place CLE-RS3 as the third CLE peptide involved in AON in *L. japonicus*.

Materials and methods

Plant materials and growth conditions

The Miyakojima MG-20 ecotype of *L. japonicus* (Kawaguchi 2000) was used as the wild-type plant in this study. A description of *har1-7* and *tml-4* plants was published previously (Takahara et al. 2013). Plants were grown with or without *Mesorhizobium loti* MAFF 303099 as previously described (Suzaki et al. 2013).

Identification of new *CLE* genes in *L. japonicus*

Five new *CLE* genes were identified using the deduced amino acid sequence of a CLE domain from CLE-RS1 as a query for a BLAST search of a database (http://mycorrhiza.nibb.ac.jp) that contains the reference sequence data set for the *L. japonicus* genome assembly Lj2.5 and the unique *de novo* assembled contigs derived from *L. japonicus* (Handa et al. 2015). The cDNA sequences of the genes were determined by rapid amplification of cDNA ends (RACE) methods using a SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer's protocol. The putative signal sequence cleavage sites were predicted by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/).

Expression analyses

The primers used for PCR are listed in Table S2. Total RNA was isolated from respective organs using the PureLink Plant RNA Reagent (Invitrogen). First-strand cDNA was prepared using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). Real-time RT-PCR was performed using a Light Cycler 96 System (Roche) with a THUNDERBIRD SYBR qPCR Mix (Toyobo) according to the manufacturer's protocol. The expression of *LjUBQ* was used as the reference.

Constructs and hairy root transformation of *L. japonicus*

The primers used for PCR are listed in Table S2. The *β-glucuronidase* (*GUS*) gene in pENTR-gus (Invitrogen) was inserted into pCAMBIA1300-GW-GFP-LjLTI6b (Suzaki et al. 2014) by the LR recombination reaction to create the vector, pCAMBIA1300-GUS-GFP-LjLTI6b. The 3.0- or 1.1-kb fragments of the promoter region of *CLE-RS3* or *LjCLE40* were respectively amplified by PCR from wild-type genomic DNA, and inserted between the *Sac*I and *Sma*I sites of pCAMBIA1300-GUS-GFP-LjLTI6b that are located upstream of the *GUS* gene. The coding sequence of *CLE-RS3* was amplified by PCR from template cDNA prepared from wild-type *L. japonicus* and cloned into the pENTR/D-TOPO vector (Invitrogen). The insert was transferred into pH7WG2D,1 (Karimi et al.

2002) by the LR recombination reaction to make the *p35S::CLE-RS3* construct. The plasmids used for the constitutive expression of *CLE-RS1*, *CLE-RS2* or *GUS* were previously described (Okamoto et al. 2009). The resulting constructs were introduced into *L. japonicus* plants by *Agrobacterium rhizogenes*-mediated hairy root transformation as previously described (Suzaki et al. 2012).

Stable transformation of *L. japonicus*

The *p35S::CLE-RS3* described above or *p35S::GUS* (Okamoto et al. 2009) plasmids were introduced into *L. japonicus* plants by *A. tumefaciens*-mediated transformation as previously described (Suzaki et al. 2012). Transformed plants were identified by amplifying the *HYGROMYCIN PHOSPHOTRANSFERASE* (*HPT*) gene. The primers used for PCR are listed in Table S2.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: CLE-RS1, AP010912; CLE-RS2, AP010911; CLE-RS3, LC120808; LjCLE39, LC120809; LjCLE40, LC120810; LjCLE41, LC120811; LjCLE42, LC120812.

Results

Identification of five new *CLE* genes in *L. japonicus*

A previous *in silico* search of the *L. japonicus* genome sequence database for genes containing a CLE domain resulted in the identification of 39 *LjCLE* genes (Okamoto et al. 2009). The ratio of the coverage of the gene space in the database, however, was estimated at 91.3% (Sato et al. 2008). Hence, it was possible that this search was insufficient to fully cover the *L. japonicus CLE* genes. In this new study, we used another database (http://mycorrhiza.nibb.ac.jp) that has a reference sequence data set containing the *L. japonicus* genome assembly Lj2.5 and the unique *de novo* assembled contigs derived from *L. japonicus* (Handa et al. 2015). A BLAST search using the amino acid sequence of a CLE domain from CLE-RS1 as a query enabled us to identify five new small proteins that show some similarity with CLE-RS1. The five genes encode small proteins with a conserved CLE domain at their C-termini; thus, they were named CLE-RS3, LjCLE39, LjCLE40, LjCLE41 and LjCLE42 (Fig. 1, Table 1, Table S1). For reasons described below, one of the genes was named CLE-RS3 because it is more likely to be involved in nodulation (see below). Of note, three *CLE* genes, *CLE-RS2*, *CLE-RS3* and *LjCLE40*, are located in tandem within the limits of about 33 kb on chromosome 3 (Fig. S1, Table 1).

Expression of *CLE-RS3* and *LjCLE40* is induced during nodulation

We first monitored the expression patterns of the *CLE* genes identified above in some vegetative and reproductive tissues. *CLE-RS3* and *LjCLE40* were expressed most specifically in roots, whereas the expression of *LjCLE39*, *LjCLE41* and *LjCLE42* were widely observed in the tissues examined (Fig. S2). The strong expressions of *CLE-RS3* and *LjCLE40* are similar to those of *CLE-RS1* and *-RS2*, which were expressed specifically in inoculated roots (Fig. S2). To gain insights into the role of the *CLE* genes during root nodule symbiosis, we examined their time-course expression patterns after inoculation of rhizobia. The known expression pattern of *NIN*, which is strongly induced in response to rhizobia (Schauser et al. 1999), served as a reference standard for the cDNA prepared for this real-time RT-PCR analysis (Fig. 2e). We found that the induction of *CLE-RS3* was detectable at 3 days after inoculation (dai) (Fig. 2a). Subsequently, the expression continued increasing until 14 dai. We also found upregulation of *LjCLE40* at 7 dai with expression becoming stronger as a function of time after inoculation (Fig. 2b). In contrast, the expression of the other *CLE* genes, *LjCLE39*, *LjCLE41*, and *LjCLE42*, was largely unaffected by rhizobial inoculation (Fig. S3). Although it is known that the *CLE-RS1* and *-RS2* genes are rapidly upregulated by rhizobial inoculation (Okamoto et al. 2009), their expression patterns at later nodulation stages were unknown. Therefore, we determined the expression patterns of *CLE-RS1/2* along a longer

time course; expression levels were highest at 3 or 5 dai and then gradually decreased with time after inoculation (Fig. 2c, d). Previous studies have shown that *LjCLE3* and *LjCLE16* expression is upregulated in nodulated roots, although their detailed expression patterns remain unknown (Handa et al. 2015; Okamoto et al. 2009). In summary, *L. japonicus* has at least 6 *CLE* genes, *CLE-RS1/2/3*, *LjCLE3*, *LjCLE16* and *LjCLE40*, whose expression patterns are differentially regulated during nodulation.

The spatial expression patterns of *CLE-RS3* and *LjCLE40* were next determined using transgenic hairy roots that were transformed with either the *ProCLE-RS3::GUS* or *ProLjCLE40::GUS* constructs, in which a 3.0- or 1.1-kb fragment of the promoter region of the respective gene was inserted upstream of the *GUS* reporter gene. The *M. loti* strain, which constitutively expresses *DsRED*, was used to visualize rhizobia enabling us to find infection foci. In *ProCLE-RS3::GUS* roots, GUS activity was observable at the site of presumptive incipient nodule primordia beneath root hairs with infection threads, where the bulges of nodule primordia were not yet visible (Fig. 3a). In contrast, at the corresponding site in *ProLjCLE40::GUS* roots, GUS activity was undetectable (Fig. 3d). After the formation of nodule primordial bulges into which rhizobia start to colonize, GUS activity in both *ProCLE-RS3::GUS* and *ProLjCLE40::GUS* roots was observed within nodule primordia (Fig. 3b-f). These results suggest that *CLE-RS3* and *LjCLE40* are primarily expressed along the nodulation cell lineage, but the timing of expression seems to be different between the two genes. The relatively delayed induction of *LjCLE40* expression in comparison with *CLE-RS3* as determined by GUS activity in hairy roots agrees with the results of real-time RT-PCR (see above).

Constitutive expression of *CLE-RS3* suppresses nodulation

On the basis of the relatively earlier induction of *CLE-RS3* expression compared with *LjCLE40*, *CLE-RS3* may have a role during an earlier nodulation stage. Because the negative regulation of nodulation mediated by AON is known to occur during the early nodulation stages (Suzuki et al. 2008), we hereafter examined the potential involvement of *CLE-RS3* in AON. We first examined the effect of constitutive expression of *CLE-RS3* on nodulation using transgenic hairy roots transformed with the *35S::CLE-RS3* construct. Nodule number was significantly reduced by the constitutive expression of *CLE-RS3* (Fig. 4a-e). Since these inhibitory effects on nodulation were observed not only in transformed but also in untransformed roots (Fig. 4a, b), it is likely that *CLE-RS3* expression has a systemic effect possibly through long-distance communication between roots and shoots. To confirm these effects, we generated stable *L. japonicus* transgenic plants in which *CLE-RS3* was constitutively expressed (Fig S4). The phenotype of reduced nodule number was observed in two independent transgenic plants that

constitutively expressed *CLE-RS3* (Fig. 5). This result led us to conclude that CLE-RS3 acts as a negative factor in nodulation.

As previously shown, constitutive expression of either *CLE-RS1* or -*RS2* attenuates nodulation (Miyazawa et al. 2010; Okamoto et al. 2009; Sasaki et al. 2014; Suzaki et al. 2012; Takahara et al. 2013). In our experimental conditions, the effect of constitutive expression of *CLE-RS3* was significantly weaker in comparison with those of *CLE-RS1* or -*RS2*. Nodule development was almost completely compromised in plants that constitutively expressed the *CLE-RS1* or -*RS2* genes, whereas a few nodules were formed in plants that expressed *CLE-RS3* (Fig. 4a-g). The negative CLE-RS1/2 effect on nodulation is required for shoot-acting *HAR1* and root-acting *TML*, which respectively encode an LRR-RLK that acts as a putative receptor for the CLE peptides and a putative F-box protein. Thus, the CLE-RS1/2-mediated suppression of nodulation activity was masked in the *har1* or *tml* mutants (Okamoto et al. 2009). In order to elucidate the *HAR1*- or *TML*- dependency of CLE-RS3 action, we next constitutively expressed *CLE-RS3* in the corresponding mutants. The hypernodulating phenotype of the *har1* and *tml* plants was unaffected by the constitutive expression of *CLE-RS3* (Fig. 4h-l), suggesting that HAR1 and TML is required for the suppression of nodulation mediated by CLE-RS3.

CLE-RS3 and _LjCLE40_ expression is responsive to nitrate

Nodulation is known to be inhibited in the presence of high nitrate concentrations, and some data suggest that the nitrate-mediated inhibition of nodulation may share a partly conserved mechanism with AON; the mutants involved in AON are partially tolerant to high nitrate (Magori et al. 2009). Furthermore, the expression of *CLE-RS2* is induced by exogenous application of nitrate (Okamoto et al. 2009). In addition to the known induction of *CLE-RS2*, the expression of *CLE-RS3* and *LjCLE40* was induced 24 hours after nitrate application (Fig. 6a). On the other hand, the expression of *LjCLE39*, *LjCLE41*, and *LjCLE42* genes was unaffected by nitrate (Fig. 6a). Given that the *CLE-RS2*, *CLE-RS3* and *LjCLE40* loci are located in tandem (Table 1) and the three genes are induced both by rhizobial infection and nitrate (Fig. 2, 6a), it is possible that the genes may have duplicated from a common gene.

Cytokinin is another known factor involved in the activation of nodulation-related *CLE* genes (Mortier et al. 2012; Soyona et al. 2014). *CLE-RS1* and -*RS2* expressions are activated in response to exogenous cytokinin treatment as previously shown (Fig. 6b, Soyano et al. 2014). In contrast, the expression of other five *CLE* genes was unaffected by cytokinin (Fig. 6b).

Discussion

Prior to this investigation, only two nodulation-related *CLE* genes in *L. japonicus*, *CLE-RS1* and -*RS2*, were well characterized. Additionally, *LjCLE3* and *LjCLE16* may have roles related to nodulation because their expression is upregulated in nodulated roots (Handa et al. 2015; Okamoto et al. 2009). A previous split-root experiment using *L. japonicus* indicates that the negative effect on nodulation by AON starts to be observed at 3 dai, and full inhibition of nodulation is accomplished at 5 dai (Suzuki et al. 2008). This observation implies that production of root-derived signals should occur at a much earlier timing than 3 dai. Following the initiation of *NIN* expression at 3 hours after inoculation, the expression of *CLE-RS1/2*, direct targets of NIN, is detected at the latest at 6 hours after inoculation (Okamoto et al. 2009; Soyano et al. 2014). These immediate responses of *CLE-RS1/2* by rhizobial inoculation correspond to the expected behavior for root-derived signals. Here, we have newly identified another *L. japonicus* nodulation-related *CLE* gene, designated as *CLE-RS3*, of which expression is induced by rhizobial inoculation and constitutive expression of the gene results in a reduction in nodule number. In our experimental conditions, the activation of *CLE-RS3* expression starts to be detected at 3 dai, although the level is much less than the expression of *CLE-RS1/2*. *CLE-RS3* expression continues to increase with time after inoculation. In contrast, after 5 dai, when nodulation seems fully inhibited, *CLE-RS1/2* expression levels gradually decrease as the nodulation process proceeds. The seemingly transient, high expression of *CLE-RS1/2* before 5 dai suggests that upregulation of the genes may play a major role in the AON in order to establish immediate control of nodulation in response to rhizobial infection. On the other hand, we never observed an exponential increase in the number of nodules, even if the plants were grown for an extremely long time. Therefore, we hypothesize that there is a mechanism enabling long-term control of nodule numbers. One possibility is that in the downstream part of the CLE-RS1/2-HAR1 signaling module, there might be a mechanism to memorize the activation of the signal transduction to continuously produce SDI. Alternatively, other CLE peptides can be replaced with CLE-RS1/2 at a later stage to predominantly interact with HAR1, resulting in the production of SDI. The prolonged, higher expression of *CLE-RS3* may account for the latter case. We, however, cannot rule out the possibility that the *CLE-RS3* expression at the later stages may be related to different aspects of root nodule symbiosis other than AON, such as nitrogen fixation and utilization processes. If the *CLE* gene has a role related to innate nitrogen control, it is notable that its expression is induced by nitrate application. The expression of *LjCLE40*, another newly identified, was not detected in infection foci and started to be specifically expressed after the formation of nodule primordia bulges, suggesting that it has a role during nodulation. Nevertheless, we currently cannot reach the convincing conclusion regarding the effect of *LjCLE40*

overexpression on nodulation probably due to the unstable nature of *LjCLE40* overexpression. *LjCLE40* expression responds to nitrate application as well. The nitrate responsiveness of some nodulation-related *CLE* genes is thought to be conserved in leguminous plants because in soybean the expression of the *NITRATE-INDUCED CLE 1* gene also responds to nitrate (Reid et al. 2011). Further functional analyses focusing on the role of CLE-RS3 and LjCLE40 at the later nodulation stages may provide new insights into the role of CLE peptides in the control of root nodule symbiosis. Although rhizobial infection or nitrate treatment commonly activates the *CLE-RS2*, *CLE-RS3* and *LjCLE40* expression, cytokinin treatment can induce only *CLE-RS2* expression. Thus, there may be both common and context-dependent mechanisms with respect to the activation of these genes.

Like *CLE-RS1/2*, constitutive expression of *CLE-RS3* suppresses nodulation. On the other hand, the effect of *p35::CLE-RS3* on nodulation is weaker than those of *p35::CLE-RS1* or *p35::CLE-RS2*. It is unlikely that different expression levels of the respective genes are attributable to the differences in response because the same promoter was used in this assay. Generally, *CLE* genes encode a small protein with a conserved CLE domain at the C-terminus. In the case of CLE-RS2, the 12 amino acid peptide derived from the CLE domain can function similarly to the mature active form. In soybean, RHIZOBIA-INDUCED CLE 1 (RIC1), which has a CLE domain that is considerably conserved with those of CLE-RS1/2, has a negative effect on nodulation (Reid et al. 2011). A site-directed mutagenesis study of the CLE domain of RIC1 showed that the Arg1, Ala3, Pro4, Gly6, Pro7, Asp8, His11, and Asn12 residues are critical for its nodulation suppression activity (Reid et al. 2013). Alignment of CLE domain sequences of CLE-RS1, -RS-2, RS3 and RIC1 showed that the Arg1 residue is not conserved in CLE-RS3, whereas the other potentially important amino acid residues are mostly conserved among the CLE peptides (Fig. 1). The small difference in the CLE domain may determine the relatively weaker suppression activity of CLE-RS3. Root-specific constitutive expression of *CLE-RS3* suppressed nodulation of both transformed and untransformed roots. The result indirectly suggests that CLE-RS3 can act as a long-distance signal between roots and shoots, of which conclusion needs to be confirmed by more rigid assay such as split-root experiments. The suppression effects were masked in the *har1* mutants; therefore, HAR1 may be required for CLE-RS3 action. CLE-RS1 and -RS2 also have negative effects on nodulation in a HAR1-dependent manner (Okamoto et al. 2009). Currently, there is direct evidence that CLE-RS2 physically interacts with HAR1 (Okamoto et al. 2013). On the basis that the CLE domain of CLE-RS1 is completely identical to that of CLE-RS2 (Fig. 1), it seems reasonable to propose that HAR1 can also recognize CLE-RS1. Although 3 of 12 residues in the CLE domain of CLE-RS3 are different from those of CLE-RS1/2 (Fig. 1), is it possible for CLE-RS3 to interact with HAR1? In Arabidopsis, CLV1 can bind to CLE2 or CLE9, both of which belong to phylogenetically different clades

from CLV3 (Ogawa et al. 2008). CLV1 was recently shown to be required for CLE3-mediated control of root architecture in response to nitrogen-deficiency (Araya et al. 2014). The genetic data suggest that CLE3, which also belongs to a phylogenetically different clade than CLV3, can be recognized by CLV1. In addition, the expression of rice *FLORAL ORGAN NUMBER 2* (*FON2*), which encodes a CLE protein, can rescue the *clv3* mutant phenotype, although 3 of 12 residues of the FON2 CLE domain are different from those of CLV3 (Suzaki et al. 2006). These observations suggest that, in addition to CLV3, CLV1 can recognize other CLE peptides with some affinity within a permissible range. It is therefore possible that CLE-RS3 acts as a negative regulator of nodulation through interaction with HAR1.

With respect to the genetic relationship between *CLE-RS3* and known components of AON, in addition to HAR1, TML may be required for the CLE-RS3 action, because the *tml* mutation suppresses the effect of *CLE-RS3* overexpression. Given that *klv* nodulation phenotype is almost identical to *har1* and KLV can physically interact with HAR1 (Miyazawa et al. 2010), we can presume that KLV can be required for the CLE-RS3 action.

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Figure legends

Fig. 1 Amino acid alignment of the CLE domains of related proteins. Conserved amino acid residues are highlighted.

Fig. 2 Real-time RT-PCR analysis of *CLE-RS3* (a), *LjCLE40* (b), *CLE-RS1* (c), *CLE-RS2* (d) and *NIN* (e) expression in wild-type non-inoculated roots (0) and 1, 3, 5, 7 and 14 days after inoculation (dai). Each cDNA sample was prepared from total RNA derived from the entire root. *LjUBQ* was used to assess the relative expression of each gene. Error bars indicate SE (n = 3–4 independent pools of roots).

Fig. 3 Spatial expression patterns of the *CLE-RS3* and *LjCLE40* genes. Blue staining indicates *GUS* activity under the control of the *CLE-RS3* (a–c) and *LjCLE40* (d–f) promoters at the site of presumptive incipient nodule primordia (a and d) and nodule primordia (b, c, e and f) of wild-type plants. GUS activity was observed at 7 dai (a, b, d and e) or 10 dai (c and f). The *M. loti* strain that constitutively expresses *DsRED* was used for these experiments. *DsRED* fluorescence shown on the right panels represents infection foci. Scale bars: 200 μm.

Fig. 4 The effect of constitutive expression of *CLE-RS3*, *CLE-RS1*, *CLE-RS2* or *GUS* on nodulation. The number of nodules formed on transformed (a), untransformed (b) and total (c) roots of the wild-type plants that have transgenic hairy roots constitutively expressing the respective genes (n = 14–20 plants). The nodulation phenotype of the plants that have transgenic hairy roots constitutively expressing *GUS* (d), *CLE-RS3* (e), *CLE-RS1* (f) and *CLE-RS2* (g). The number of nodules formed on all the roots of the *har1-7* and *tml-4* plants that have transgenic hairy roots constitutively expressing the *GUS* or *CLE-RS3* genes (n = 11–20 plants) (h). The nodulation phenotype of the plants that have transgenic hairy roots constitutively expressing *GUS* (i, k) or *CLE-RS3* (j, l) in the respective mutants. Transgenic roots were identified by GFP fluorescence. The nodulation phenotype was observed at 21 dai. The *M. loti* strain that constitutively expresses *DsRED* was used in these experiments. Error bars indicate SE. Scale bars: 2 mm. **P* = 0.05 by Student's *t* test.

Fig. 5 Number of nodules in stably transformed *L. japonicus* transgenic plants that were constitutively expressing *CLE-RS3* or *GUS*. The nodulation phenotype was observed at 21 dai (n = 11–12 plants). Error bars indicate SE. **P* = 0.05 by Student's *t* test.

Fig. 6 Effect of nitrate and cytokinin on *CLE* genes expression. Wild-type plants were grown with 10 mM (black bars) or without (white bars) KNO_3 for 24 hours (a). Wild-type plants were grown with 50 nM (black bars) or without (white bars) benzylaminopurine (BAP) for 24 hours (b). Expression of each gene was determined by real-time RT-PCR. Each cDNA was prepared from total RNA derived from the entire root. *LjUBQ* was used to assess the relative expression of each gene. Error bars indicate SE (n = 3 independent pools of roots).

Fig.S1 Schematic structure of the genomic region harboring *CLE-RS2*, *CLE-RS3* and *LjCLE40*.

Fig.S2 Real-time RT-PCR analysis of *CLE-RS3* (a), *LjCLE40* (b), *CLE-RS1* (c), *CLE-RS2* (d), *LjCLE39* (e), *LjCLE41* (f) and *LjCLE42* (g) expression in wild-type. Each cDNA sample was prepared from total RNA derived from the flower, leaf, stem, shoot apex, non-inoculated (-) and 1 dai (+) roots. The expression patterns of *CLE-RS1* and *CLE-RS2* in the tissues other than inoculated roots are shown as inset (c,d). *LjUBQ* was used to assess the relative expression of each gene. Error bars indicate SE (n = 3 independent pools of respective organs).

Fig. S3 Real-time RT-PCR analysis of *LjCLE39* (a), *LjCLE41* (b) and *LjCLE42* (c) expression in wild-type non-inoculated roots (0) and 1, 3, 5, 7 and 14 dai. Each cDNA was prepared from total RNA derived from the entire root. *LjUBQ* was used to assess the relative expression of each gene. Error bars indicate SE (n = 3–4 independent pools of roots).

Fig.S4 Real-time RT-PCR analysis of *CLE-RS3* expression in stably transformed *L. japonicus* transgenic plants that were constitutively expressing *CLE-RS3* or *GUS*. Each cDNA was prepared from total RNA derived from the entire root. *LjUBQ* was used to assess the relative expression of each gene. Error bars indicate SE (n = 3 independent pools of roots).

Table 1 Features of the *CLE* genes identified in this study.

Name	Chromosome location	Orientation	Number of amino acid residues	Predicted intron	SP cleavage site
LjCLE-RS3	Chr3:35048895-35049113	Forward	72	N	29
LjCLE39	Chr1:50801117-50801398	Reverse	93	N	28
LjCLE40	Chr3:35016355-35016597	Forward	80	N	23
LjCLE41	Chr2:21357987-21360256	Forward	98	Y	37
LjCLE42	Chr4:10320058-10320393	Forward	111	N	42
LjCLE-RS1	unknown	Reverse	116	N	24
LjCLE-RS2	Chr3:35039780-35040025	Forward	81	N	29

The genetic location, number of amino acid residues of the deduced protein, presence of introns and putative signal peptide (SP) cleavage sites of the deduced protein are shown. The genetic location is based on pseudomolecule data obtained from miyakogusa.jp (http://www.kazusa.or.jp/lotus/index.html). Putative SP cleavage sites were predicted by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0).

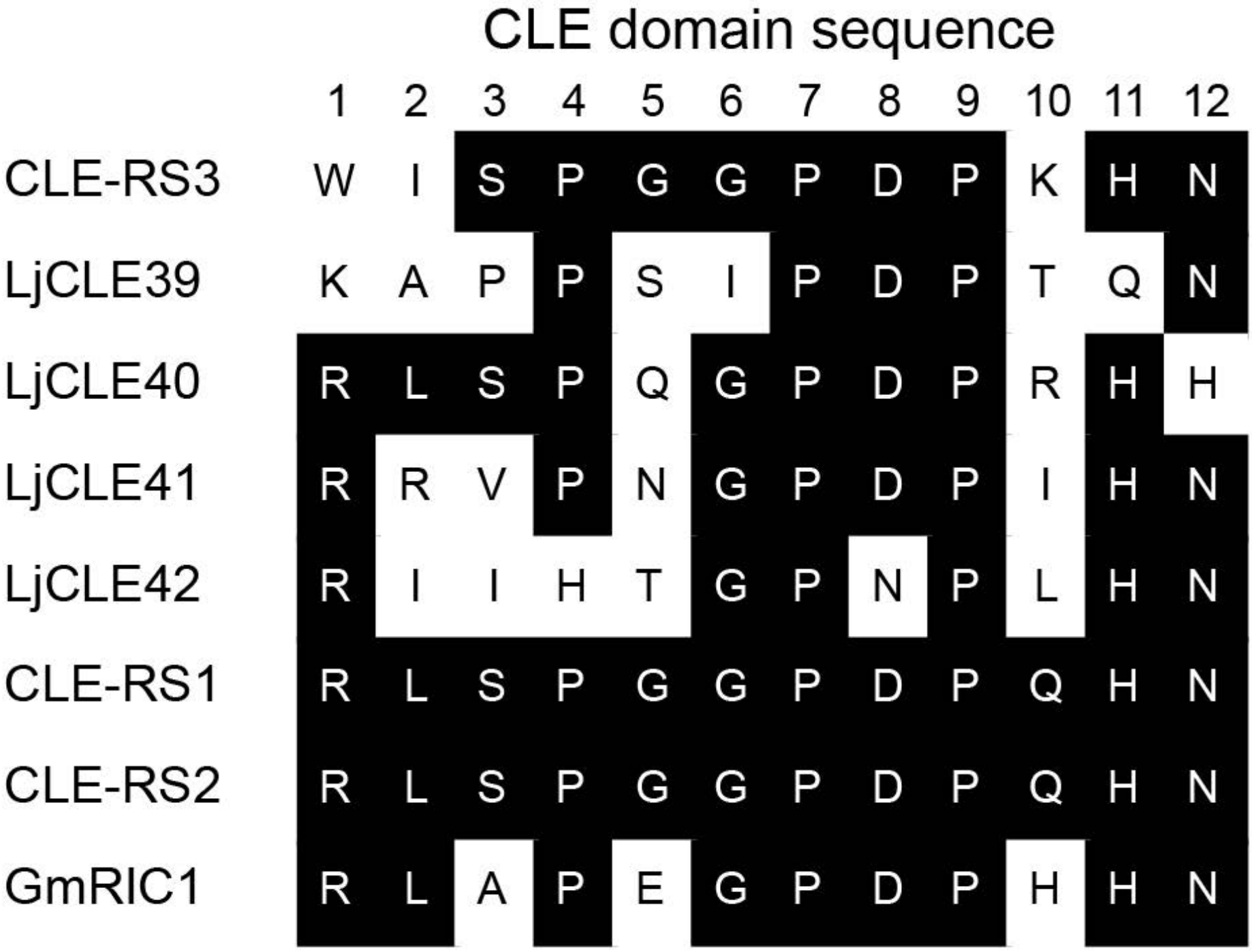
CLE domain sequence
1 2 3 4 5 6 7 8 9 10 11 12
CLE-RS3 W I S P G G P D P K H N
LjCLE39 K A P P S I P D P T Q N
LjCLE40 R L S P Q G P D P R H H
LjCLE41 R R V P N G P D P I H N
LjCLE42 R I I H T G P N P L H N
CLE-RS1 R L S P G G P D P Q H N
CLE-RS2 R L S P G G P D P Q H N
GmRIC1 R L A P E G P D P H H N

Fig. 1

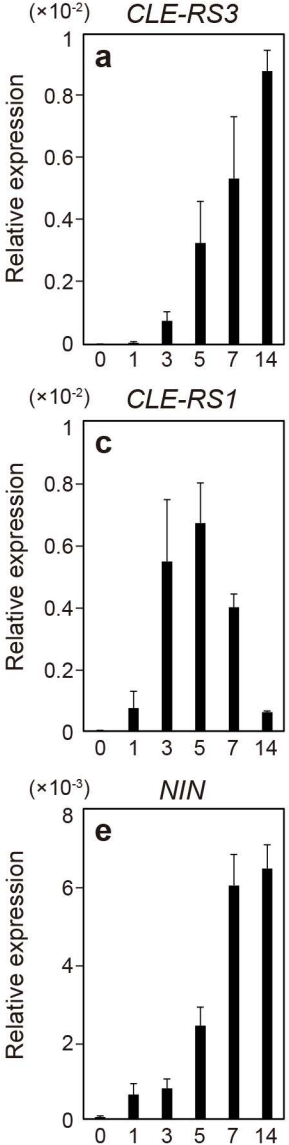
(×10-2)
CLE-RS3
a
Relative expression
0
0.2
0.4
0.6
0.8
1
0 1 3 5 7 14
(×10-4)
LjCLE40
b
Relative expression
0
1
2
3
4
5
6
0 1 3 5 7 14
(×10-2)
CLE-RS1
c
Relative expression
0
0.2
0.4
0.6
0.8
1
0 1 3 5 7 14
(×10-3)
CLE-RS2
d
Relative expression
0
1.5
1
1.5
2
2.5
3
0 1 3 5 7 14
(×10-3)
NIN
e
Relative expression
0
2
4
6
8
0 1 3 5 7 14

Fig. 2

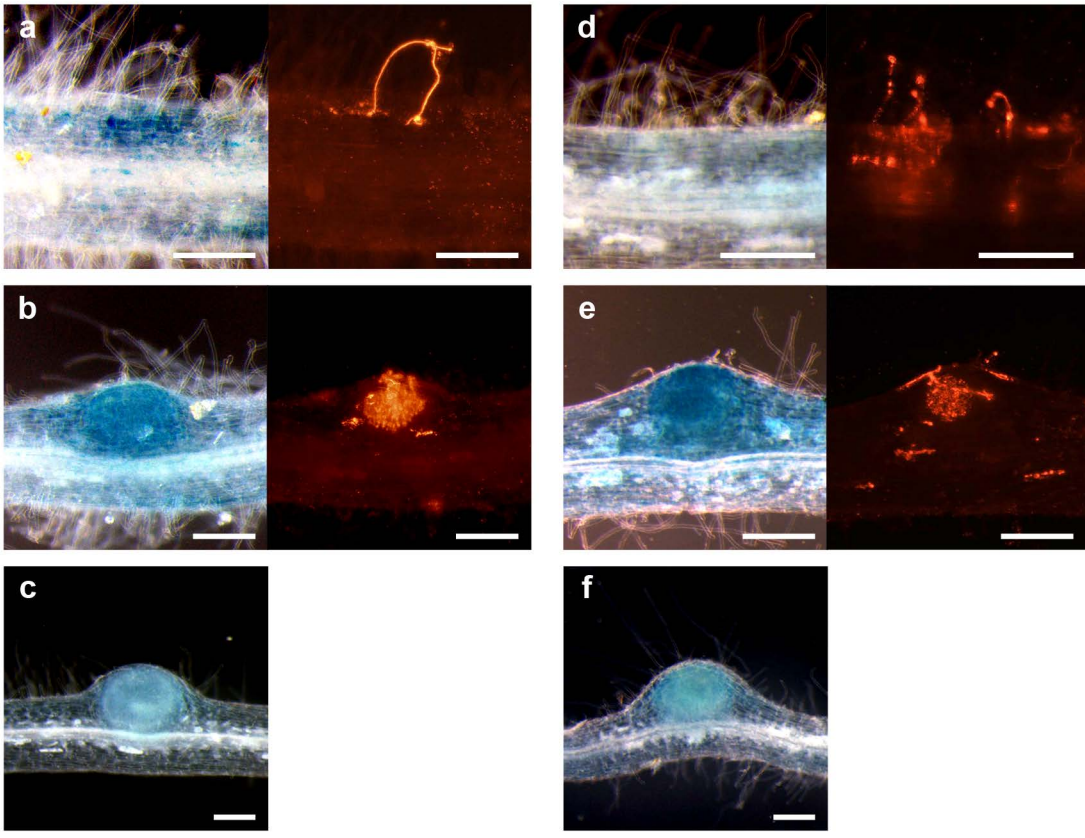

Fig. 3

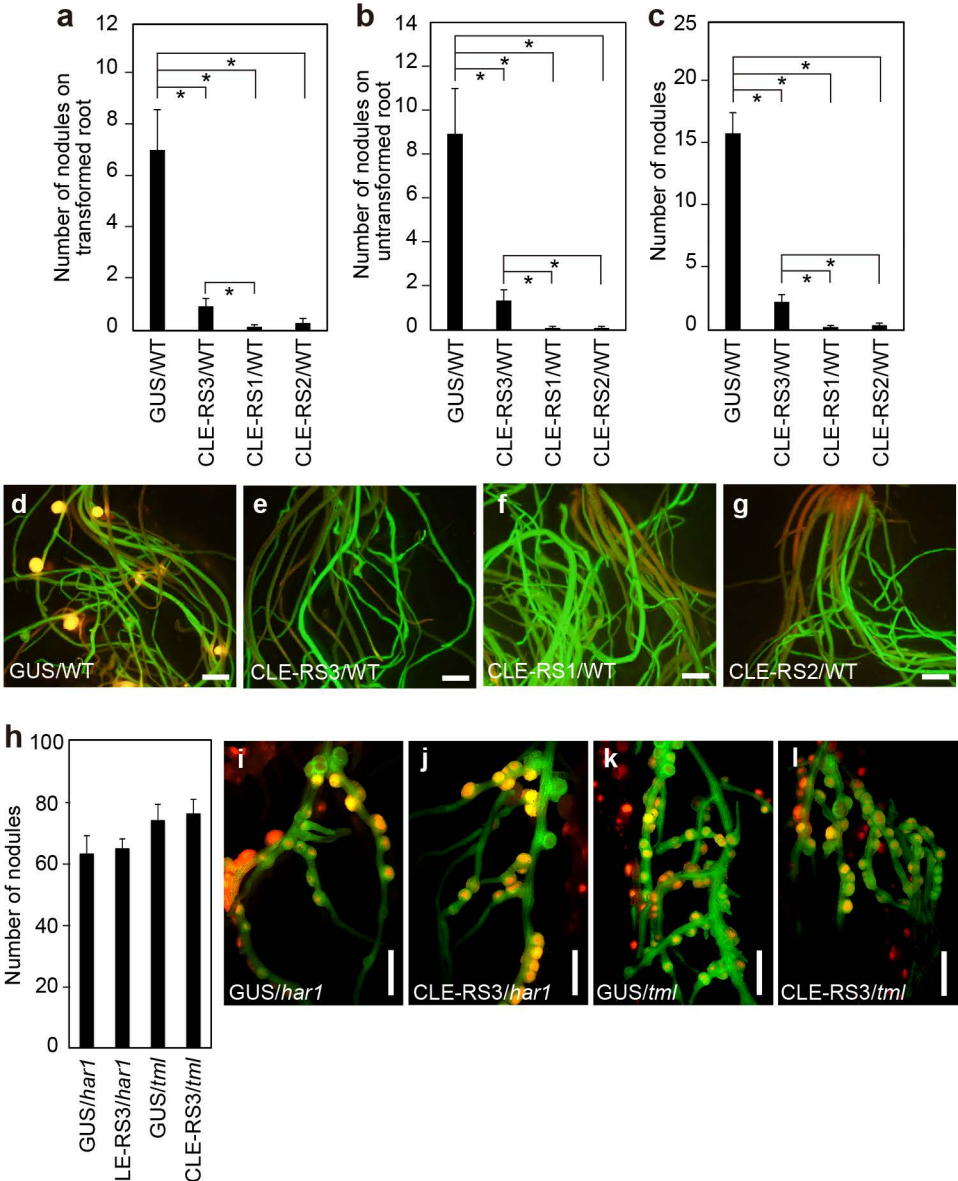
a
Number of nodules on transformed root
12
10
8
6
4
2
0
GUS/WT
CLE-RS3/WT
CLE-RS1/WT
CLE-RS2/WT
b
Number of nodules on untransformed root
14
12
10
8
6
4
2
0
GUS/WT
CLE-RS3/WT
CLE-RS1/WT
CLE-RS2/WT
c
Number of nodules
25
20
15
10
5
0
GUS/WT
CLE-RS3/WT
CLE-RS1/WT
CLE-RS2/WT
d
GUS/WT
e
CLE-RS3/WT
f
CLE-RS1/WT
g
CLE-RS2/WT
h
Number of nodules
100
80
60
40
20
0
GUS/har1
CLE-RS3/har1
GUS/tml
CLE-RS3/tml
i
GUS/har1
j
CLE-RS3/har1
k
GUS/tml
l
CLE-RS3/tml

Fig. 4

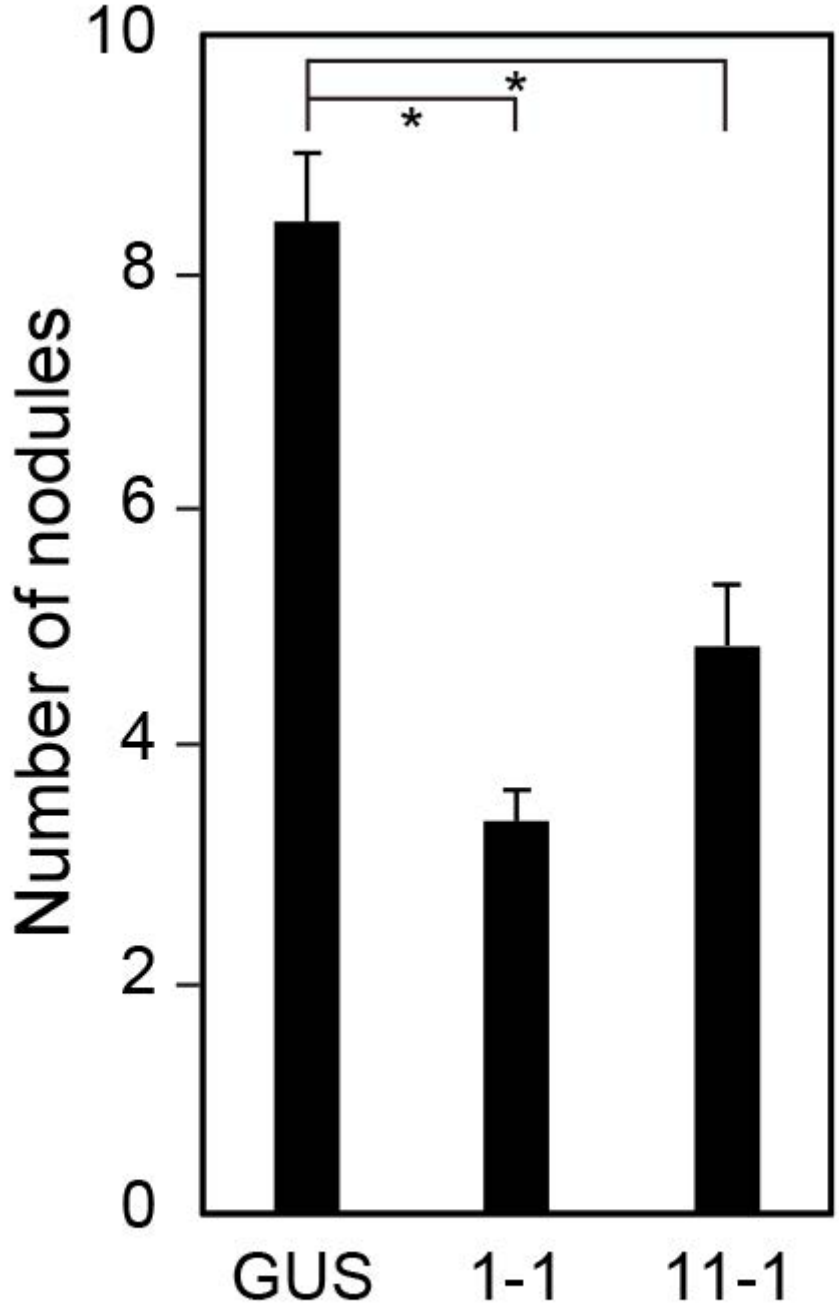

Fig. 5

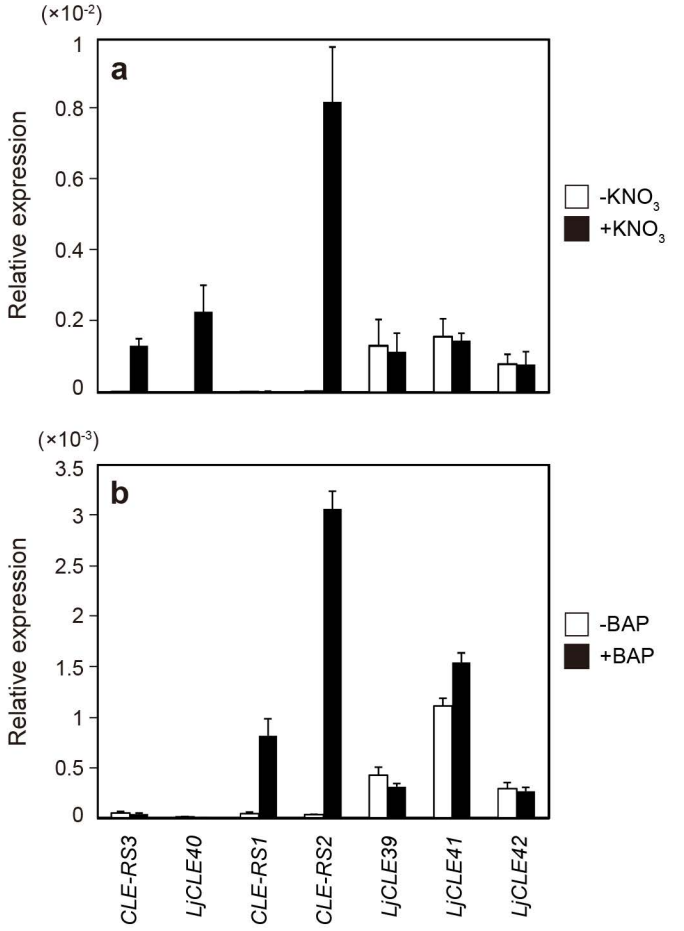
(×10-2)
a
Relative expression
1
0.8
0.6
0.4
0.2
0
-KNO3
+KNO3
(×10-3)
b
Relative expression
3.5
3
2.5
2
1.5
1
0.5
0
-BAP
+BAP
CLE-RS3
LjCLE40
CLE-RS1
CLE-RS2
LjCLE39
LjCLE41
LjCLE42

Fig.6

Table S1 Amino acid sequences of the CLE proteins identified in this study.

Name	Amino acid sequence
LjCLE-RS3	MANASRIMRVFIVMLMMSTLFIMSLVQARSLERRVDSQHLLQMMKHKHPRRALED**WISPGGPDPKHN**KQGHG
LjCLE39	MKITSCRVLAAILALVLVHFLLFSSFCLHHRQSFSRETSLPVSRKLLSSSSTSSASFFTRFGKISGSRNQNRKTVEPSLR**KAPPSIPDPTQN**K
LjCLE40	MANSKQVTCLVLLMLLISKMESRSLEASMETKKISAKGGSQELIQKSQLLKASFAKGGNIFPNLYDTN**RLSPQGPDPRHH**
LjCLE41	MGTSSSSFLPRFFFRVLVVVWLVCVLVLGSLGSGGATRELATQWSSEGLKHEQVVGRDMPVNHAELDFNYMSK**RRVPNGPDPIHN**RRAGNSGRPPGQT
LjCLE42	MIGFSFSREKERTSSRSSETITRLSLARAAIFSLWVMLVFALISLLFSINNHDQIQTTHSTPRRVLNKKHSFSTTLFHPPSSSSAQNIAENTALYGDDK**RIIHTGPNPLHN**

Underline indicates the putative signal sequence as predicted by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0). Amino acid sequences of the CLE domains are shown in bold.

Table S2 Primers used in this work.

LjUBQ RT-PCR			
	UBQ-F	5'-ATGCAGATCTTCGTCAAGACCTTG-3'	
	UBQ-R	5'-ACCTCCCCTCAGACGAAG-3'	
CLE-RS1 RT-PCR			
	RS1-F	5'-TGCAAGTGTCGATGCTCATAGC-3'	
	RS1-R	5'-GATGTTTTGCTGAACCAAGGGATA-3'	
CLE-RS2 RT-PCR			
	RS2-F	5'-GCTCGTAATCTCCAAATCATTCACA-3'	
	RS2-R	5'-GGTGAGAGTCTTTGCTGTTGATATCC-3'	
CLE-RS3 RT-PCR			
	RS3-F	5'-GCAGGCTCGAAGTCTAGAGAGAC-3'	
	RS3-R	5'-GTGAGATCCAATCTTCCAAAGCT-3'	
LjCLE39 RT-PCR			
	CLE39-F	5'-AGTGGAAGCAGGAATCAGAACAGGA-3'	
	CLE39-R	5'-CTACTTGTTCTGGGTAGGATCTGGGATG-3'	
LjCLE40 RT-PCR			
	CLE40-F	5'-ATCACAGTTACTGAAGGCAAG-3'	
	CLE40-R	5'-GTCTATTCGTATCGTAGAGATTTGGA-3'	
LjCLE41 RT-PCR			
	CLE41-F	5'-GCCTGTGAACCATGCAGAATTGGA-3'	
	CLE41-R	5'-CTAAGTCTGGCCAGGAGGTCTACCAGA-3'	
LjCLE42 RT-PCR			
	CLE42-F	5'-CCTTCTTCTTCAAGTGCTCAGA-3'	
	CLE42-R	5'-CTAGTTGTGCAGAGGATTTGGACCGGTGTG-3'	
NIN RT-PCR			
	NIN-F	5'-CAATGCTCTTGATCAGGCTGTTGA-3'	
	NIN-R	5'-GAGTGCTAATGGCAAATTGTGTGTC-3'	
Cloning of *CLE-RS3* promoter			
	proRS3-F	5'-CAAGAGCTCCTCTCTCTCATTCTCATTCT-3'	*SacI* site is added
	proRS3-R	5'-CAACCCGGGTCTATATCCCAAGGGCGCAT-3'	*SmaI* site is added
Cloning of *LjCLE40* promoter			
	proCLE40-F	5'-CAAGAGCTCCCACCTCTCCACACCTCATT-3'	*SacI* site is added
	proCLE40-R	5'-CAACCCGGGGCATGCAATGGAAGTTTAAA-3'	*SmaI* site is added
Cloning of *CLE-RS3* CDS			
	RS3 CDS-F	5'-CACCATGGCGAATGCAAGTAGAATAATGCG-3'	
	RS3 CDS-R	5'-TCAACCATGACCCTGCTTATTATGCTTA-3'	
HPT PCR			
	HPT-F	5'-CTATTCCTTTGCCCTCGGAC-3'	
	HPT-R	5'-ATGAAAAAGCCTGAACTCAC-3'	

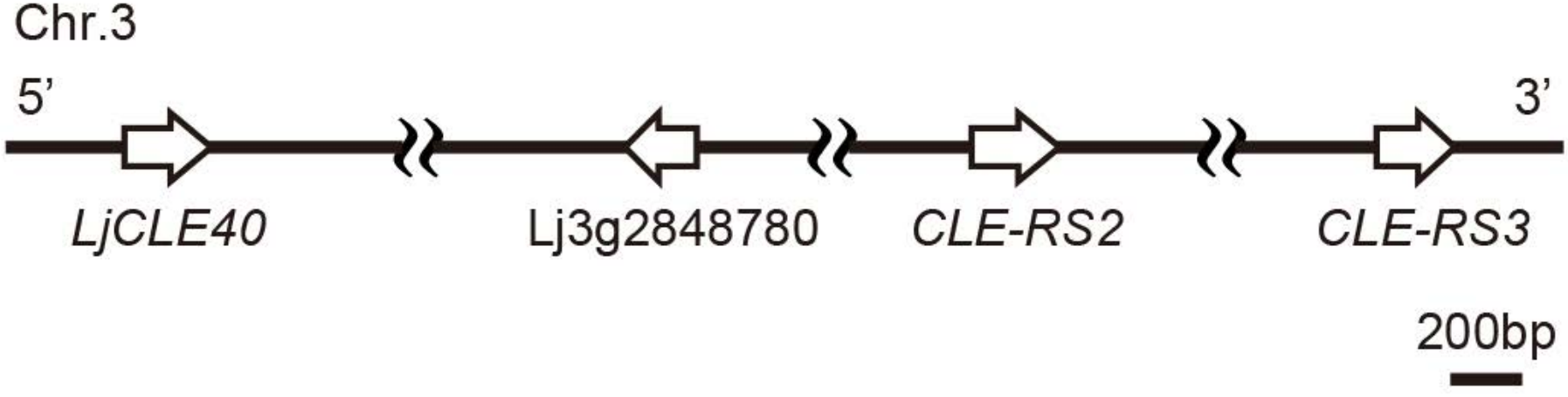

Fig.S1

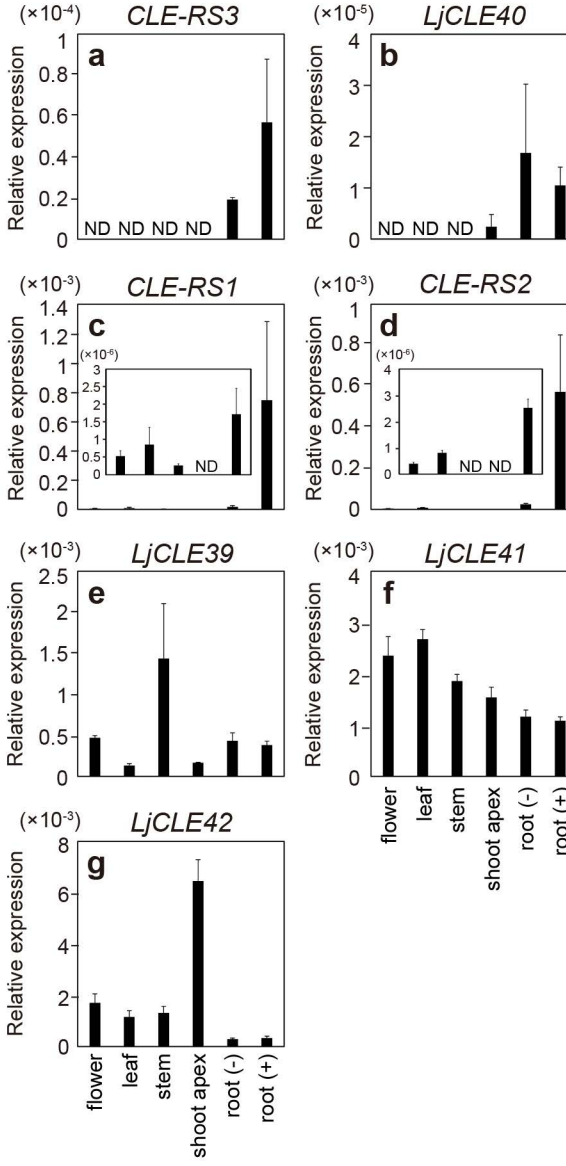
(×10-4)
CLE-RS3
a
Relative expression
ND ND ND ND
(×10-5)
LjCLE40
b
Relative expression
ND ND ND
(×10-3)
CLE-RS1
c
(×10-6)
ND
Relative expression
(×10-3)
CLE-RS2
d
(×10-6)
ND ND
Relative expression
(×10-3)
LjCLE39
e
Relative expression
(×10-3)
LjCLE41
f
Relative expression
(×10-3)
LjCLE42
g
Relative expression
flower
leaf
stem
shoot apex
root (-)
root (+)

Fig.S2

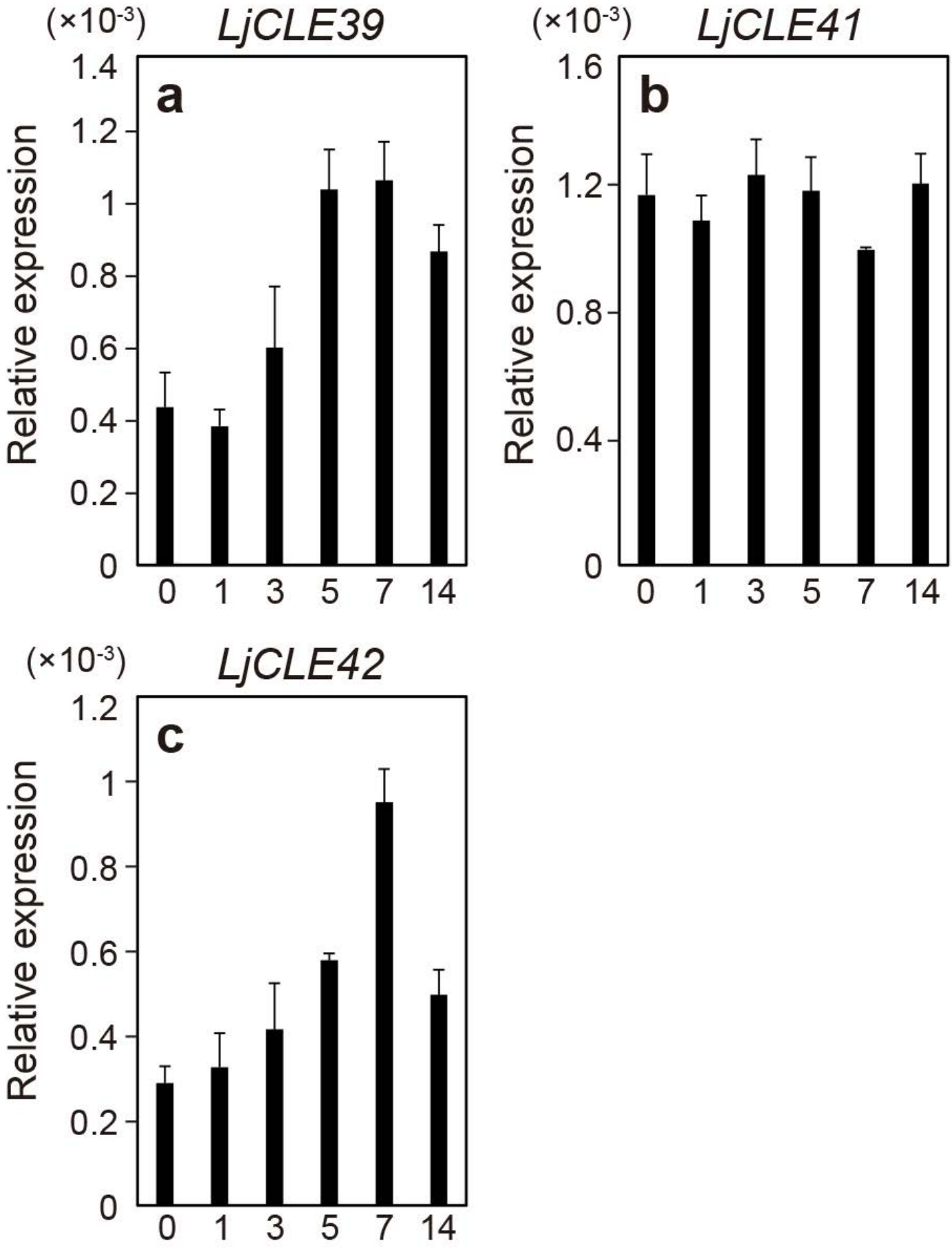
(×10-3)
LjCLE39
a
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 3 5 7 14
(×10-3)
LjCLE41
b
Relative expression
1.6
1.2
0.8
0.4
0
0 1 3 5 7 14
(×10-3)
LjCLE42
c
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
0 1 3 5 7 14

Fig. S3

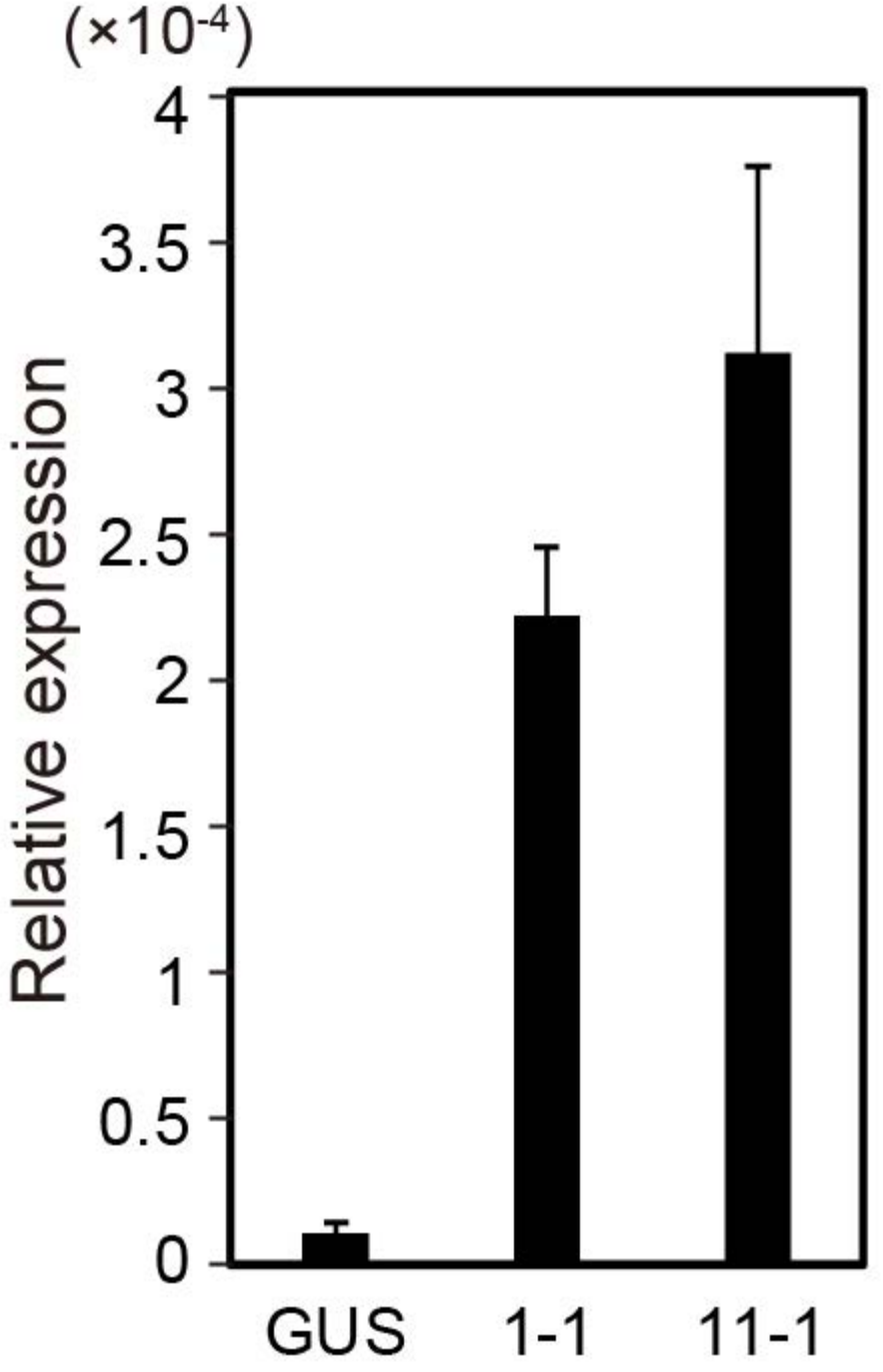

Fig.S4