Doctoral dissertation

博士学位論文

Studies on systemic regulatory mechanisms of root nodulation in *Lotus japonicus*

ミヤコグサにおける根粒形成の全身的抑制機構に関する研究

Takema Sasaki

佐々木　武馬

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General introduction

Legume-rhizobia symbiosis

Like animals, plants have active strategies for collecting necessary nutrients. For example, flowering plants show phototropism, and their roots respond to nutrient-rich soil patches by the proliferation of lateral roots (Alvarez et al., 2012; Goyal et al., 2013). However, unlike animals, the movements of plants are extremely limited. Thus, plants have developed numerous strategies to gain nutrients, such as modifications of the roots to uptake nutrients in nutrient states and the establishment of interactions with other organisms to obtain lacking nutrients (Bulgarelli et al., 2013; Girin et al., 2010).

Endosymbiosis is a symbiosis in which one symbiont lives within the tissues of the other. These interactions are common in nature (Venkateshwaran et al., 2013; Wernegreen, 2004); for example, arbuscular mycorrhizal (AM) fungi can colonize in 70-90% of land plant species and facilitate the nutrient uptake of host plants (Schussler et al., 2001). There is a variety of other plant-microbe interactions in nature.

Legume-rhizobia symbiosis is one of the most successful typical examples of endosymbiosis. In this symbiosis, legumes supply photosynthates in exchange for nitrogen that is derived from N_2 that is fixed by rhizobia. Nitrogen is one of the most important nutrients for organisms, and the earth's atmosphere contains abundant N_2, but plants cannot use it directly. Nitrogen fixation by rhizobia is very powerful; approximately 200 metric million tons of nitrogen is produced annually worldwide, of which 90 million metric tons are produced in agricultural land. In contrast, industrial nitrogen fixation generates 50 million metric tons (Ohyama et al., 2014). By this powerful nitrogen-fixing activity, legumes can grow under oligotrophic soil conditions.

In response to rhizobial infection, legumes form a plant-derived specific organ called the nodule, which is formed by cortical cell divisions in the roots.

Rhizobial infection and Nodulation

Reception of rhizobia

The initiation of the legume-rhizobia interaction is the perception of rhizobia-derived molecules called Nod factors (NFs), which are produced in rhizobia in response to host plant-derived flavonoids (D'Haeze and Holsters, 2002). NFs are lipochitooligosaccharides that are highly host specific due to their distinct structural modifications in different rhizobial species (Felle et al., 1995, 1998; Lerouge et al., 1990; Long, 1996). The structural difference between flavonoids and NFs causes a high host specificity in legume-rhizobia interactions. NFs are required for several events in the invasion of rhizobia.

A genetic approach using the model legume *Lotus japonicus* has unraveled the mechanisms regarding NF perception; in *L. japonicus*, two receptor-like kinases (RLKs), termed NFR1 and NFR5, perceive NFs on epidermal cells (Radutoiu et al., 2003). NF receptors consist of an intracellular serine/threonine kinase domain, a transmembrane domain and an extracellular portion (Radutoiu et al., 2003). NFR1 has a typical serine/threonine kinase domain, whereas NFR5 lacks the activation loop in the serine/threonine kinase domain (Limpens et al., 2003; Radutoiu et al., 2003). The activation loop is the site of phosphorylation in most eukaryotic protein kinases. In *L. japonicus*, previous reports have indicated that NFR1 and NFR5 have been assembled into a heterodimeric receptor (Radutoiu et al., 2003). The receptor-like kinase SYMRK is also localized at the plasma membrane (Den Herder et al., 2012; Stracke et al., 2002)

and is predicted to function in NF perception (Stracke et al., 2002), but its function is not clearly defined.

Invasion of rhizobia

The reception of NFs by RLKs activates ion channels and induces physiological responses, such as ion influx and calcium spiking, which are transient increases in intracellular Ca^{2+} levels. Two cation channels, CASTOR and POLLUX (Ane et al., 2004; Charpentier et al., 2008; Imaizumi-Anraku et al., 2005) and the nucleoporins NUP85 and NUP133 (Groth et al., 2010; Kanamori et al., 2006; Saito et al., 2007) are required in the physiological responses. Approximately 10 min after NF reception, calcium spiking is induced in root hairs (Wais et al., 2000; Walker et al., 2000). Calmodulin-dependent protein kinase (CCaMK) is a strong candidate decoder of calcium spiking because CCaMK has a C–terminal visinin-like domain with three EF hands and a calmodulin (CaM)-binding domain (Mitra et al., 2004; Patil and Poovaiah, 1995). CCaMK also has a serine/threonine protein kinase with an N–terminal kinase domain that may transmit symbiotic signals to following factors (Mitra et al., 2004). CYCLOPS, which is a protein with a C terminal coiled-coil domain, has also been predicted to decode the calcium spiking signaling (Messinese et al., 2007; Yano et al., 2008). CYCLOPS was shown to interact directly with CCaMK (Yano et al., 2008). SYMRK, CASTOR, POLLUX, NUP85, NUP133, CCAMK and CYCLOPS are shared with AM symbiosis in what is termed as the “common SYM pathway”.

The reception of NFs induces root hair curling, and rhizobia are entrapped in the curl, which is called the infection pocket (Esseling et al., 2003; Geurts et al., 2005). In response to calcium spiking, tubular structures called infection threads (ITs) are

formed from this infection pocket toward the nodule primordium. The ITs conduct rhizobia inside the plant tissues.

Nodulation

Concurrently with IT formation, cortical cell divisions for nodule primordium formation initiate. CCaMK is involved in not only formation of IT but also the incitation of nodulation because the roots of *Snf1*, a gain-of-function mutant of CCaMK, are induced to spontaneously form nodules without rhizobia (Tirichine et al., 2006). The NF-dependent activation of CCaMK leads to the accumulation of cytokinins (CKs) and stimulates the division of cortical cells for nodule primordium formation (Frugier et al., 2008). CKs, which are phytohormones, are necessary and sufficient to form nodule primordia because the application of CKs forms nodule-like primordia in *L. japonicus* roots (Heckmann et al., 2011). Additionally, the CK receptor LHK1, which has a histidine kinase domain, functions in the root cortex and is required for cell divisions during nodulation (Gonzalez-Rizzo et al., 2006; Tirichine et al., 2007), while *Snf2*, a gain-of-function mutant of LHK1, results in a spontaneous nodulation phenotype in the absence of rhizobia (Tirichine et al., 2007). Moreover, the LHK1 mutant has restricted nodulation (Murray et al., 2007). Consequently, LHK1-dependent CK signaling plays a primary role in nodulation. In addition, LHK1 functions partially redundantly with other CK receptors, such as LHK1A and LHK3, to mediate cell division for the formation of nodule primordium (Held et al., 2014). CK signaling activates several key transcription factors that are required for nodulation, such as NSP1, NSP2, NIN and NF-Y (Cornbier et al., 2008; Heckmann et al., 2006; Kalo et al., 2005; Laloum et al., 2013; Marsh et al., 2007; Murakami et al., 2006; Schauser et al., 1999; Smit et al., 2005; Soyano et al.,

2013). These transcription factors act not only in nodulation but also in the formation of ITs. Thus, nodulation is a complex pathway.

As described above, the formation of ITs and nodule primordia progresses in parallel in the epidermis and cortex, respectively. When the ITs reach the nodule primordia, rhizobia are released into nodule primordia and are endocytosed by cells of the nodule primordia. Then, the rhizobia differentiate into bacteroids, which are enfolded by the peribacteroid membrane (Brewin, 2004).

Regulation of the symbiotic balance

While a symbiotic relationship is generally beneficial to both partners, the formation of excessive numbers of nodules suppresses the growth of the host plants. To avoid this effect, plants regulate the nodule number.

A number of genes have been identified as negative regulators of nodule number. The loss of function of MtEFD, an ethylene response factor, results in increased nodule number, possibly by altering CK signaling (Vernie et al., 2008). *LjASTRAY*, which encodes a bZIP transcription factor, was also reported as a negative regulator of the nodule number (Nishimura et al., 2002b). Ethylene is also known to inhibit nodulation (Ferguson et al., 2011; Lorteau et al., 2001). The application of ethylene suppresses nodulation and the loss of function of genes that are involved in ethylene sensitivity, such as *LjETR1* and *LjEIN2*, results in increased nodule number (Gresshoff et al., 2009; Lohar et al., 2009; Penmetsa et al., 2008).

In addition, it has been proposed that autoregulation of nodulation (AON) is systemic regulation of the nodule number (Caetano-Anolles and Gresshoff, 1991). AON is a long-distance negative feedback system involving root-shoot communication

(Ferguson et al., 2010; Magori and Kawaguchi, 2009; Oka-Kira and Kawaguchi, 2006). In *Lotus japonicus*, two leucine-rich repeat receptor-like kinases, HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1) and KLAVIER (KLV), have been identified as key components of the AON and function in the shoots (Krusell et al., 2002; Miyazawa et al., 2010; Nishimura et al., 2002a; Oka-Kira et al., 2005; Wopereis et al., 2000). These two proteins are orthologous to *Arabidopsis thaliana* CLAVATA1 and RECEPTOR-LIKE PROTEIN KINASE PROTEIN 2, respectively, which are involved in the maintenance of stem cell populations in shoot apical meristems via short-range cell-to-cell communication (Clark et al., 1997; Kinoshita et al., 2010). As underlying mechanisms of AON, it has been postulated that signaling substances are produced in the roots upon rhizobial infection that are subsequently transported to the shoot (Ferguson et al., 2010; Oka-Kira and Kawaguchi, 2006). The perception of these primary signals in the shoot generates secondary signals. These shoot-derived signals, which are called shoot-derived inhibitors (SDI), are transported to the roots, where they inhibit the initiation of new nodule development (Ferguson et al., 2010; Kenjo et al., 2010; Lin et al., 2010; Oka-Kira and Kawaguchi, 2006; Yamaya and Arima, 2010a, b). In *L. japonicus*, the two peptides, CLE-ROOT SIGNAL 1 (CLE-RS1) and CLE-RS2, are strong candidates for root-derived mobile signaling molecules. The expression of the corresponding genes is induced specifically in infected roots, and CLE-RS2 glycopeptides are transported in the xylem to the shoot where they directly bind to HAR1(Okamoto et al., 2009; Okamoto et al., 2013). Application of arabinosylated CLE-RS peptides to the shoots suppresses nodulation in a HAR1-dependent manner (Okamoto et al., 2013). Furthermore, the TOO MUCH LOVE (TML) F-box protein has been identified as a root-acting AON factor that inhibits the

nodulation downstream of HAR1 (Magori et al., 2009; Takahara et al., 2013).

An overview of the pathways that are involved in rhizobial infection, nodulation and AON is provided in Fig. 1.

Introduction

Autoregulation of nodulation (AON) systemically controls the nodule number. In *L. japonicas*, this regulation requires functional LRR-RLKs, such as HAR1, CLV2 and KLV in the shoots (Broghammer et al., 2012; Nishimura et al., 2002a) and acts by long-distance communication between the shoots and the roots. For root-shoot communication, AON is believed to consist of two long-distance signals, which are root-derived signals (RDSs) and shoot-derived inhibitors (SDIs). As described in the general introduction, CLE-RS1 and CLE- RS2 are strong candidates for RDSs, whereas the SDIs are currently unidentified. Many studies have attempted to identify SDIs, and several candidates have been proposed. For example, the foliar application of methyl jasmonate inhibited the nodulation of *L. japonicus* (Nakagawa and Kawaguchi, 2006), and the foliar application of brassinosteroids also inhibited the nodulation of En6500, the supernodulating soybean mutant (Terakado et al., 2005). Additionally, recent studies successfully purified the substances inhibiting nodulation from the crude extract of soybean shoots, but the chemical structure has not been determined (Kenjo et al., 2010; Lin et al., 2010; Lin et al., 2011; Yamaya and Arima, 2010a, b). These candidates affect the inhibition of nodulation. However, it has not been concluded that this negative effect arises in AON signaling because these reports cannot indicate involvement between the negative effect and AON signaling.

The *har1* mutant has defects in AON, namely defects in SDI production in the shoot. In contrast, the constitutive expression of *CLE-RS1* or *CLE-RS2* activates AON, which is derived from increased SDI production in the shoot. Thus, the SDI levels in the shoots should differ between *har1* mutants and *CLE-RS1* or *CLE-RS2* constitutive expression lines. Consequently, I hypothesized that candidates of SDIs should be

identifiable by comparing the transcriptome and metabolite profiles between wild type, *har1* mutants and *CLE-RS* constitutive expression lines. Thus, in this study, I compared transcriptome and metabolite profiles between these lines.

I show that the cytokinin (CK) level is decreased in *har1* shoots and increased in the shoots of the *CLE-RS1* or *CLE-RS2* constitutive expression lines compared to that of the shoots of wild type. In addition, the results of this study indicate that the production of CKs is activated by rhizobial infection and that the application of exogenous CKs to the shoots can inhibit nodulation in a TML-dependent manner. Consequently, the results of this study suggest that shoot-derived CKs systemically regulate root nodulation in AON.

Results

1. Phenotypic analyses in the *CLE-RS1/2* overexpression lines and the *har1* mutant

I generated stable *L. japonicus* transgenic plants in which either *CLE-RS1* or *CLE-RS2* was overexpressed under the control of the CaMV 35S promoter (*CLE-RS1/2*ox, Fig. 2a). As controls, transgenic plants expressing the *GUS* gene were generated (MG-20 background). Control (*GUS*ox), *har1-7* mutants, *CLE-RS1*ox and *CLE-RS2*ox plants were inoculated with *Mesorhizobium loti*, and the nodules were counted two weeks after inoculation. *har1-7* plants formed approximately three times more nodules than did the control, whereas *CLE-RS1*ox and *CLE-RS2*ox did not form nodules (Fig. 2b,f). This result is consistent with previous observations of nodulation inhibition by root-specific *CLE-RS1* and *CLE-RS2* expression following hairy root transformation (Okamoto et al., 2009). In addition, I found that the lateral root numbers in *CLE-RS1*ox and *CLE-RS2*ox were decreased to 18% and 27% of those in the control, respectively (Fig. 2c,g). In addition, *har1-7* plants formed approximately twice as many lateral roots as did the control plants (Fig. 2c,g). Thus, in terms of the nodule and lateral root numbers, the *CLE-RS1/2*ox phenotypes were opposite of those of the *har1-7* mutant. To determine whether the nodulation inhibitory effects of *CLE-RS1/2* overexpression are mediated through the shoots, I performed a reciprocal grafting of the shoots and roots between MG-20 (wild type), *har1-7* mutants, *CLE-RS1*ox and *CLE-RS2*ox plants (Fig. 2d). The shoots of *CLE-RS1*ox and *CLE-RS2*ox inhibited nodulation in both wild-type and *har1-7* mutant rootstocks, indicating that *CLE-RS1* and *CLE-RS2*ox expression in the shoots is sufficient to block nodulation. Furthermore, the inhibition of nodulation in the rootstocks overexpressing *CLE-RS1/2* was suppressed by the shoots of *har1-7* mutants. These results indicate that CLE-RS1/2 inhibited

nodulation through shoot-root communication in a HAR1-dependent manner, as previously observed (Okamoto et al., 2009). Likewise, the shoots from *CLE-RS1*ox plants inhibited lateral root formation in both wild-type and *har1-7* mutant rootstocks (Fig. 2e), suggesting that lateral root formation was inhibited by *CLE-RS1* expression in the shoot.

2. Transcriptome analysis of *CLE-RS1/2* overexpression lines and the *har1* mutant

RNA-Seq libraries were generated from RNA samples of MG-20, *har1-7* mutants, *CLE-RS1*ox and *CLE-RS2*ox shoots. RNA-Seq was performed on Illumina HiSeq 2000 machine in three independent biological repeats. The raw sequence data have been deposited in the DDBJ Sequence Read Archive (Accession number: DRA002563). After removing the low-quality reads, I obtained at least more than 9.8 million high-quality reads (Table 1). More than 76% of these high-quality reads could be mapped to reference genome sequences of *L. japonicus* (Table 1).

To identify the genes that are associated with AON, differentially expressed genes (DEGs) were identified by comparison between each dataset and the MG-20 dataset. The average expression of each mapped gene was calculated using the FPKM method (Trapnell et al., 2010). The genes that met the following criteria were treated as DEGs: expression between two lines differs by more than 2-fold change and FDR < 0.05. In DEGs, the expression levels of 379 and 206 genes were upregulated in *CLE-RS1*ox and *CLE-RS2*ox, respectively. In contrast, 329 and 299 genes were downregulated in *CLE-RS1*ox and *CLE-RS2*ox, respectively. In these DEGs, 68 genes were upregulated in both *CLE-RS1*ox and *CLE-RS2*ox, and 108 genes were downregulated in both *CLE-RS1*ox and *CLE-RS2*ox (Fig. 3). In a dataset of the *har1*

mutant, 167 genes were upregulated, and 108 genes were downregulated. DEGs that are upregulated in *CLE-RS1/2*ox and downregulated in *har1* are likely to be regulated by AON signaling. One DEG (CM0553.180) involving AON was identified (Table 2). The sequence of CM0553.180 on *L. japonicus* genome (Lj2.5 genome) has lacked portions, thus the complemented sequence was constructed using Trinity software (accession number: LC013384). The transcript is 612 base pairs in length and the length of an ORF was 77 amino acids. I performed blast analysis of the complemented CM0554.180 but there is no homologue in *A. thaliana* and other organisms.

To identify the property of DEGs that are induced by CLE-RS1/2 peptides, I performed a gene ontology (GO) analysis on DEGs. First, I performed a BLAST analysis between *L. japonicus* protein sequences and *A. thaliana* protein sequences, and the gene ID of DEGs was replaced with a gene ID of *A. thaliana*. A GO analysis was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7. GO terms that are involved in biotic stress, such as response to organic substances, defense response and immune response, were identified by upregulated DEGs (Table 3). In addition, GO terms that are involved in response to phytohormone, endogenous and organic stimuli were identified by downregulated DEGs (Table 3).

3. Quantification of phytohormones in *CLE-RS1/2* overexpression lines and the *har1* mutant

RNA-seq analysis suggested that AON influences gene expression in response to hormones. Additionally, it was recently reported that SDI is a small amphiphilic compound (possible molecular mass < 10 kDa) that is unlikely to be a protein or an RNA molecule (Kenjo et al., 2010; Lin et al., 2010; Yamaya and Arima, 2010a, b). The

har1 mutant exhibits developmental alterations, including short primary roots and enhanced lateral root formation (Wopereis et al., 2000). In contrast, the number of lateral roots decreased in *CLE-RS1*ox and *CLE-RS2*ox plants (Fig. 2c). Therefore, I assumed that SDI might affect plant development in addition to its effects on nodule formation. Plant development is controlled by phytohormones, which are low-molecular-mass compounds (Lin et al., 2010). Some phytohormones act as long-distance signals in the integrative regulation of development and in defense responses (Cheng et al., 2013; Heil and Ton, 2008; Kudo et al., 2010). Thus, next I compared phytohormone compositions in MG-20 shoots with those of the *har1-7* mutant and *CLE-RS1/2*ox shoots. Given that SDI is produced downstream of CLE-RS1/2 and HAR1 (Oka-Kira and Kawaguchi, 2006; Okamoto et al., 2009), I focused on phytohormones by combining two trends, namely increased levels in both *CLE-RS1*ox and *CLE-RS2*ox shoots and decreased levels in *har1-7* mutant shoots. In three independent analyses, only one of 39 of the examined compounds consistently exhibited the two trends, namely N^6-(Δ^2-isopentenyl) adenine riboside 5'-phosphates (iPRPs), which are intermediates of CK biosynthesis (Table 4).

4. Cytokinin levels increased in response to rhizobial infection

The amount of iPRPs in the MG-20 shoots significantly increased by approximately two-fold in response to rhizobial infection compared to that of non-infected plants (Fig. 4). It is believed that CKs are transferred from the roots to the shoots through the xylem (Ko et al., 2014; Zhang et al., 2014), and tZ-type CKs are major components that are involved in the xylem-mediated CKs translocation (Hirose et al., 2008). The tZ-type CK levels in the shoots were unaffected by rhizobial infection

(Fig. 4). Thus, it is probable that the increased iPRPs levels are caused by CK synthesis in the shoots rather than by transferred CKs from the roots. In *A. thaliana*, the production of iPRPs include the initiation step of CK biosynthesis and involve the total volume of CKs (Miyawaki et al., 2004). Taken together, my results suggest that during nodulation, the production of CKs is induced in the shoots through the activation of the CLE-RS1/2-HAR1 signaling pathway.

5. Feeding CK to the shoots inhibits nodulation and lateral root formation

To investigate whether CKs accumulating in the shoots have SDI-like activities, I applied various concentrations of 6-benzylaminopurine (BAP) to *L. japonicus* MG-20, *har1* and *tml* seedlings via the cut surfaces of cotyledons. The application of 10^{-6} M and 10^{-5} M BAP to MG-20 seedlings decreased the number of nodules to 44% and 13% compared to that of the buffer-only control treatment (Fig. 5a). Similarly, BAP inhibited nodulation in *har1-7* seedlings in a dose-dependent manner (Fig. 5a). Evidently, CK applied to the shoots inhibits nodulation through a mechanism that is downstream of HAR1 action. The inhibitory effect was not observed in plants carrying the *tml* mutation (Fig. 5a), indicating that *TML*, which functions in roots downstream of HAR1, is required for the inhibitory action of CK.

I further examined the effects of CK feeding on lateral root formation, which was affected in the *CLE-RS1/2*ox, as well as on nodule formation (Fig. 2c). The formation of lateral roots was inhibited by BAP fed to the shoots; the lateral root numbers in MG-20 decreased to 40% and 20% of the control by 10^{-7} M and 10^{-6} M BAP, respectively (Fig. 5b). The application of BAP to the shoot also inhibited lateral root formation in *har1-7* mutants (Fig. 5b). *tml* mutants have similar lateral root numbers

with MG-20, but their formation was not inhibited by BAP treatment (Fig. 5b), suggesting that shoot-derived CK controls lateral root formation as well as nodulation in a *TML*-dependent manner.

In *A. thaliana*, the phloem is capable of transporting large amounts of CKs (Bishopp et al., 2011). I applied labeled CK (Tokunaga et al., 2012) to determine whether the CK that is fed to the leaves is transported to the roots in *L. japonicus*. In fact, the labeled CKs were soon detected in the root tips (Table 5). These results indicate that the CKs in the cotyledon are transported to the root tip in *L. japonicus*; thus, it is likely that the BAP that was applied in Fig. 5 is transported to the root.

6. Expression analyses of *L. japonicus IPT* genes in the shoots during nodulation

The quantification of phytohormones demonstrated that *CLE-RS1/2*ox shoots accumulated significantly larger amounts of iPRPs compared to MG-20 shoots. iPRPs are synthesized during the initial steps of the CKs biosynthetic pathway in a reaction that is catalyzed by isopentenyltransferase (IPT). The steps are rate-limiting in CK production, suggesting that the production of iPRPs and the CK levels are regulated by the expression of *ITPs* (Miyawaki et al., 2004; Takei et al., 2001). Six *IPT* genes (*LjIPT*) can be found in the *L. japonicus* genomic database (Chen et al., 2013). I examined the expression patterns of these *LjIPT* genes in the shoots of MG-20, *har1-7* mutants and *CLE-RS1/2*ox plants during nodulation (Fig. 6a). Among the five *LjIPT* genes for which activity could be detected, *LjIPT1* and *LjIPT3* showed increased expression in MG-20 shoots starting at 1 and 3 days after inoculation, respectively. The other three *LjIPT* genes showed no response (Fig. 6a). The responses of the *LjIPT1* and *LjIPT3* genes were not observed in the *har1-7* shoots (Fig. 6a). Moreover, the expression of *LjIPT3*

constitutively increased in *CLE-RS1*ox and *CLE-RS2*ox plants in the absence of rhizobia (Fig. 6b). These findings suggest that the activation of *LjIPT3* in the shoot is closely related to AON activation.

I next examined the expression pattern of *LjIPT3* in the roots. The expression of *LjIPT3* is induced in MG-20 roots by rhizobial infection, as recently shown (Fig. 6c) (Chen et al., 2013). This infection-dependent activation is also observed in *har1-7* (Fig. 6c). Thus, this result suggests that, unlike in the shoots, HAR1 is not required for the activation of *LjIPT3* in the roots. Additionally, the timing of *LjIPT3* activation is different between the shoots and roots, starting 3 days after inoculation in the shoots but as early as 1 day after inoculation in the roots (Fig. 6a,c).

I examined the spatial expression patterns of *LjIPT3* using transgenic *ProLjIPT3:GUS* plants, in which a 2-kb fragment of the *LjIPT3* promoter region was inserted upstream of the *GUS* reporter gene. The GUS signals were detected along a subset of leaf veins in the shoots and bumble cells in the roots (Fig.7a,b,e,f). Detailed analyses of the leaf cross-sections showed the signal being restricted to the phloem tissue (Fig. 7c,d). This pattern resembled that of *HAR1* that was previously reported (Nontachaiyapoom et al., 2007).

7. Functional analysis of the *LjIPT3* gene

To elucidate the function of *LjIPT3* in AON, I identified mutant lines with *LORE1* retrotransposon inserts in the coding sequence of *LjIPT3* (Fig. 8a) (Fukai et al., 2012; Urbanski et al., 2012). In addition, I produced transgenic plants overexpressing *LjIPT3* under the control of the CaMV 35S promoter (Fig. 8b). In *LjIPT3*ox shoots, the amount of iPRPs increased significantly by approximately five-fold in the MG-20

shoots in the absence of rhizobia (Fig. 8c), suggesting that the overexpression of *LjIPT3* is sufficient to increase iPRP levels. *LjIPT3*ox lines and *Ljipt3* mutants exhibited opposite phenotypes in terms of the nodule number. *LjIPT3*ox lines formed only 30-40% of the nodule number that was found in MG-20, whereas the nodule numbers in the *Ljipt3* mutants exceeded those in Gifu B-129 by 58% (Fig. 8d,e). Evidently, *LjIPT3* expression negatively influences nodulation. Furthermore, reciprocal grafting analysis showed that *Ljipt3* shoots increased the number of nodules that formed on Gifu rootstocks, whereas the nodule numbers were comparable between *Ljipt3* and Gifu rootstocks carrying Gifu shoots (Fig. 8f). These results indicate that *LjIPT3* functions in the shoot-dependent inhibition of nodulation. The negative effect of *LjIPT3* expression in the shoot was confirmed by grafting *LjIPT3*ox shoots onto MG-20 wild-type rootstocks (Fig. 8g). As I also observed a reduction of nodule numbers in *LjIPT3*oxrootstocks carrying MG-20 shoots, the expression of *LjIPT3* in roots appeared to inhibit nodulation as well (Fig. 8g).

8. Analyses of the site of AON action in the root

To gain insight into the potential site of AON action in the root, I next examined the interaction between CLE-RS and CK signaling involving LHK1. Proper nodule development requires *LHK1*, which encodes a putative CK receptor (Murray et al., 2007; Tirichine et al., 2007). Initially, I performed grafting using rootstocks of *hit1* and *snf2* mutants, which are loss-of- and gain-of-function mutants of *LHK1*, respectively. It seems that the *hit1* mutation reduces the number of nodules due to a defect in CK signaling in the roots (Murray et al., 2007), and the *snf2* mutation causes the formation of nodule-like structures, termed spontaneous nodules, in the absence of

rhizobia (Tirichine et al., 2007). Grafting analysis showed that *CLE-RS1*ox shoots inhibit nodulation in *hit1-1* and cause spontaneous nodulation in *snf2* rootstocks (Fig. 9a,b). Additionally, CKs that are applied to the shoots inhibit the nodulation of *hit1* mutants (Fig. 9c). These results suggest that the site of AON action in the roots may be downstream of LHK1. In *Medicago truncatula*, it is likely that the CK signaling involving MtCRE1, a counterpart of LHK1, may be involved in nodulation by mediating *NSP2* expression (Ariel et al., 2012). Therefore, I investigated the relationship between the expression of *NSP2* and *CLE-RS1/2* overexpression. In *CLE-RS1/2*ox plants, *NSP2* was downregulated to 15% and 40% of that in the control (MG-20), respectively (Fig. 9d). Additionally, a grafting experiment suggested that TML is necessary for the downregulation of *NSP2* in roots (Fig. 9e). Thus, AON seems to suppress the expression of *NSP2* downstream of LHK1 in TML-dependent manner.

Discussion

AON systemically regulates the number of root nodules through shoot-root communication (Ferguson et al., 2010; Oka-Kira and Kawaguchi, 2006). Inhibition of the formation of excess root nodules by a shoot-derived signal is a key element of AON, but the molecular mechanisms that are involved in the production of the shoot-derived signal are poorly understood. In this dissertation, I have demonstrated that CLE-RS1/2-HAR1 signaling activates CK production in the shoots and that the shoot-derived CKs inhibit nodulation. TML, a component of AON acting in the roots, is required for the inhibitory action of CKs. Thus, these results suggest that the inhibition of nodulation that is caused by CKs is under the control of AON, especially between HAR1 and TML (Fig. 10).

Expression and function of *LjIPT3* and shoot-derived CKs

The results of this study also indicate that LjIPT3 is involved in CK production during nodulation. *LjIPT3* expression is activated in the shoots in an HAR1-dependent manner. Genetic analysis indicates that *LjIPT3* acts as a negative regulator of nodulation. Because the nodulation phenotype of *Ljipt3* knockout mutants is milder than that of canonical AON mutants, several *LjIPT* genes may function redundantly in the control of nodulation. Generally, *A. thaliana IPT* genes seem to have overlapping functions in diverse processes of plant development (Miyawaki et al., 2006). Promoter-reporter analyses showed that the *LjIPT3* promoter is active specifically in phloem cells, suggesting that CKs may be synthesized in the shoot phloem upon rhizobial infection of the roots (Fig. 7). In *A. thaliana*, iP-type CKs are transported from the shoot to the root via phloem sieve tubes (Hirose et al., 2008; Sakakibara, 2006), and I demonstrated that

CKs are transported from the cotyledons to the root tips in *L. japonicus*. Additionally, in grafting experiments, nodulation was inhibited when *LjIPT3*ox plants were used as scions (Fig. 8), which is consistent with the notion that shoot-derived CKs inhibit nodulation in roots.

Dual role of CKs in nodulation

CKs have long been suggested to act as positive regulators of nodule development. For example, a nodulation-deficient *Rhizobium* mutant could be recovered by introducing a gene that is related to *trans*-zeatin secretion (Cooper and Long, 1994), and recent genetic analyses demonstrated that the activation of CK signaling in host plants is necessary and sufficient to form nodule primordia (Heckmann et al., 2011; Murray et al., 2007; Tirichine et al., 2007). Moreover, LjIPT3 is involved in CK production in roots with positive effects on nodulation (Chen et al., 2013). In contrast, my results showed a negative effect of shoot-derived CKs on nodulation, as CKs act systemically to restrict the number of nodules. The production of CKs in both the shoots and the roots during nodulation is induced by the expression of *LjIPT3*, but the timing of *LjIPT3* activation is different between the shoots and the roots. *LjIPT3* exhibited increased expression in MG-20 shoots starting 3 days after inoculation (Fig. 6a). This timing for the induction is consistent with that of the initiation of AON that was previously reported (Suzuki et al., 2008). In contrast, *LjIPT3* induction was observed in MG-20 roots starting 1 day after inoculation (Fig. 6c).

Additionally, the results of this study also indicate that *LjIPT3* expression in the shoots is induced in an HAR1-dependent manner, whereas HAR1 is not required for the activation of *LjIPT3* in the roots. These findings suggest that CKs, which negatively

affect nodulation, may be produced following AON signaling in the shoots, whereas CKs with positive effects on nodulation may be produced independently of AON signaling in the roots. Therefore, different induction mechanisms, including the differential timing of CK production and HAR1-dependence, can provide CKs with a dual role in nodulation.

The CKs with a negative and positive effect on nodulation are expected to accumulate in different tissues of the infected roots. It is suggested that CKs with a positive effect on nodulation accumulate in epidermal or cortical cells, whereas the results of this study suggest that CKs with a negative effect are transported through phloem cells to the roots from the shoots (Ferguson et al., 2010). Thus, the difference in accumulating cells is likely to result in a dual role of CKs in nodulation.

Loss-of-function phenotypes of LjIPT3

The visible loss-of-function phenotypes of LjIPT3 might differ drastically between different lines due to the dual role of CKs. RNA interference knockdown lines of *LjIPT3* decreased nodule number, most likely because the nodulation-promoting function of LjIPT3 is predominantly affected (Chen et al., 2013). In contrast, the knockout lines that were used in the present study (*Ljipt3-1* and *Ljipt3-2*) exhibited increased nodule numbers, presumably due to defects in the nodulation-restricting role of LjIPT3 in the shoots. In this case, any inhibition of the positive effects of LjIPT3 in roots does not become apparent. In *Ljipt3* mutants, the CKs with positive effects should be accumulated by an unknown factor other than LjIPT3, because *Ljipt3* mutants can form nodules. Thus, the different contribution rates of CK accumulation by LjIPT3 in the shoots or roots are likely to provide loss-of-function lines of LjIPT3 with different

phenotypes. Additionally, off-target effects by RNA interference should be considered, because the genome sequence of *L. japonicus* has not been completed. Thus, the expression of unidentified *LjIPT* having positive effects for nodulation may be downregulated in RNA interference knockdown lines of *LjIPT3*. In this case, the difference of visible loss-of-function phenotypes of LjIPT3 is thought to result from off-target effects by RNA interference.

Site of AON action

NSP2 is a component that constitutes Nod factor signaling and encodes a GRAS transcription factor that is required for nodule initiation (Kaló et al. 2005, Murakami et al. 2006, Hirsch et al. 2006). A grafting experiment and expression analysis suggest that AON may suppress *NSP2* expression downstream of LHK1 and/or in a TML-dependent manner. CK application to the roots significantly downregulates *NSP2* expression after transient upregulation (Ariel et al., 2012). Similarly, shoot-derived CKs may control the number of nodules by downregulating *NSP2* expression. A grafting experiment showed that AON retains its inhibitory effects on nodulation in the LHK1 mutant background. Additionally, feeding experiments indicate that the CKs that were applied to shoots also inhibited nodulation in the LHK1 mutant background. *L. japonicus* has three putative CKs receptors with overlapping functions in the control of nodulation (Held et al., 2014). Based on these findings, I hypothesize that CKs with negative effects on nodulation may suppress the expression of *NSP2* via CK receptor(s) other than LHK1. This differential function of CK receptors may explain the dual role of CKs.

CKs and lateral root formation

Auxin and CKs interact in a complex manner to control many aspects of growth and differentiation (Su et al., 2011). In lateral root formation, the accumulation of auxin is an initiation step, while CK signaling inhibits the accumulation of auxin by interacting with factors that are involved in the accumulation of auxin, such as SHY2 and PIN (Desbrosses and Stougaard, 2011; Perianez-Rodriguez et al., 2014). Thus, CKs are negative factors in the formation of lateral roots. In this dissertation, I demonstrate that the CKs that are produced in the shoots block the formation of lateral roots in a TML-dependent manner, indicating that nodulation and lateral root formation are systemically regulated via the same pathway that is downstream of CK production in the shoot. Although *tml-4* mutants were insensitive to shoot-applied CKs with regard to the inhibition of lateral root formation, the lateral root numbers of the mutants were similar to those of the wild type, suggesting that the TML regulation of lateral root development involves additional factors. For instance, nitrate influences both lateral root development and nodulation (Alvarez et al., 2012; Cho and Harper, 1991; Zhang et al., 1999). These additional factors may regulate lateral root number in TML-independent manner. Otherwise, TML itself may not be involved in lateral root formation. TML homologs are well conserved, even in non-leguminous plants, such as *Arabidopsis*. Thus, a functional analysis of TML homologs in *L. japonicus* and *Arabidopsis* is necessary.

Transcriptome analysis

A transcriptome analysis indicated that the expression of a gene (accession number: LC013384) of unknown function is regulated by AON signaling in the shoots,

but there is no homologue in *A. thaliana*. The transcript has only one short ORF (77 amino acids). mRNA coding a short ORF functions as the RNA itself and/or the template for the oligopeptide. The oligopeptides may act as signals in development (Lindsey et al., 2002). RNA can play an important role in the function of the gene, and they are called non-coding RNAs (Furini et al., 1997; Erdmann et al., 2001; MacIntosh et al., 2001). Thus, LC013384 may function as oligopeptide or non-coding RNA on AON. In the shoots, the only genes that have been identified as involved in AON are LRR-RLKs, such as HAR1, CLV2 and KLV. The molecular mechanism of AON in the shoots is mostly unknown. In further works, an analysis of LC013384 may help reveal the mechanism of AON in the shoots.

General discussion

The results of this study suggest that shoot-derived CKs act as shoot-derived signals for the inhibition of nodulation. In addition, these results surround the signaling regulation and growth of plants, which are not limited to the regulation of the nodule number.

I indicate that TML, a component that is downstream of HAR1 in the AON, is required for the inhibition of nodulation by shoot-derived CKs. In *A. thaliana*, KMD, a Kelch repeat F-box protein that resembles TML (Fig. 11), forms an S-PHASE KINASE-ASSOCIATED PROTEIN 1 SKP1/Cullin/F-box protein (SCF) E3 ubiquitin ligase complex and degrades a type-B response regulator of CK signaling (Kim et al., 2013b; Takahara et al., 2013). SCF complexes catalyze the ubiquitination and proteasomal degradation of target proteins, and F-box proteins constructing the SCF complex act as receptors of phytohormones, such as auxin, gibberellin and jasmonic acid; the SCF complex interacting with each plant phytohormone activates this hormone signaling via the degradation of inhibitors, such as IAA, DELLA and JAZ (Santner and Estelle, 2009). As an attractive hypothesis, CKs and TML may also be applied to the framework between phytohormones and the F-box protein on the SCF complex, namely that TML proteins interacting with shoot-derived CKs may suppress nodulation by the ubiquitin-mediated degradation of the type-B response regulator. Type-A and type-B response regulators are positive factors for nodulation (Gonzalez-Rizzo et al., 2006; Op den Camp et al., 2011). In this study, I suggest that AON suppresses the expression of *NSP2* downstream of LHK1; *NSP2* has a potential response regulator binding site (Ariel et al., 2012). The site of AON action may be a response regulator of CK signaling that is targeted by TML (Fig. 12a). CKs are widely involved in plant development; therefore,

the finding that an F-box protein is a CK receptor provides insight into plant developmental research. I expect that a study of TML would elucidate the relationship between CKs and F-box proteins.

Nitrate influences both lateral root development and nodulation (Alvarez et al., 2012; Cho and Harper, 1991; Zhang et al., 1999). Nodulation is suppressed by nitrate, and AON factors, such as HAR1, KLV and TML, are involved in this inhibitory regulation (Caetano-Anolles and Gresshoff, 1991; Magori et al., 2009; Oka-Kira and Kawaguchi, 2006; Oka-Kira et al., 2005; Wopereis et al., 2000). Nitrate systemically regulates the root architecture and induces *IPT3* expression in *A. thaliana* shoots (Alvarez et al., 2012; Ruffel et al., 2011; Takei et al., 2004). Thus, CKs seem to play a central role in the systemic regulation of root lateral organ development. However, the underlying mechanisms remain obscure. Interactions between CKs and TML may be essential for the negative regulation of nodulation as well as lateral root development by CKs. Further investigations focusing on the interaction between CKs and TML will elucidate how CKs achieve their dual function in the control of nodule development and may provide insight into the long-distance regulatory mechanism that is required for nodulation and lateral root development. In addition, AON mutants, such as *har1* and *klv,* exhibit morphological change of not only the roots but also the shoots (Miyazawa et al., 2010; Nishimura et al., 2002a). The transcriptome analysis in this study indicated that the genes that are involved in the growth of plants, such as the genes that are involved in response to phytohormones and the production of the cell wall, are upregulated in *CLE-RS1/2*ox shoots. These findings suggest that AON affects the growth of shoots. It has been proposed that CKs are long-distance signals that regulate the balance between the shoots and the roots (Beck, 1996). Therefore, AON may be a

mechanism to regulate the balance between the growth of plants and the amount of lateral roots and nodules (Fig. 12b).

The findings of this study add essential components to understanding of how legumes control nodulation to balance nutritional requirements and the energy status and will be valuable for understanding the molecular mechanisms involving the systemic regulation of CKs. In future work, understanding of the relationship between CKs and SCF/proteasome-mediated degradation by TML during nodulation will help to reveal general and novel roles of CKs.

Materials and methods

Plant materials and growth conditions

I used *L. japonicus* accessions Miyakojima MG-20 and Gifu B-129 as the wild type and the *Ljipt3-1* and *Ljipt3-2LORE1* retrotransposon insertion lines with plant IDs 30001893 and 30012123, respectively. The hypernodulation mutants were *har1-7*, *tml-1* and *tml-4* (Magori et al., 2009; Takahara et al., 2013). *tml-1* is the large deletion allele that is produced by carbon-ion-beam irradiation, and mutation *tml-4* is a single-base substitution that is produced by ethyl methane sulfonate mutagenesis. Mutants of *LHK1* were *hit1-1* (Murray et al., 2007) and *snf2* (Miyazawa et al., 2010). The plants were grown in autoclaved vermiculite that was supplemented with Broughton and Dilworth (B&D) solution (Broughton and Dilworth, 1971) with 0.5 mM KNO_3 under 16-h light/8-h dark cycles. The *Rhizobium* strain *Mesorhizobium loti* MAFF 303099 was used for nodulation.

Constructs and transformation

p35S-CLE-RS1, p35S-CLE-RS2 and p35S-GUS constructs reported previously(Okamoto et al., 2009) were used to produce stably transformed plants that constitutively expressed either *CLE-RS1* or *CLE-RS2*.

For overexpressing *LjIPT3*, the *LjIPT3* ORF fragment was amplified from MG-20 genomic DNA using the specific primer set (5'-CACCGATCAGATACCAATTTTGCA-3' and 5'-ACCACCCTCTATGAACATAACTAAC-3'), and cloned into the pENTR/D-TOPO vector (Invitrogen). The insert was transferred downstream of p35S promoter of pH7WG2D(Karimi et al., 2002) by LR clonase (Invitrogen).

For promoter analysis of *LjIPT3*, the 2 kb DNA fragment upstream of the putative translation initiation codon was cloned into the pENTR/D-TOPO vector, and transferred to pGWB3(Nakagawa et al., 2007) by Gateway LR reaction. The promoter fragment was amplified from MG-20 genomic DNA using the specific primer set (5'-CACCTTTGGTAATTGAATTTAATGGGCA-3' and 5'-GGTGAATTGCAAAATTGGTATCTGATC-3'). Resultant binary vectors were introduced into *Agrobacterium tumefaciens* strain AGL1 and transformed into MG-20 as described previously (Sasaki et al., 2013).

Grafting experiments

The seeds were sown on vertical 1% agar plates and grown for 2 days in darkness. The plants were further cultured for 2 days in 16-h light/8-h dark cycles. Root stocks were produced by cutting off the hypocotyl at its base. A short vertical slit (approximately 2 mm) was made in the basal hypocotyl stump with a scalpel blade. A shoot scion was inserted into this vertical slit. The grafted plants were grown on filter papers that were saturated with sterilized water in Petri dishes for 4 days before transfer to vermiculite that was supplemented with B&D solution and 0.5 mM KNO_3 (Magori et al., 2009).

Isolation of the Total RNA and Next-generation Sequencing

The shoots of 2-week-old plants were collected 3 days after inoculation with *M. loti*. The total RNA was isolated using PureLink^{TM} Plant RNA Reagent (Invitrogen). To eliminate genomic DNA, DNase I (Ambion) was used, and the total RNA was repurified over RNeasy mini columns (QIAGEN). Library preparation for next generation sequencing was performed using the Illumina TrueSeq library preparation kit.

RNA-Seq was performed on an Illumina HiSeq 2000 machine by single-end sequencing in three independent biological repeats. The read length was 50 bp.

Analysis of RNA-seq datasets

High-quality reads were defined as reads containing more than 90% of the bases with a Phred score > 20. The selected high-quality reads were aligned with the reference transcriptome (ftp://ftp.kazusa.or.jp/pub/lotus/lotus_r2.5/Lj2.5_genome.contigs.fna.gz) by Tophat version 2.0.13 (Kim et al., 2013a; Trapnell et al., 2009). To generate FPRM, CuffDiff of CuffLinks version 2.1.1 was used, and CuffDiff was used to calculate the DEGs (Trapnell et al., 2012; Trapnell et al., 2010). Blast was performed by Protein-Protein BLAST version 2.2.29+ (Altschul et al., 1997). GO analysis was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7. De-novo transcriptome assembly was performed by Trinity program as described elsewhere (Grabherr et al., 2011).

Quantification of phytohormones

Shoots of 2-week old plants were collected 3 days after inoculation with *M. loti*, frozen in liquid N_2 and stored at -80°C. Preparation of crude extracts form 100 mg shoot tissues and determination of primary metabolites was conducted in three independent biological repeats according to Kojima et al. (Kojima et al., 2009). Determined Phytohormones were *trans*-zeatin (tZ); tZ riboside (tZR); tZR 5'-phosphates (tZRPs); *cis*-zeatin (cZ); cZ riboside (cZR); cZR 5'-phosphates (cZRPs); dihydrozeatin (DZ); DZ riboside (DZR); DZ riboside 5'-phosphates (DZRPs); N^6-(Δ^2-isopentenyl) adenine (iP); iP riboside (iPR); iPR 5'-phosphates (iPRPs); tZ-7-*N*-glucoside (tZ7G);

tZ-9-*N*-glucoside (tZ9G); tZ-*O*-glucoside (tZOG); cZ-*O*-glucoside (cZOG); tZR-*O*-glucoside (tZROG); cZR-*O*-glucoside (cZROG); tZRPs-*O*-glucoside (tZRPsOG); cZRPs-*O*-glucoside (cZRPsOG); DZ-9-*N*-glucoside (DZ9G); iP-7-*N*-glucoside (iP7G); iP-9-*N*-glucoside (iP9G); Gibberellin (GA); Salicylic acid (SA); Jasmonic acid (JA); indole-3-acetic acid (IAA); Abscisic acid (ABA).

Quantification of cytokinins

The shoots of 2-week old plants were collected 5 days after inoculation with *M. loti* (infection sample) or treatment with B&D solution in the absence of rhizobia (non-infection sample). The preparation of crude extracts from 100-mg shoot tissues and the determination of the CK contents were conducted according to Kojima et al. (Kojima et al., 2009).

CK feeding assay

The distal half of a cotyledon was removed from 2-day-old seedlings that were grown on vermiculite. The remaining cotyledon stump was inserted into a plastic tube that was filled with 10 mM MES (pH 6.2) containing 6-benzylaminopurine (BAP). On the following day, the plants were inoculated with rhizobia. Nodules were counted 2 weeks after inoculation.

Expression analysis

Total RNA was isolated from plants using the Concert Plant RNA Reagent (Invitrogen). First-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time RT-PCR was performed on an ABI prism 7000 sequence detection

system (Applied Biosystems) using the THUNDERBIRD SYBR qPCR Mix (Toyobo) according to the manufacturer's protocol. The following primers were used in the expression analysis: *ubiquitin*, 5'-ATGTGCATTTTAAGACAGGG-3' and 5'-GAACGTAGAAGATTGCCTGAA-3'; *LjIPT1*, 5'-AAAGGACTCGACACCACCAC-3' and 5'-GGGTGAAATCACCGAGAGAA-3'; *LjIPT3*, 5'-CTGAGCATGAGGCAGATTCA-3' and 5'-TTTCTGAGGGGAAACAGGTG-3'; *LjIPT4*, 5'-AGCCAAACACTTCCAACCAG-3' and 5'-GTGGGATTCCACCACACTCT-3'; *LjIPT5*, 5'-GTGGGCATGGAAAACGTATC-3' and 5'-TAGAATGCTCCCGCTTCTGT-3'; *LjIPT9*, 5'-GCCGTGTTCCCATAGTTGTT-3' and 5'-GCAGAGGCTTTAGGCACATC-3'; *CLE-RS1*, 5'-TGCAAGTGTCGATGCTCATAGC-3' and 5'-GATGTTTTGCTGAACCAAGGGATA-3'; *CLE-RS2*, 5'-GCTCGTAATCTCCAAATCATTCACA-3' and 5'-GGTGAGAGTCTTTGCTGTTGATATCC-3'; *NSP2*, 5'-CAAACAAGAACCGCGAACTG- 3' and 5'-TTGCTGCTGCTATTGTACGC-3'. Expression of *ubiquitin* served as the reference. Data are shown as the mean ± s.d. of three biological replicates.

GUS assay

Leaves were incubated in 90% acetone for 15 min at -20°C and then incubated with GUS staining buffer (0.5 mg ml^{-1} X-gluc, 100 mM phosphate buffer)(Takeda et al., 2012) for 24 h at 37°C. The samples were washed twice with 100 mM $NaPO_4$ (pH 7.0), bleached with 70% ethanol at room temperature, and dehydrated in 100% ethanol. Then,

the samples were embedded in Technovit 7100 resin (Haraeus Kulzer) according to the manufacturer's protocol and sectioned (5-μm thickness) using a Microtome RM2255 (Leica). The sections were stained with 0.02% toluidine blue (SIGMA) before observation.

CK transport assay

The distal half of each cotyledon was excised from seedlings that were grown for 3 days on a 1% agar plate. Three microliters of 0.5% agar containing 100 pmol isotope-labeled iPR, [$^{13}C_{10}$,$^{15}N_5$]N^6-(Δ^2-isopentyl) adenine riboside (Sakakibara laboratory, RIKEN Center for Sustainable Resource Science) (Tokunaga et al., 2012) were placed on the cut surface of each cotyledon. The plants were placed vertically on sterilized filter paper that was saturated with sterilized water in Petri dishes for 4 h. Twenty root tips (2 mm) were collected and stored at -80°C. The CK contents were quantified as previously described (Kojima et al., 2009).

Accession number

The nucleotide sequences reported in this paper have been submitted to the NCBI database with accession numbers: *LjIPT1*, DQ436462; *LjIPT2*, DQ436463; *LjIPT3*, DQ436464; *LjIPT4*, DQ436465; *LjIPT5*, ABW77761; *LjIPT9*, EEE85226.2.

Tables and Figures

Table 1 Summary of mapping result.

	Replicate	Total HQ reads	Mapped reads	Multiposition match
MG-20	1	17764081	14053106 (79.1%)	1928922 (13.7%)
	2	19801069	15613590 (78.9%)	1693640 (10.8%)
	3	9861836	7858577 (79.7%)	1029192 (13.1%)
har1-7	1	20217972	16020134 (79.2%)	2017954 (12.6%)
	2	16572101	13136134 (79.3%)	1529227 (11.6%)
	3	20860582	16412542 (78.7%)	1775307 (10.8%)
*CLE-RS1*ox	1	17624470	13633383 (77.4%)	1374863 (10.1%)
	2	22298870	17351898 (77.8%)	1620342 (9.3%)
	3	13255374	10377333 (78.3%)	1165992 (11.2%)
*CLE-RS2*ox	1	12897486	10164253 (78.8%)	1183670 (11.6%)
	2	13671300	10770797 (78.8%)	1154118 (10.7%)
	3	19478473	14928593 (76.6%)	1698572 (11.4%)

The quality of the RNA-Seq dataset is assessed by gene coverage, which is the percentage of a gene covered by reads. This value is determined as the ratio of the base number in a gene covered by unique mapping reads to the total bases number of that gene.

Table 2 Expression propaties of CM0553.180

	MG	har1	RS1	RS2
RPM (Replicate 1)	2.14	0.25	31.26	40.47
RPM (Replicate 2)	1.62	0.24	34.67	34.82
RPM (Replicate 3)	0.61	0.34	49.87	31.93
Fold change (log_2)		-2.40	4.73	4.62
FDR		0.00302845	0.00172977	0.00221183

DEGs were identified as genes with more that 2-fold change and FDR < 0.05 compared with MG-20 dataset. FDRs were culculated by CuffDiff of CullLinks version 2.1.1.

Table 3 GO enrinchment analysis of DGEs.

GO terms for upregulated DEGs

	GO term	Count	P-Value	Benjamini
BP	response to organic substance	10	1.20×10^{-3}	2.30×10^{-1}
BP	defense response	9	2.20×10^{-3}	2.10×10^{-1}
BP	cell death	5	2.90×10^{-3}	1.90×10^{-1}
BP	death	5	2.90×10^{-3}	1.90×10^{-1}
BP	response to chitin	4	3.30×10^{-3}	1.60×10^{-1}
BP	immune response	5	4.80×10^{-3}	1.90×10^{-1}
BP	response to carbohydrate stimulus	4	1.10×10^{-2}	3.40×10^{-1}
BP	response to oxidative stress	4	3.00×10^{-2}	6.00×10^{-1}
BP	phenylpropanoid biosynthetic process	3	3.60×10^{-2}	6.30×10^{-1}
BP	cell wall modification	3	4.00×10^{-2}	6.30×10^{-1}
BP	response to wounding	3	4.70×10^{-2}	6.40×10^{-1}
MF	transcription regulator activity	9	3.30×10^{-2}	9.10×10^{-1}
MF	transcription factor activity	8	4.70×10^{-2}	8.30×10^{-1}

GO terms for downregulated DEGs

	GO term	Count	P-value	Benjamini
BP	response to hormone stimulus	9	2.20×10^{-3}	3.60×10^{-1}
BP	response to endogenous stimulus	9	3.40×10^{-3}	2.90×10^{-1}
BP	response to organic substance	9	1.00×10^{-2}	5.10×10^{-1}
BP	response to abscisic acid stimulus	4	3.90×10^{-2}	8.70×10^{-1}
BP	response to gibberellin stimulus	3	3.90×10^{-2}	8.10×10^{-1}
BP	cellular glucan metabolic process	3	4.20×10^{-2}	7.70×10^{-1}
CC	cell wall	10	9.90×10^{-7}	3.30×10^{-5}
CC	external encapsulating structure	10	1.10×10^{-6}	1.80×10^{-5}
CC	plant-type cell wall	6	1.30×10^{-4}	1.40×10^{-3}
CC	apoplast	6	6.50×10^{-4}	5.30×10^{-3}
CC	extracellular region	8	6.30×10^{-3}	4.10×10^{-2}
MF	xyloglucan:xyloglucosyl transferase activity	3	2.00×10^{-3}	1.50×10^{-1}
MF	protein dimerization activity	4	1.40×10^{-2}	4.30×10^{-1}
MF	transcription regulator activity	9	3.30×10^{-2}	5.80×10^{-1}
MF	transcription factor activity	8	4.70×10^{-2}	6.10×10^{-1}

GO enrichments for biological processes (GO), cellulr compoment (CC) and molecular function (MF) were prepared using DAVID. Figure shows GO term, number of genes (count), and P-values for EASE score (P-value < 0.05) and Benjamini adjustment.

Table 4

Comparison of phytohormone levels between shoots of MG20, *har1-7* mutants and *CLE-RS1/2*ox plants.

	MG-20	*har1-7*	*CLE-RS1*ox	*CLE-RS2*ox
tZ	0.70±0.42	0.53±0.23	1.12±0.55	N.D.
tZR	0.22±0.01	0.15±0.01 dec**	0.26±0.04	0.23±0.03
tZRPs	0.45±0.19	0.42±0.10	1.04±0.20 inc*	0.64±0.13
cZ	N.D.	N.D.	N.D.	N.D.
cZR	N.D.	N.D.	N.D.	N.D.
cZRPs	0.07±0.02	0.08±0.02	0.11±0.03	0.10±0.04
DZ	N.D.	N.D.	N.D.	N.D.
DZR	N.D.	N.D.	N.D.	N.D.
DZRPs	0.55±0.09	0.48±0.15	0.25±0.03 dec*	0.36±0.04
iP	0.47±0.34	0.48±0.37	1.20±0.95	0.92±0.74
iPR	0.15±0.08	0.09±0.04	0.36±0.19	0.25±0.12
iPRPs	3.60±0.29	2.40±0.15 dec*	12.66±0.16 inc***	7.34±1.54 inc*
tZ7G	N.D.	N.D.	N.D.	N.D.
tZ9G	N.D.	N.D.	N.D.	N.D.
tZOG	4.97±2.41	4.21±2.56	5.17±2.14	3.30±1.93
cZOG	N.D.	N.D.	N.D.	N.D.
tZROG	0.15±0.07	0.07±0.04	0.24±0.08	0.09±0.03
cZROG	0.16±0.06	0.08±0.03	0.12±0.08	0.23±0.18
tZRPsOG	N.D.	N.D.	N.D.	N.D.
cZRPsOG	N.D.	N.D.	N.D.	N.D.
DZ9G	0.87±0.01	1.07±0.12	0.42±0.10 dec*	0.48±0.21
iP7G	N.D.	N.D.	N.D.	N.D.
iP9G	0.54±0.13	0.37±0.08	1.05±0.33	0.69±0.15
GA1	2.24±0.96	1.34±0.35	0.84±0.15	2.21±0.72
GA3	N.D.	N.D.	N.D.	N.D.
GA4	N.D.	N.D.	N.D.	N.D.
GA7	N.D.	N.D.	N.D.	N.D.
GA8	9.06±1.57	6.31±0.80	7.21±0.74	7.42±1.91
GA9	N.D.	N.D.	N.D.	N.D.
GA19	61.03±8.61	67.37±2.84	44.98±0.48	68.83±5.57
GA20	8.50±1.19	5.51±0.94	6.22±1.07	11.37±0.58
GA24	0.73±0.14	1.02±0.11	0.58±0.10	0.54±0.03 inc*
GA44	1.68±0.33	0.85±0.09 dec*	1.51±0.22	2.19±0.32
GA53	4.10±0.68	4.62±0.52	2.23±0.09 dec*	3.74±0.43
SA	2077.71±227.77	4083.6±838.25 inc*	3746.33±648.02 inc*	3382.37±1429.06
JA	104.12±1.42	91.37±21.15	93.29±47.40	88.73±16.20
IAA	205.7±12.00	331.64±146.73	258.37±31.78	321.73±107.61
ABA	44.61±4.31	43.44±4.72	52.67±10.18	51.76±13.60

pmol/gFW

Phytohormones were analyzed by mass spectrometry. Means ± SE from three independent experiments are shown. Asterisks indicate that contents of the compound were significantly increased (inc) or decreased (dec) compared to MG20 (*P <0.05, **P <0.01, ***P <0.001; Student's t-test). N. D., not detected.

Table 5 Labeled CK applied to the cotyledon is transported to the root tip.

	Control1	Control2	Control3	Sample1	Sample2	Sample3
labeled iP, iPR and iPRPs	N.D.	N.D.	N.D.	1.0448	0.6634	1.8270
labeled tZ, tZR and tZRPs	N.D.	N.D.	N.D.	0.0237	0.0152	0.0341
labeled cZ, cZR and cZRPs	N.D.	N.D.	N.D.	0.0081	0.0037	0.0066

pmol/1sample

Labeled iPR, [$^{13}C_{10}$,$^{15}N_5$]N^6-(Δ^2-isopentyl) adenine riboside, was applied to cut edges of cotyledons (3-days-old seedlings). Root tips (2 mm) were collected 4 h after the treatment. Labeled and authentic CKs were analyzed by mass spectrometry. The applied iPR is metabolized to iP, iPRPs, tZ-type CKs and cZ-type CKs. CK contents of untreated plants were determined as control. Data from three independent replicates are shown. N. D., not detected.

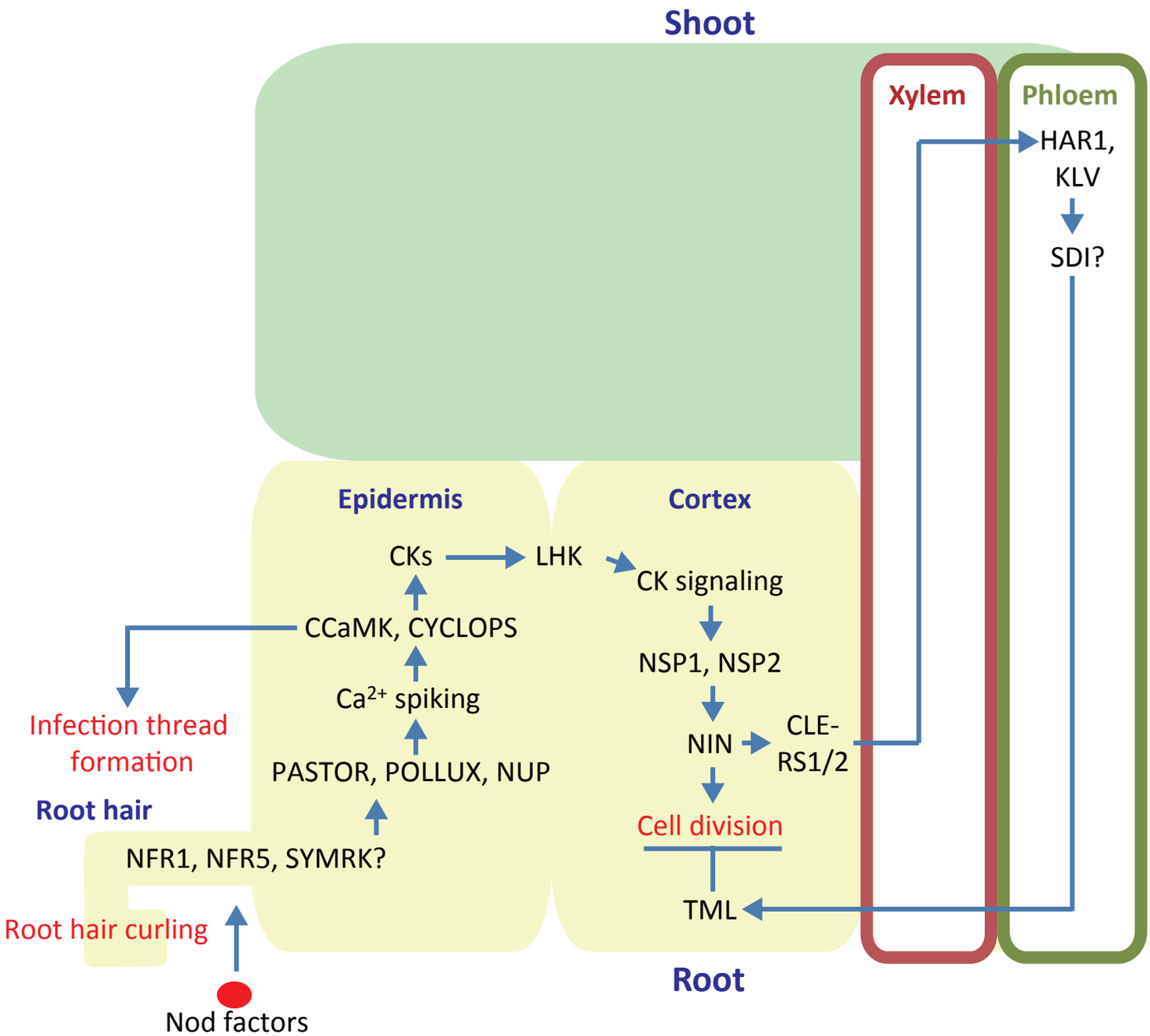

Figure 1 Overview of pathways involved in nodulation. See general introduction for details.

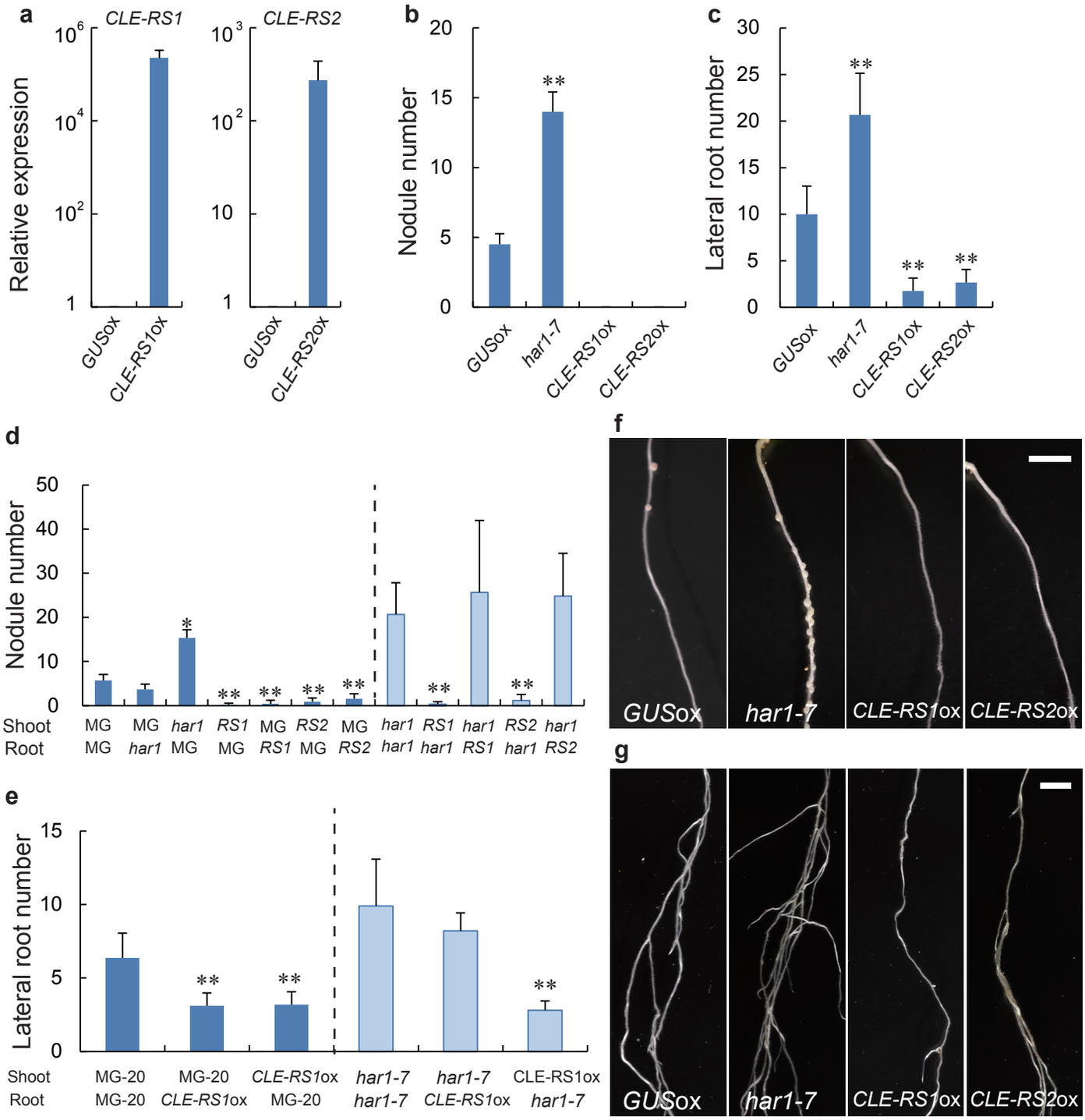

Figure 2 Phenotypic analysis of *CLE-RS1* and *RS2* (a) Expression of *CLE-RS1/2* in transformed plants. Expression levels relative to *GUS*ox controls are shown. Data presented are means ± SD from three biological repeats. Transcript amounts in different samples were normalized to those of *UBIQUITIN*. (b) Nodule number in *GUS*ox, *har1-7* mutant and *CLE-RS1/2*ox plants (n = 6). Nodules were counted 14 days after infection with *M. loti*. (c) Lateral root numbers (n = 6-10) at 21 days after germination in the absence of rhizobia. (d,e) Shoot-to-root reciprocal grafting between MG-20, *har1-7* mutant and *CLE-RS1/2*ox plants (n = 6-9). (d) Nodules were counted 14 days after infection. (e) Lateral roots were counted 21 days after grafting (n = 10-13). Plants were grown in the absence of rhizobia. Bars in (a-e) represent means ± SD. Asterisks indicate statistically significant differences from the control (a, b: *GUS*ox, c, d: MG-20 or *har1-7*) at P<0.05 (*) and 0.01 (**), according to Student's t-test (a, b) and Tukey HSD (c, d). (f-g) Nodulation phenotypes (f) and root phenotypes (g). Bars: 5 mm in (f) , 1 cm in (g).

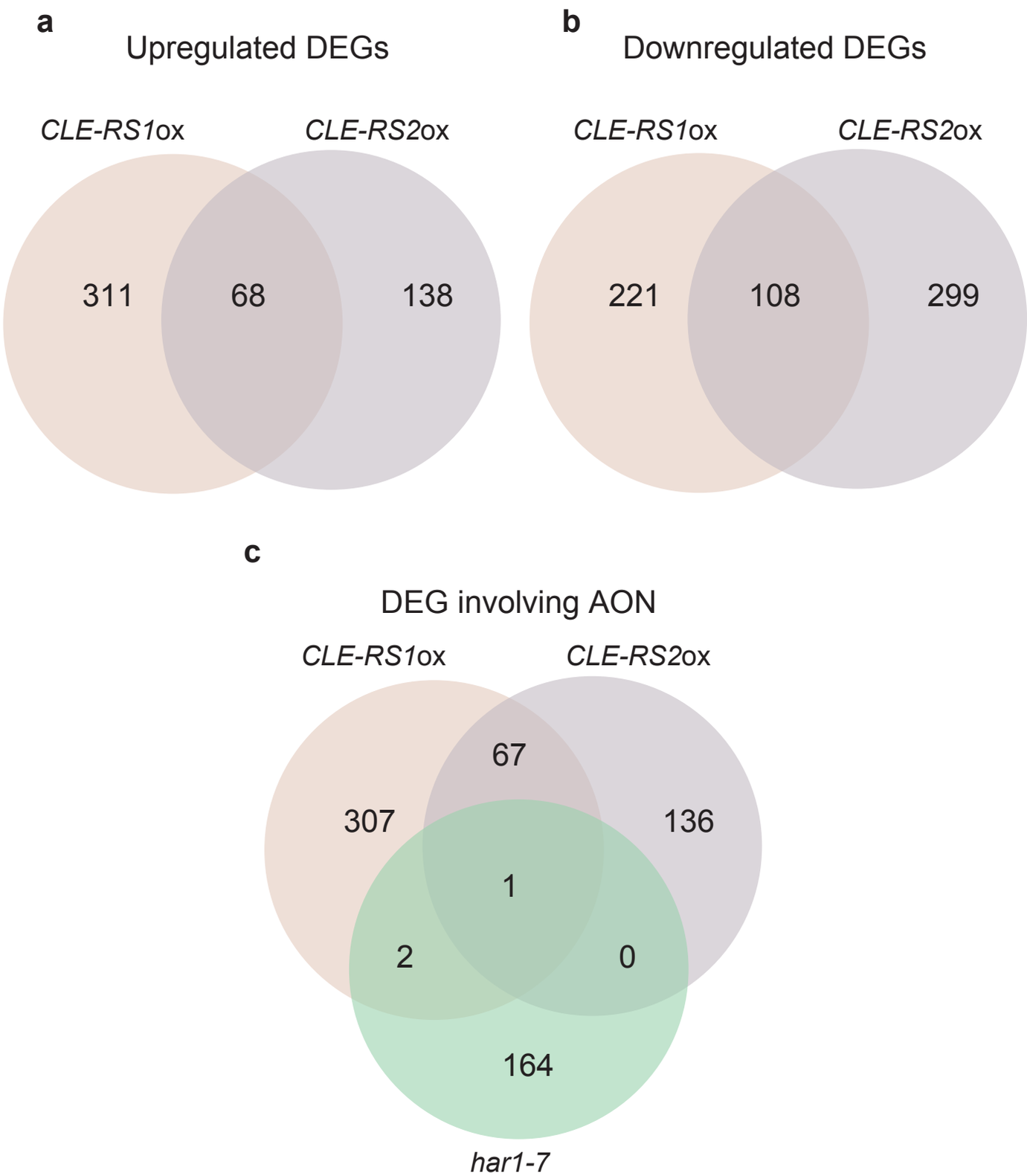

Figure 3 Venn diagrams based on the RNA-seq results. Intersection between upregulated DEGs compared with MG-20 (a), downregulated DEGs compared with MG-20 and upregulated DEGs in *CLE-RS1/2*ox and downregulated DEGs in *har1-7* compared with MG-20 (c). DEGs were identified as genes with more that 2-fold change and FDR < 0.05 compared with MG-20 dataset.

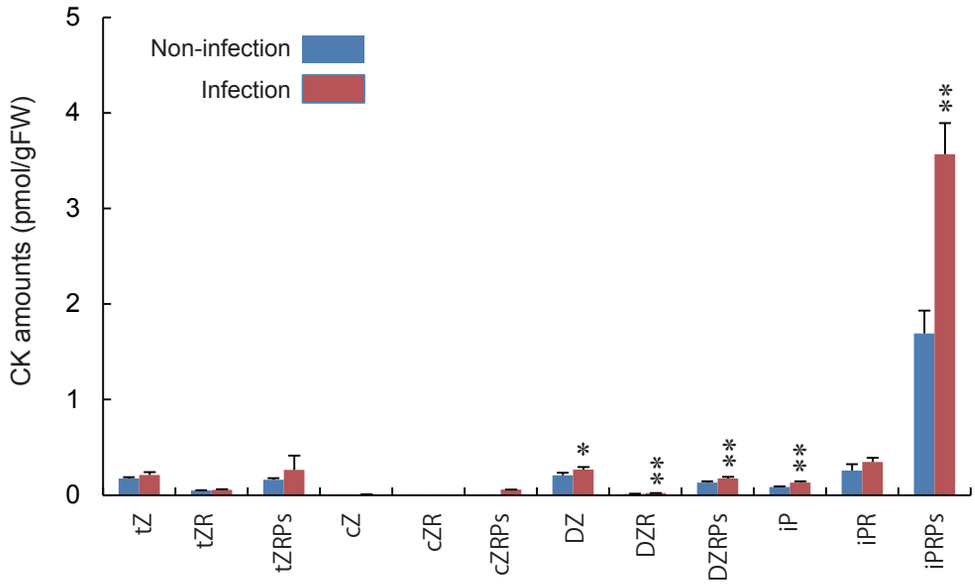

Figure 4 iPRPs level in shoots is increased by rhizobial infection. CK levels in shoots were determined at five days after infection with *M. loti*. (infection) or in the absence of rhizobia (non-infection). Means ± SD from three independent experiments are shown. Asterisks indicate that contents of the compound show statistically significant differences compared with non-infection (*P <0.05, **P <0.01; Student's t-test).

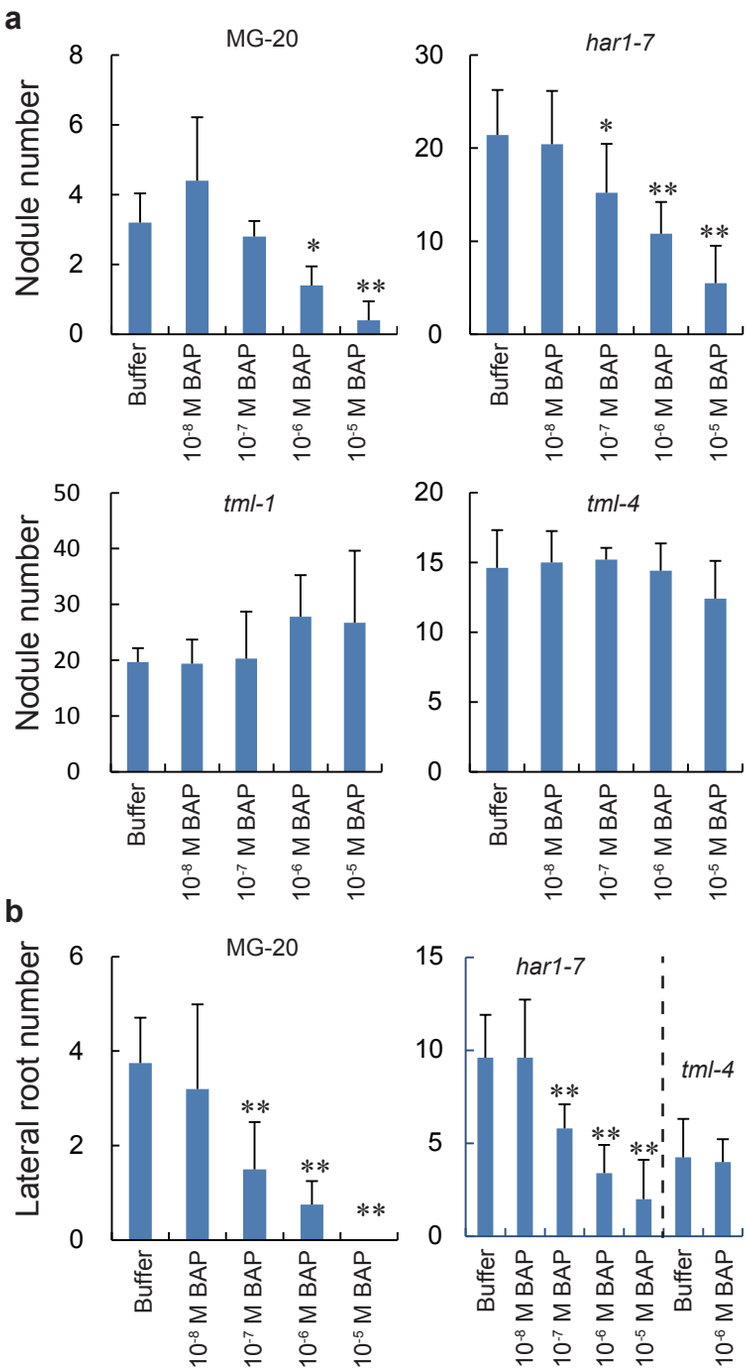

Figure 5 BAP suppresses nodulation and lateral root formation in a TML-dependent manner. (a) Effects on nodulation of BAP application to shoots in MG-20 and *har1-7*, *tml-1* and *tml-4* mutants (n = 5). Distal halves of cotyledons were removed at 2 days after germination, and BAP was fed to the cut surfaces. Plants were inoculated with *M. loti* one day after starting BAP application. Nodules were counted 14 days after infection. (b) Effects on lateral root formation of BAP application to shoots in MG-20, *har1-7* and *tml-4* mutants (n = 5). Lateral roots were counted 21 days after starting BAP application in the absence of rhizobia. Data presented are means ± SD. Asterisks indicate statistically significant differences at P<0.05 (*) and P<0.01 (**), according to Student's t-test.

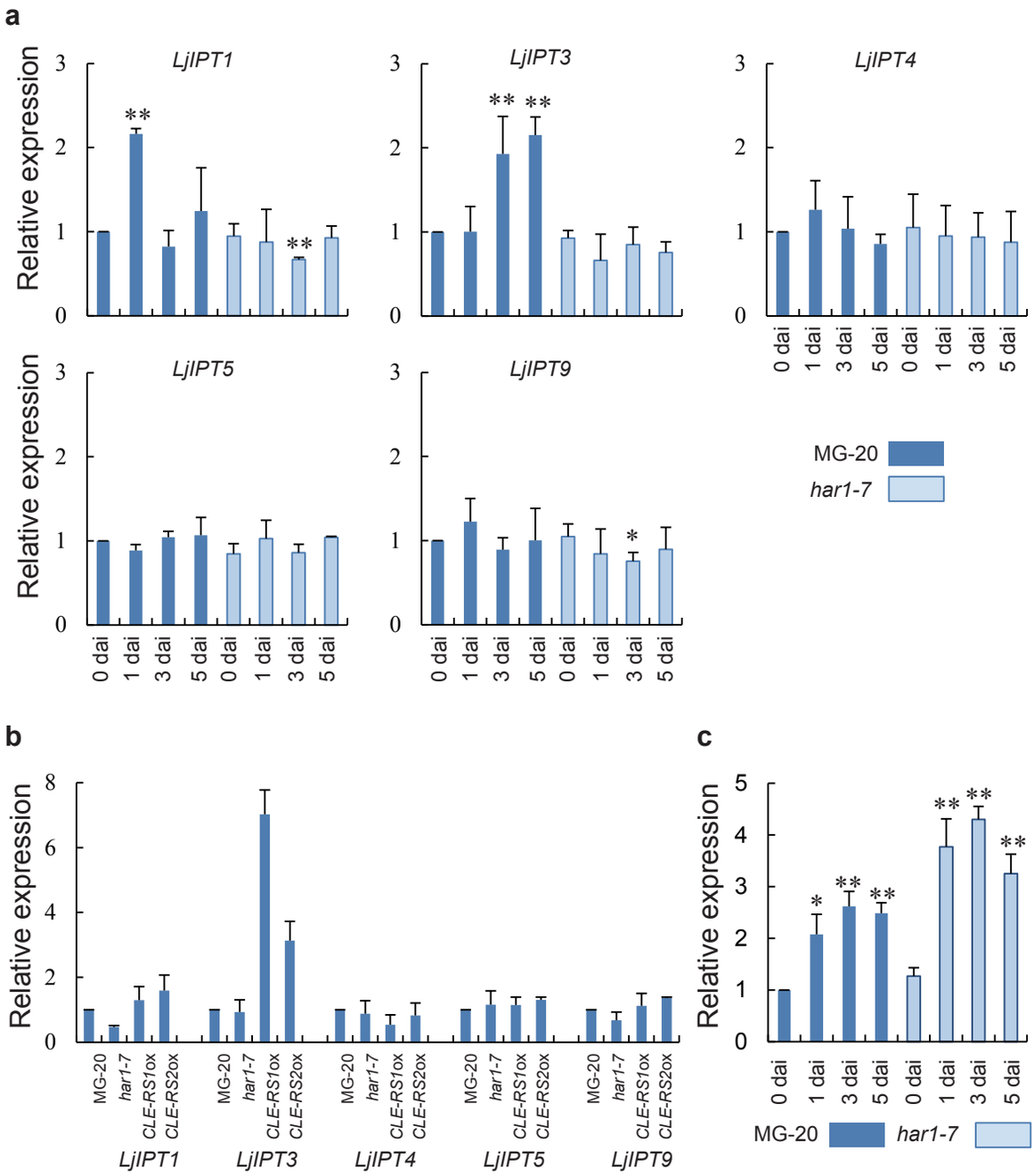

Figure 6 Expression analyses of *LjIPTs* genes. (a) Relative expression of *LjIPTs* genes in MG-20 and *har1-7* shoots at different times after infection with *M. loti* (dai). (b) Relative expression of *LjIPTs* genes in shoots of various plant lines grown in the absence of rhizobia. (c) Relative expression of *LjIPT3* in MG-20 and *har1-7* roots at different times after infection with *M. loti* (dai). (a-c) Data presented are means ± SD of three biological repeats. Transcript amounts in different samples were normalized to those of *UBIQUITIN*. Asterisks indicate statistically significant differences at P<0.05 (*) and P<0.01 (**), according to Student's t-test.

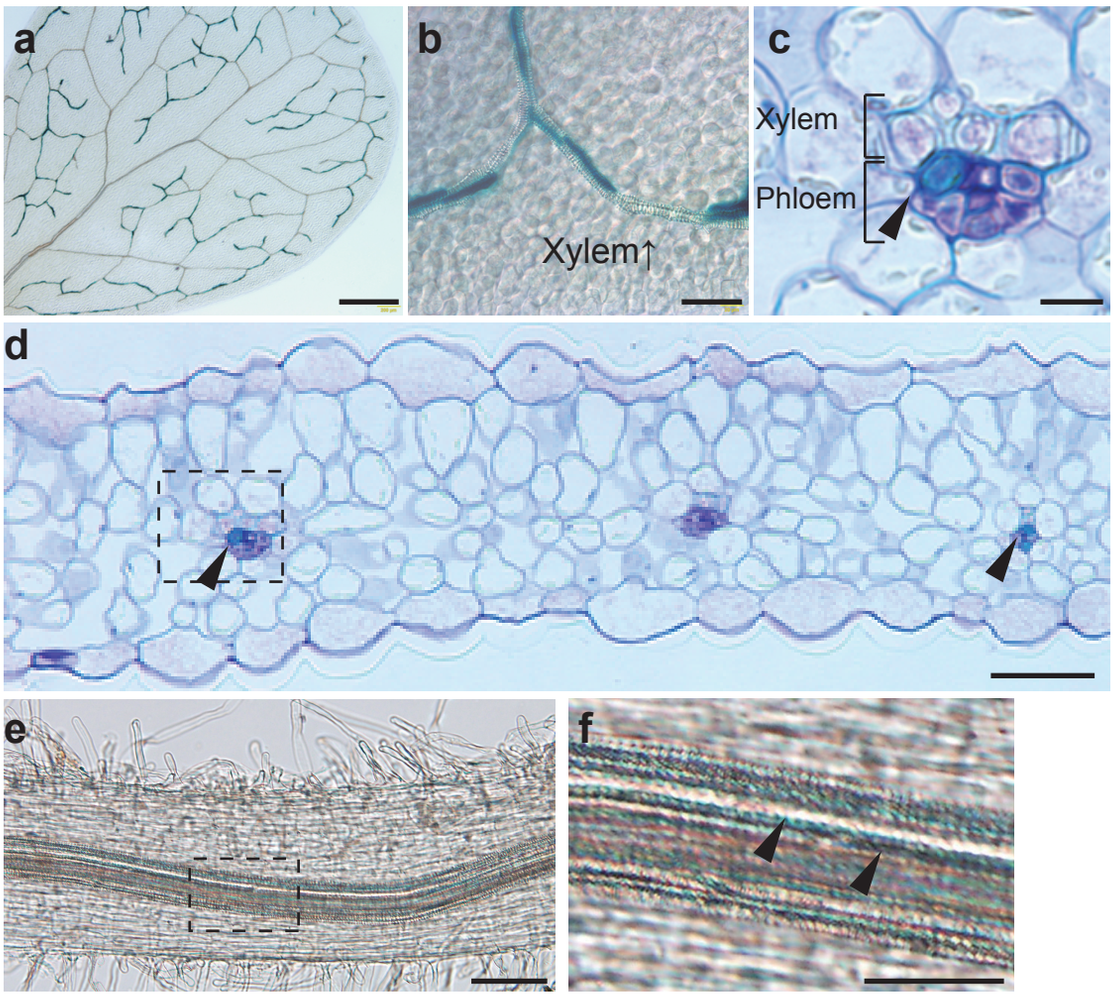

Figure 7 The *LjIPT3* promoter is active in phloem cells. (a,b) GUS staining in leaves of *ProLjIPT3:GUS* plants at three days after infection with *M. loti*. (c,d) Cross section of a leaf stained with 0.05% toluidine blue. (e,f) GUS staining in roots of *ProLjIPT3:GUS* plants.The arrowhead highlights an area showing the GUS signal. (c) Magnified image of the boxed region in (d). (f) Magnified image of the boxed region in (e). Bars: 0.5 mm (a), 50 µm (b,d,f), 10 µm (c), 100 µm (e).

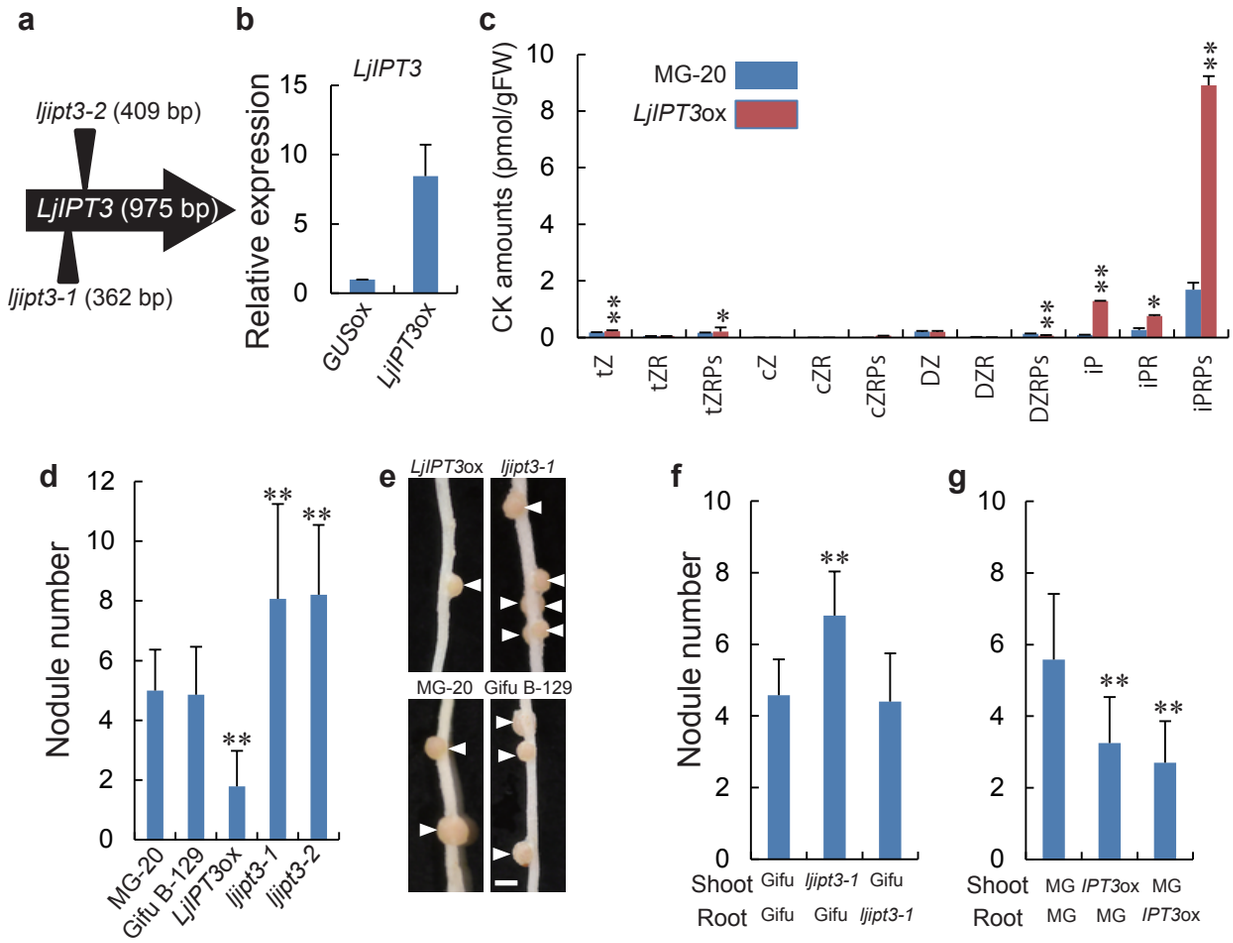

Figure 8 LjIPT3 effects on nodule number and accumulation of CKs. (a) Schematic illustration of LORE1 retrotransposon insertions in *LjIPT3*. Arrowheads indicate positions of LORE1 inserts in *ljipt3-1* (Plant ID, 30001893) and *ljipt3-2* (30012123). Numbers of nucleotides from the translation initiation site are also shown. (b) Expression of *LjIPT3* in transformed plants. Expression levels relative to *GUS*ox controls are shown. Data presented are means ± SD from three biological repeats. Transcript amounts in different samples were normalized to those of *UBIQUITIN*. (c) Comparison of CK levels between shoots of MG20 and *LjIPT3*ox plants. CK levels in shoots grown in the absence of rhizobia were determined. Means ± SD from three independent experiments are shown. (d) Nodule number of wild type (MG-20 and Gifu B-129), *LjIPT3*ox plants (MG-20 background) and *ljipt3* mutants (Gifu B-129 background) (n = 14-15). (e) Nodule phenotypes of each line. Arrowheads indicate nodules. Bar: 1 mm. (f,g) Nodule numbers in shoot-to-root reciprocal grafts between MG20 and *LjIPT3*ox (n = 10-12) (f) Nodules were counted 21 days after infection with M. loti. Data presented are means ± SD. (g) and between Gifu B-129 and ljipt3-1 mutants (n = 10-12). (c,d,f,g) Asterisks indicate that contents of the compound show statistically significant differences compared with MG-20 (*P <0.05, **P <0.01; Student's t-test).

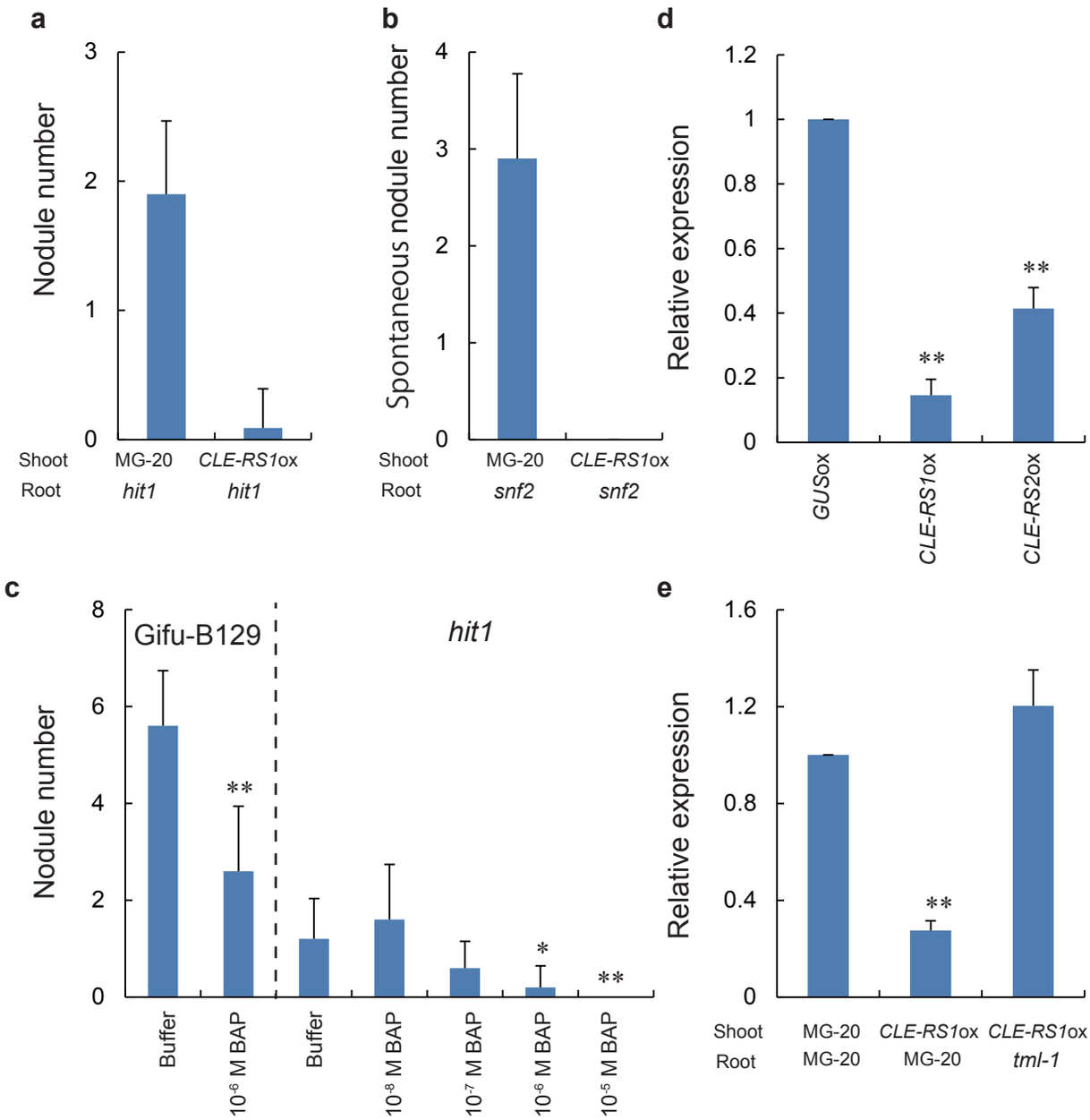

Figure 9 Overexpression of *CLE-RS1/2* suppresses nodulation of *hit1-1* mutats, formation of spontaneous nodules of *snf2* mutants and expression analyses of *NSP2* in roots. (a, b) Nodule and spontaneous nodule numbers in shoot-to-root grafts between shoots of MG20 or *CLE-RS1*ox and roots of *hit1*-1 or *snf2* mutants. (a) In *hit1-1* roots, nodules were counted 21 days after granting and infection with *M. loti* (n = 10-12). (b) In *snf2* roots, spontaneous nodules were counted 28 days after grafting in the absence of rhizobia. Data presented are means ± SD. (c) Effects on nodulation of BAP application to shoots in Gifu-B129 and *hit1* mutants (n = 5). Distal halves of cotyledons were removed at 2 days after germination, and BAP was fed to the cut surfaces. Plants were inoculated with *M. loti* one day after starting BAP application. Nodules were counted 14 days after infection. (d) Relative expression of *NSP2* in *GUS*ox, *CLE-RS1*ox and *CLE-RS2*ox of roots were detected in the absence of rhizobia (1-week-old seedlings). (e) Relative expression of *NSP2* in shoot-to-root grafts between shoots of MG20 or *CLE-RS1*ox and roots of *hit1*-1 or *snf2* mutants were detected in the absence of rhizobia. Data presented are means ± SD of three biological repeats. Asterisks indicate statistically significant differences at P<0.05 (*) and P<0.01 (**), according to Student's t-test. Transcript amounts in different samples were normalized to those of *UBIQUITIN*.

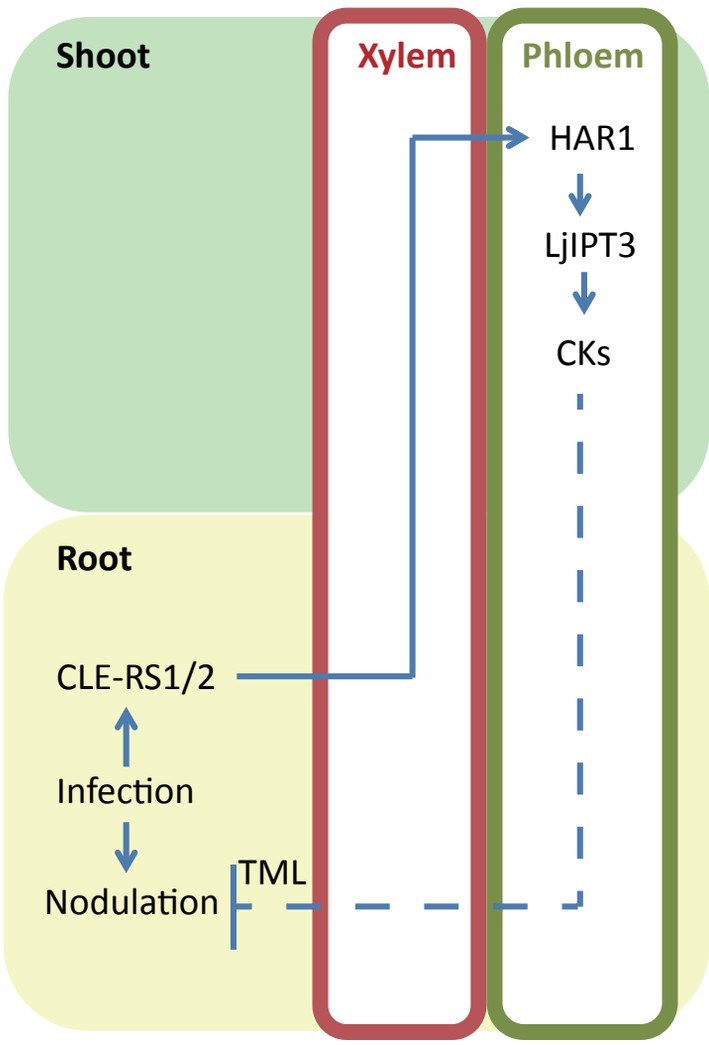

Figure 10 Schematic illustration of the proposed AON model. Demonstrated and putative regulatory relationships are indicated by bold and dotted lines, respectively. See text for details.

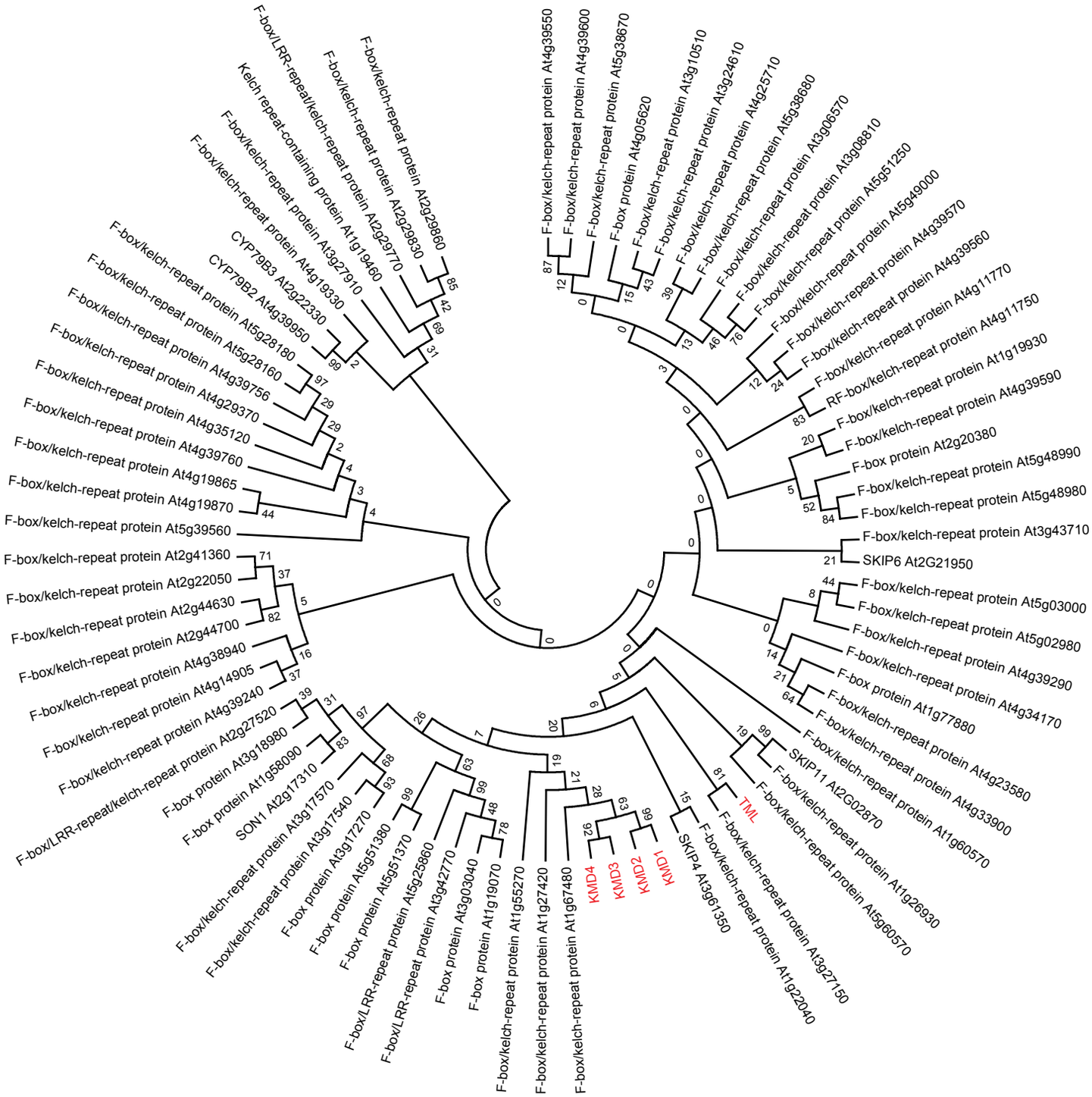

Figure 11 Phylogenetic analysis. Deduced amino acid sequences of F-box domain in *TML*-related proteins were aligned and the phylogenetic tree was constructed by neighbor-joining. A node was supported in 1000 bootstrap pseudoreplications. Tree is shown with bootstrap confidence values expressed in percentage. TML and KMDs were indicated in the red.

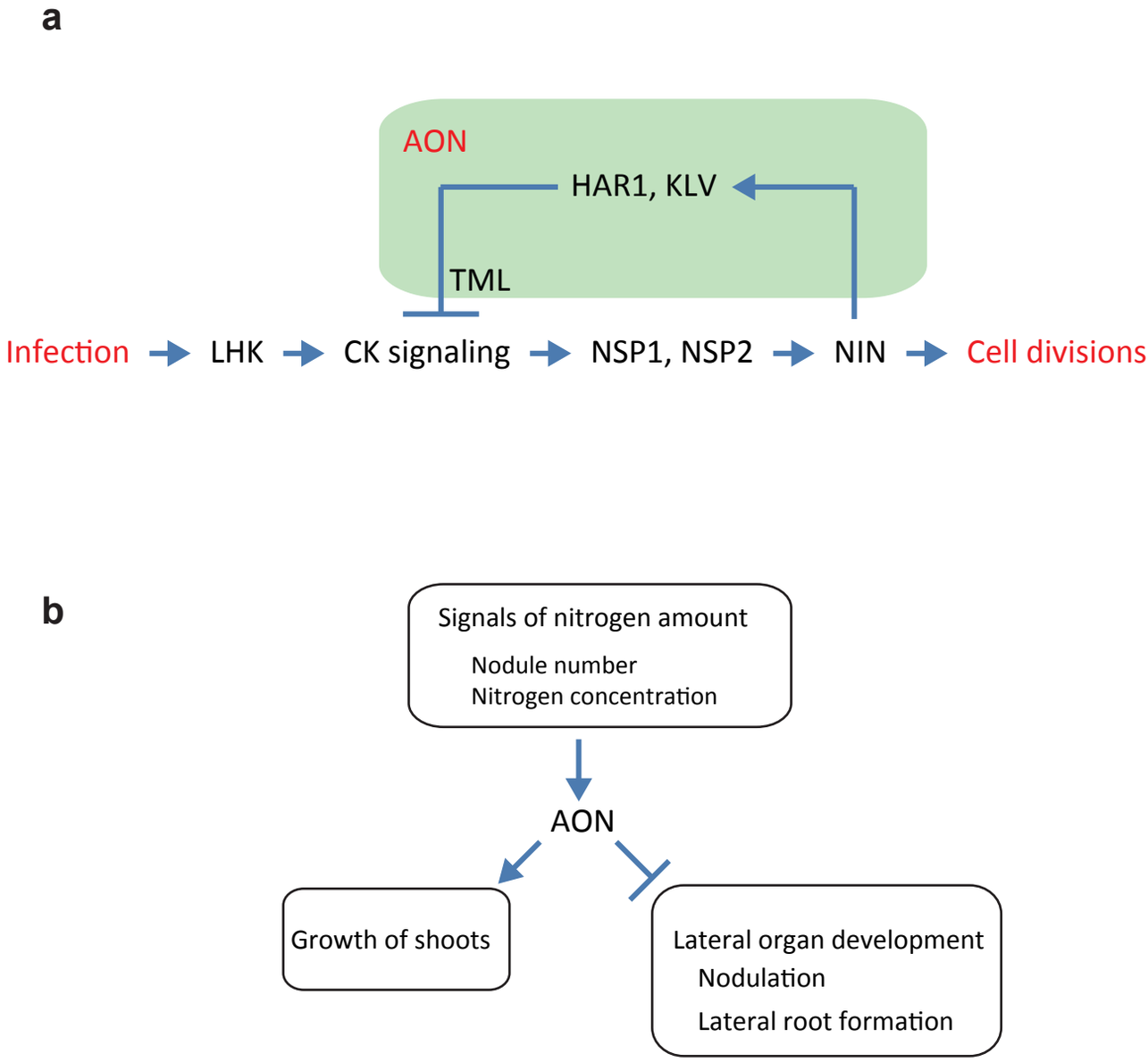

Figure 12 Summary of the AON model. (a) About the proposed site of AON. (b) About the relationship between AON and growth of host plants.

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