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The receptor-like kinase KLAVIER mediates systemic regulation of nodulation and non-symbiotic shoot development in *Lotus japonicus*

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SUMMARY

In legumes, the number of symbiotic root nodules is controlled by long-distance communication between the shoot and the root. Mutants defective in this feedback mechanism exhibit a hypernodulating phenotype. Here, we report the identification of a novel leucine-rich repeat receptor-like kinase (LRR-RLK), KLAVIER (KLV), which mediates the systemic negative regulation of nodulation in *Lotus japonicus*. In leaf, *KLV* is predominantly expressed in the vascular tissues, as with another LRR-RLK gene, *HAR1*, which also regulates nodule number. A double-mutant analysis indicated that *KLV* and *HAR1* function in the same genetic pathway that governs the negative regulation of nodulation. LjCLE-RS1 and LjCLE-RS2 represent potential root-derived mobile signals for the HAR1-mediated systemic regulation of nodulation. Overexpression of *LjCLE-RS1* or *LjCLE-RS2* did not suppress the hypernodulation phenotype of the *klv* mutant, indicating that *KLV* is required and acts downstream of *LjCLE-RS1* and *LjCLE-RS2*. In addition to the role of *KLV* in symbiosis, complementation tests and expression analyses indicated that *KLV* plays multiple roles in shoot development, including maintenance of shoot apical meristem, vascular continuity, shoot growth and promotion of flowering. Biochemical analyses using transient expression in *Nicotiana benthamiana* revealed that KLV has the ability to interact with HAR1 and with itself. Together, these results suggest that the potential KLV-HAR1 receptor complex regulates symbiotic nodule development and that KLV is also a key component in other signal transduction pathways that mediate non-symbiotic shoot development.

KEY WORDS: *Lotus japonicus*, Hypernodulation, KLAVIER, Long-distance signaling, Leucine-rich repeat receptor-like kinase, Shoot development

INTRODUCTION

Legumes are capable of establishing root symbiosis with soil bacteria commonly known as rhizobia. Within root-derived organs called nodules, rhizobia fix atmospheric nitrogen, which allows the host plant to grow independently of soil nitrogen.

The development of nodules and the subsequent nitrogen fixation carry a metabolic cost to the host plants. Thus, the maintenance of an appropriate nodule number is crucial for plants to avoid excessive nodulation. To this end, legume plants have evolved systemic feedback regulation termed 'autoregulation of nodulation', in which earlier nodulation suppresses the subsequent nodulation events via long-distance signaling (Nutman, 1952; Pierce and Bauer, 1983; Caetano-Anollés and Gresshoff, 1991; van Brussel et al., 2002; Oka-Kira and Kawaguchi, 2006). Mutants defective in this mechanism develop an increased number of nodules and nodule primordia within a drastically enhanced nodulation zone (termed a hypernodulation or supernodulation phenotype) (Jacobsen and Feenstra, 1984; Carroll et al., 1985a; Carroll et al., 1985b; Delves et al., 1986; Gremaud and Harper, 1989; Olsson et al., 1989; Akao and Kouchi, 1992; Sagan and Duc, 1996; Schauser et al., 1998; Szczyglowski et al., 1998; Wopereis et al., 2000; Kawaguchi et al., 2002; Penmetsa et al., 2003; Oka-Kira et al., 2005; Magori et al., 2009).

Several hypernodulation mutants have been isolated in *Glycine max*, *Pisum sativum* and the two model legumes *Lotus japonicus* and *Medicago truncatula*. However, only a limited number of the causative genes have been cloned. The only genes that have been identified to date are *HYPERNODULATION ABERRANT ROOT FORMATION 1* (*HAR1*) of *Lotus* (Krusell et al., 2002; Nishimura et al., 2002), and its orthologs *NTS1* (also known as *NARK*) (Nishimura et al., 2002; Searle et al., 2003), *SYM29* (Krusell et al., 2002) and *SUNN* (Schnabel et al., 2005) in *G. max*, *P. sativum* and *M. truncatula*, respectively. All these genes encode a receptor-like kinase (RLK) protein consisting of N-terminal leucine-rich repeats (LRRs), a single transmembrane domain, and a C-terminal serine/threonine kinase domain. Grafting experiments have shown that *HAR1* and its orthologs function in the shoot to control root nodule number. Thus, *HAR1* is likely to be involved, directly or indirectly, in the generation of a shoot-derived mobile signal that

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inhibits further nodulation (Krusell et al., 2002; Nishimura et al., 2002; Jiang and Gresshoff, 2002; Buzas and Gresshoff, 2007; Lin et al., 2010).

The *Arabidopsis* gene with the highest similarity to *HAR1* is *CLAVATA1* (*CLV1*), which controls the size of the shoot apical meristem (SAM) (Clark et al., 1997). However, the *L. japonicus* mutant *har1* and other legumes carrying deleterious mutations in the *HAR1* orthologous loci do not show any *clv1*-like shoot phenotypes. This indicates that unlike *CLV1*, these *CLV1*-like genes in legumes play a specific role in the regulation of nodule development but not in the control of SAM size.

Although components involved in the regulation of the SAM, in place of CLV1, remain to be identified in legumes, several hypernodulation mutants are known to exhibit *clv*-like phenotypes. For example, the *Lotus* mutant *klavier* (*klv*) exhibits not only a typical hypernodulation phenotype when inoculated with the symbiotic bacteria of *Lotus*, *Mesorhizobium loti*, but also non-symbiotic phenotypes, such as abnormal leaf venation, extremely delayed flowering, and dwarf shoot, even in the absence of *M. loti* (Oka-Kira et al., 2005). In addition, *clv*-like phenotypes, such as fasciated stems, an increased number of flowers per peduncle and bifurcated pistils, which do not occur in *har1*, are frequently observed in the *klv* mutant. Grafting between *klv* shoots and wild-type roots has demonstrated that *KLV* functions in the shoots to control nodule number (Oka-Kira et al., 2005). Similarly, the pea mutants *sym28* (Sagan and Duc, 1996) and *nod4* (Sidorova and Shumnyi, 2003) also exhibit shoot-regulated hypernodulation, fasciated stems and increased flower numbers. Together, these observations suggest a potential link between the systemic regulation of nodule number and SAM maintenance in legume plants. However, which gene(s) are responsible for hypernodulation in *klv*, and whether these pleiotropic phenotypes of *klv* are regulated by just a single gene, are questions that remain to be addressed.

Here, we report the molecular identification of *KLV*, which encodes a putative LRR-RLK. *KLV* controls not only the development of root nodules, but also multiple aspects of shoot development, including maintenance of the SAM, leaf vascular continuity, shoot growth and promotion of flowering time. Genetic analyses reveal that *KLV* and *HAR1* mediate the systemic regulation of nodulation in the same genetic pathway. Furthermore, biochemical data demonstrate that KLV interacts with HAR1 and itself to form potential receptor complexes. These results suggest that KLV plays important roles in the regulation of symbiotic and non-symbiotic development.

MATERIALS AND METHODS

Plant materials and growth conditions

Lotus japonicus ecotype Miyakojima MG-20 (Kawaguchi, 2000) was used as the wild type. After overnight water absorption, plants were grown with or without *Mesorhizobium loti* MAFF 30-3099 in autoclaved vermiculite supplied with Broughton and Dilworth (B&D) solution (Broughton and Dilworth, 1971) containing 0.5 mM KNO_3, under 16-hour light/8-hour dark cycles at a light intensity of 150 μE/s/m^2 at 22°C in a Biotron LH-300 incubator (Nihon-ika, Osaka, Japan). For analysis of bifurcation of stems, plants were grown in a 1:3 mixture of horticultural soil (Kureha, Tokyo, Japan) and vermiculite.

Sequencing of the *KLV* locus

The genomic sequences of the *KLV* open reading frame (ORF) (see Fig. S1 in the supplementary material) in wild type and *klv* were determined by direct sequencing using the following primers: PCR-Fw1, 5′-TCCTCATCATCATCATCACTGTTCTC-3′; PCR-Rv1, 5′-ACCCAACGTCAGTGAACACTGTGACT-3′; Sequence-Fw1, 5′-CTGTGTGGGTAGTGGAGGGGCTTTG-3′; Sequence-Fw2, 5′-CTGAGCTTGGGCATTGTATGG-3′; Sequence-Fw3, 5′-CTGAATTCTCTGGTAACGCATGTC-3′; Sequence-Rv1, 5′-TGCCTGTATCTTCTGGTGGTGCAGC-3′; PCR-Fw2, 5′-TCTGTTCTTCTAGCCCTAATTGTCC-3′; PCR-Rv2, 5′-GTCAACTGATGGGTGGCACA-3′; and Sequence-Fw4, 5′-ATGATCAGTGCGTACCGCGAG-3′. SMART and Pfam programs were used for domain prediction (http://www.sanger.ac.uk/Software/Pfam/ and http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Vector construction for complementation test

A 10.6 kb genomic fragment containing a 3.4 kb ORF of the LRR-RLK and 5.1 kb upstream and 2.1 kb downstream sequences was subcloned into the pART27 binary vector plasmid (Gleave, 1992) as a transformation vector for the complementation test. First, the 3.7 kb fragment containing an upstream region of the gene was amplified using a forward primer containing a *Not*I adaptor sequence (5′-AAGGAAAAAAGCGGCCGCGTGGATTGGCTGGTTAGTGC-3′) and a reverse primer (5′-CCATAAGTTATGTATCATAAGCGG-3′). The resulting PCR product was digested and ligated into the *Not*I and *Spe*I sites of pART27. This plasmid was digested with *Spe*I and ligated to the 6.9 kb *Spe*I fragment of a TAC clone LjT09A08 (provided by the Kazusa DNA Research Institute) containing the LRR-RLK coding region and the downstream sequence.

Transformation for complementation test

The vector containing the LRR-RLK and an empty pART27 vector were transformed into *Agrobacterium tumefaciens* AGL1, and introduced into *klv* using *A. tumefaciens*-mediated hypocotyl transformation. Transformation was performed as described previously (Stiller et al., 1997), with slight modifications. The T_2 plants were used for analyses. A primer set for *NPTII* (forward, 5′-ATGGCAATTACCTTATCCGC-3′; reverse, 5′-TCAGAACAATCCGTCAAGAA-3′) was used to check for the presence of the transgene.

Morphological observation of the SAM

Leaves and leaf primordia were removed from wild-type and *klv* shoot apexes 4 days after germination (DAG), and the exposed SAMs were stained with the vital fluorescent membrane probe FM4-64 (Molecular Probes) at 50 mM for 15 minutes, and observed with a confocal laser-scanning microscope (TCS SP, Leica). Graphics were scanned and edited by Leica Confocal Software, version 2.5.

Phylogenetic analysis of KLV-related proteins

Deduced amino acid sequences of KLV-related proteins were searched by BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/) or Phytozome (http://www.phytozome.net/) and aligned using Clustal X (version 1.83) and BioEdit (version 7.0.5.2) software. The phylogenetic tree was constructed by NJplot.

Expression analysis

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was prepared using a QuantiTect Reverse Transcription Kit (Qiagen). Real-time RT-PCR was performed using ABI Prism 7000 (Applied Biosystems) with a QuanTitect SYBR Green RT-PCR Kit (Qiagen). All samples were tested for genomic DNA contamination using non-reverse transcriptase controls. *ATP synthase* (AW719841) was amplified as a reference gene. Each value represents the mean (± s.d.) of three biological replicates. The following primers were used in expression analysis: *ATP synthase*, 5′-ACATGCTTGCACCATACCAA-3′ and 5′-TCCCCAACTCCAGCAAATAC-3′; *KLV*, 5′-CCTGCACTTGGCTGTTGTTTGT-3′ and 5′-AGCACAGCCAGCCTCACCAT-3′; *HAR1*, 5′-TTTGTATGACCCTGGTGCTTCTC-3′ and 5′-CGTCACTCTTCTCGTCCACTTTC-3′.

Sample preparation for laser microdissection (LMD)

The pieces of the first leaves of 14-day-old plants inoculated with *M. loti* were fixed in Farmer's fixative (3:1 ethanol:acetic acid) overnight at 4°C. Dehydration and paraffin embedding were performed as described previously (Inada and Wildermuth, 2005) using a microwave processor. Paraffin-embedded sections were cut to 14 μm and mounted on PEN

membrane glass slides (Molecular Devices) for LMD. To remove paraffin, slides were immersed twice in HistoClear II (National Diagnostics) for 5 minutes and then air-dried completely at room temperature. Two or three individual pieces of leaf were used for each LMD experiment. LMD was performed using the Veritas Laser Microdissection System LCC1704 (Molecular Devices). Selected areas were captured by an infrared laser onto CapSure Macro LCM Caps (Molecular Devices), and were subsequently cut by a UV laser. Tissues were dissected from 150-200 transverse sections of each sample.

RNA extraction and amplification for tissue-selective RT-PCR

Total RNA was extracted from laser-microdissected cells using the PicoPure RNA Isolation Kit (Molecular Devices). Quant-iT RiboGreen RNA Reagent and Kit (Invitrogen) were used for RNA quantification. One nanogram of total RNA was amplified using the WT-Ovation RNA Amplification System (NuGEN Technologies). Normalized samples were diluted and subject to real-time RT-PCR.

Double-mutant analysis

For generation of the double mutant, the two hypernodulation mutants *klv* and *har1-7* were crossed. The F_2 plants were first grown for 3 weeks without rhizobia and the length of each shoot measured. Then, plants were inoculated with *M. loti* for 3 weeks. Genotyping of the *har1-7* mutation was performed using a dCAPS marker (forward primer, 5′-CAAATCTCGAAACGCTTCAGGCTTG-3′; reverse primer, 5′-TGAGTGACCTACACTCGCCGATT-3′) and digestion with *Sml*I (New England BioLabs), which cuts only *har1-7* (203 bp and 22 bp). Genotyping of *klv* was performed using a dCAPS marker (forward primer, 5′-GATGATGATTATAATGCTTATCTATCTTA-3′; reverse primer, 5′-AACGCCTTCTTGTCTGAGAG-3′) and digestion with *Afl*II (New England BioLabs), which cuts *klv* (26 bp and 173 bp).

For generation of the *klv snf2* double mutant, we isolated a spontaneous nodule formation (*snf*) mutant from MG-20 by ethylmethane sulfonate (EMS) mutagenesis. Sequencing analysis revealed that this *snf* mutant possesses the same mutation in *LHK1* as the previously reported *snf2* mutant (Tirichine et al., 2007). Thus, we decided to use this mutant as another allelic mutant of *snf2*. For spontaneous nodule formation, plants were grown in the absence of *M. loti*. For, *klv snf2* double-mutant analysis, the *snf2* mutants were selected from the F_2 population on the basis of spontaneous nodule formation, and could have been heterozygous or homozygous for the *snf2* mutation, as it is dominant. Then, the *klv* genotypes were checked by the dCAPS marker. The numbers of spontaneous nodules were determined 5 weeks after germination.

Hairy root transformation

For overexpression of *LjCLE-RS1*, *LjCLE-RS2* and *GUS*, *Agrobacterium rhizogenes* strains harboring the corresponding vector (Okamoto et al., 2009) were used. Hairy root transformation was performed as previously described (Kumagai and Kouchi, 2003). The transformed plants were inoculated with *M. loti* and the numbers of nodules on hairy roots were counted 14 days after inoculation (DAI). Transformed hairy roots were distinguished by GFP fluorescence under epifluorescence stereomicroscopy (SZX12, Olympus).

Transient expression in *N. benthamiana* and co-immunoprecipitation

Full-length cDNAs of *KLV* and *HAR1* without stop codons were cloned into the pENTR/D-TOPO vector (Invitrogen) and transferred into the Gateway binary vectors pGWB14 and pGWB20 by the LR recombination reaction in order to express C-terminal fusions to 3xHA (HA) or 10xMyc (Myc) tags. The expression constructs were introduced into *A. tumefaciens* strain GV3101::pMP90.

For transient expression, *A. tumefaciens* strains carrying each construct together with another *Agrobacterium* strain harboring the p19 silencing suppressor gene were co-infiltrated into *N. benthamiana* leaves as previously described (Voinnet et al., 2003; Kinoshita et al., 2010).

Total protein was extracted as described (Kinoshita et al., 2010). Protein G Mag Sepharose (GE Healthcare, Little Chalfont, UK) was used for immunoprecipitation. Total and immunoprecipitated proteins were analyzed by SDS-PAGE and western blotting using anti-HA 3F10 (Roche) and anti-Myc 9E10 (Roche) antibodies. For secondary antibodies, we used anti-rat IgG-HRP or anti-mouse IgG-HRP (both GE Healthcare). Immunoblotted PVDF membranes were developed using ECL western blotting detection reagents (GE Healthcare) or Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan) and the chemiluminescence signal was detected by an LAS-4000 image analyzer (Fujifilm).

RESULTS

KLV encodes a receptor-like kinase involved in the negative regulation of nodulation

The *KLV* locus has been mapped to a region of 0.29 cM on the long arm of *L. japonicus* chromosome 1 (Oka-Kira et al., 2005). This genetic region was located within a single physical contig covered by three TAC/BAC clones (see Fig. S1 in the supplementary material). At least 38 ORFs were predicted in this genomic region. Among them, we first analyzed a gene encoding an LRR-RLK, as many LRR-type receptor kinases are known to play pivotal roles in plant development. Sequencing analysis revealed a deletion-substitution mutation in the *klv* mutant (CTTTG to A) 3000 bp downstream from the presumed ATG start codon (see Fig. 3A). This appears to cause a frameshift followed by a premature stop codon (TAG) 49 bp downstream of the mutation site. Thus, we hypothesized that this LRR-RLK gene might represent the *KLV* locus.

To test this hypothesis, we introduced a 10.6 kb wild-type genomic fragment encompassing the entire LRR-RLK gene into *klv* plants using *Agrobacterium tumefaciens*-mediated hypocotyl transformation. Transgenic *klv* plants with the introduced gene (*klv* [*KLV*]) showed significant suppression in the hypernodulation phenotype, developing roots with a normal number of nodules and nodulation zone ratios (Fig. 1A-C). We obtained five independent transgenic lines with similar results. Based on these complementation tests, we concluded that this LRR-RLK gene indeed corresponds to the *KLV* locus.

KLV also mediates non-symbiotic shoot development

In addition to hypernodulation, *klv* mutants exhibit pleiotropic non-symbiotic phenotypes, including discontinuous leaf vascular structure (vascular islands), convex leaf veins, dwarfing of shoots and roots and late flowering. Moreover, the *klv* mutants frequently, but not always, develop fasciated stems (Oka-Kira et al., 2005). However, it is unclear whether these pleiotropic phenotypes are caused by a single gene mutation. This issue is not trivial because *klv* was isolated by ion beam irradiation, which sometimes causes large deletions or rearrangements of the genome. Therefore, we analyzed the contributions of the *KLV* gene to these pleiotropic non-symbiotic phenotypes.

First, we focused on the stem fasciation phenotype of *klv* (Oka-Kira et al., 2005). The shoots of *klv* often split into two stems of similar structure and size (bifurcated stems) just above the fasciated stem (Fig. 2A). At 30 DAG, ~70% of *klv* seedlings exhibited such bifurcation at some stem positions, whereas wild-type plants did not exhibit any bifurcation. The *KLV* gene partially rescued this bifurcation phenotype, and the extent of complementation varied between individual transgenic lines (Fig. 2B,C).

Fasciation of stems is a phenotype that is often observed in mutants defective in SAM size control (Clark et al., 1993; Clark et al., 1995). To compare the SAMs of wild-type and *klv* plants, 4-day-old seedlings were stained with the fluorescent dye FM4-64 and observed using confocal laser-scanning microscopy. In

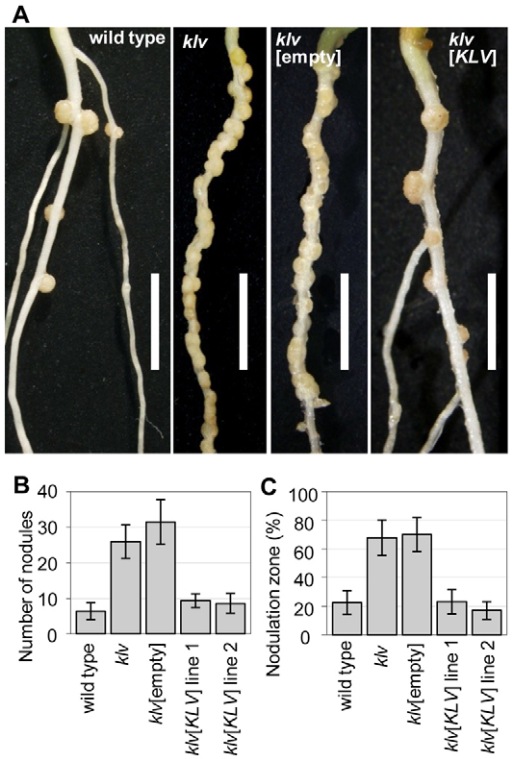

Fig. 1. Complementation of *klv* hypernodulation. (**A**) Nodulated roots of a wild-type *L. japonicus* plant, the *klv* mutant, a *klv* mutant transformed with an empty binary vector (*klv* [empty]), and a *klv* mutant transformed with the gene encoding the LRR-RLK (*klv* [*KLV*]) at 14 days after inoculation (DAI) with *Mesorhizobium loti* MAFF30-3099. Scale bars: 1 cm. (**B**,**C**) Nodule numbers per plant and nodulation zone ratios at 14 DAI ($n \geq 10$ for each value). The nodulation zone ratio is the calculated length of the nodulated region normalized to the primary root length. Two independent lines of *klv* [*KLV*] are shown (lines 1 and 2). Error bars indicate mean ± s.d.

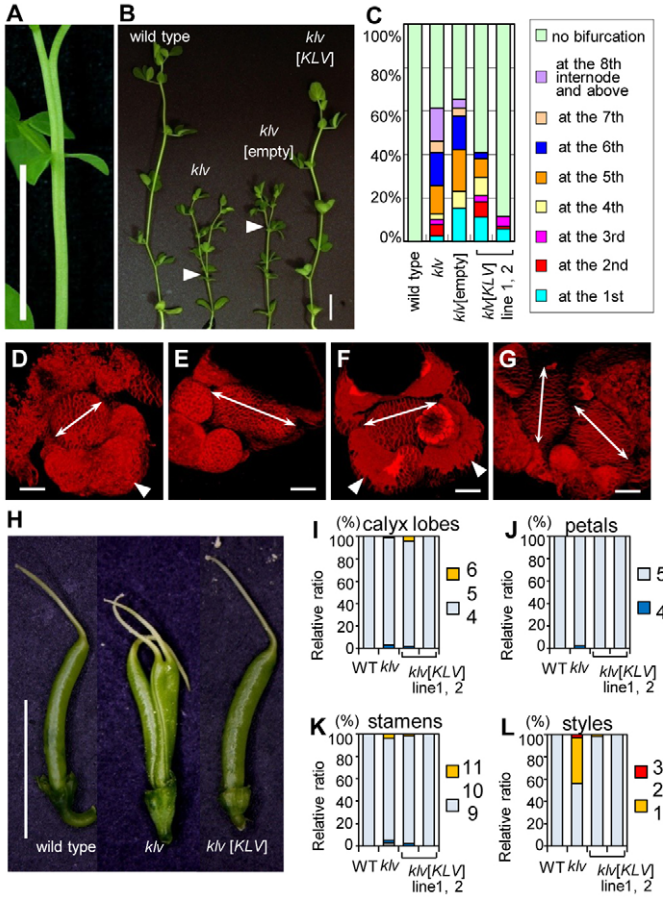

Fig. 2. Morphological defects in the shoot apical meristem of *klv* and complementation of the *klv* bifurcation. (**A**) The bifurcated stem of a *L. japonicus klv* plant. (**B**) Shoot structures of uninoculated plants 30 days after germination (DAG). Each arrowhead indicates bifurcation of a stem. (**C**) The percentages of plants that show bifurcation at 30 DAG. The lowest bifurcating position in each seedling was recorded (wild type, 26 plants; *klv*, 23 plants; *klv* [empty], 26 plants; *klv* [*KLV*] line 1, 71 plants; *klv* [*KLV*] line 2, 87 plants). The first internode is between the cotyledon and the first true leaf just above the hypocotyl, and internodes above that were numbered in order. (**D-G**) Structures of SAMs in wild-type (D) and *klv* (E-G) plants at 4 DAG. Arrows indicate the SAM regions and arrowheads indicate leaf primordia. (**H**) Magnified images of pistils. (**I-L**) The number of floral organs. A total of 50, 100, 50 and 38 flowers were examined for wild type (WT), *klv*, *klv* [*KLV*] line 1 and *klv* [*KLV*] line 2, respectively. Scale bars: 1 cm in A,B; 40 μm in D-G; 5 mm in H.

contrast to the rounded SAMs of the wild-type plants (Fig. 2D), the SAMs of *klv* plants were often oval shaped or composed of two units (Fig. 2E,G). Furthermore, the *klv* mutants frequently showed aberrant phyllotaxy. In the wild type, each leaf primordium was generated on the opposite side of the SAM from the previously formed primordium. However, in *klv* plants the two leaf primordia were often adjacent to each other (Fig. 2F). This aberrant phyllotaxy in *klv* mutants might be due to a defect in SAM function.

Furthermore, we analyzed the number of floral organs to examine whether *KLV* is involved in floral meristem maintenance (Clark et al., 1993; Kayes and Clark, 1998; Suzaki et al., 2004; Müller et al., 2008). A *Lotus* flower comprises five fused sepals and five petals. Two of the petals are fused, forming a keel that encloses ten stamens (nine fused and one free stamen) and a pistil. The *klv* flowers often developed three or three pistils that were occasionally fused at the base (Fig. 2H,L). By contrast, we observed little difference between *klv* and wild type in the number of the other floral organs (Fig. 2I-K). Introduction of the *KLV* gene into *klv* rescued its pistil phenotype, indicating that *KLV* also regulates the number of pistils (Fig. 2H,L). These results suggest that *KLV* functions in the maintenance of both the shoot and floral meristems in *Lotus*.

Additional roles of *KLV* were also analyzed. The *klv* mutants started to flower 131±24 (s.d.) DAG, whereas wild-type plants began to flower 68±9 DAG. The average flowering times of *klv* [*KLV*] plants were 73±7 (line 1) and 78±4 (line 2) DAG. Thus, the late-flowering phenotype of *klv* was also complemented by the *KLV* gene. The retardation of shoot and root growth in *klv* was partially rescued by *KLV*, in both *M. loti*-inoculated and uninoculated plants (see Fig. S2A-D in the supplementary material).

Microscopic observations of cleared leaflets revealed that *klv* formed many vascular islands, an indication of a defect in vascular development (see Fig. S3A in the supplementary material). In *klv*, the number of vascular islands was more than one order of

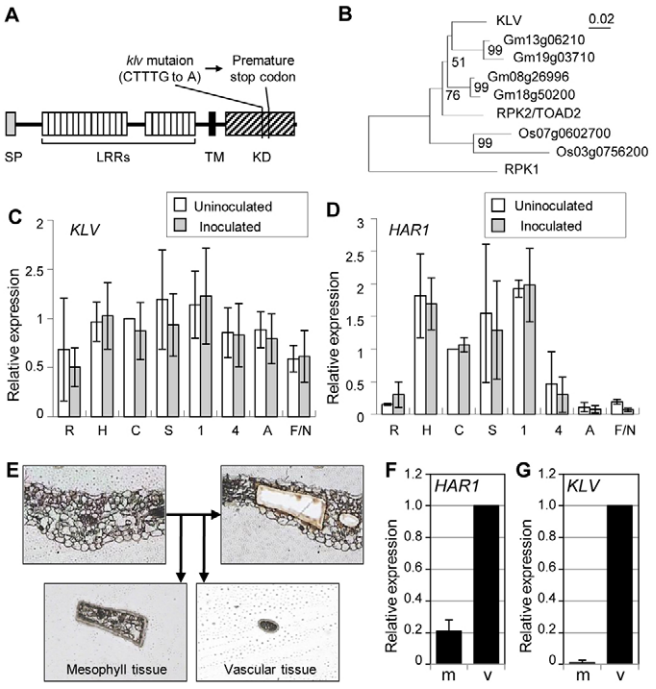

Fig. 3. Characteristics of the KLV protein and expression of the *KLV* gene. (**A**) Predicted protein structure of *L. japonicus* KLV. SP, signal peptide; LRR, leucine-rich repeat; TM, transmembrane domain; KD, Ser/Thr kinase domain. The *klv* mutant carries a nucleotide deletion-substitution of CTTTG to A in the kinase domain that leads to a frameshift and a premature stop codon. (**B**) A phylogenetic tree of KLV-related proteins based on the amino acid sequences of the kinase domains. RPK1 of *A. thaliana* was used as an outgroup. The scale bar indicates the genetic distance based on branch length. (**C**,**D**) Steady-state levels of *KLV* and *HAR1* mRNAs in different organs of uninoculated (white bars) and inoculated (gray bars) plants. R, roots; H, hypocotyls; C, cotyledons; S, shoots; 1, first (oldest) true leaves; 4, fourth (young) true leaves; A, shoot apexes; F (white bar), flowers of uninoculated plants; N (gray bar), nodules. Error bars indicate mean ± s.d. (**E**) The workflow of tissue sampling using laser microdissection (LMD). (**F**,**G**) Steady-state levels of *HAR1* and *KLV* mRNAs in mesophyll tissues (m) and vascular tissues (v) collected by LMD. Error bars indicate mean + s.d.

magnitude higher than in wild-type plants, regardless of leaf age (see Fig. S3B in the supplementary material). This phenotype was also complemented by *KLV*; the *klv* mutant transformed with the *KLV* gene developed a normal number of vascular islands (see Fig. S3B in the supplementary material). To determine whether the vascular islands in the *klv* leaves are a consequence of ectopic and excessive differentiation of tracheary elements or of a defect in proper vessel connection, we examined the early development of the leaf veins. Compared with wild-type leaves of the same age, young *klv* leaves showed underdeveloped leaf vessels, especially in the lateral leaflets of the first true leaves at 7 DAG (see Fig. S3C,D in the supplementary material). This suggests that *KLV* positively regulates vascular development. Furthermore, the convex leaf veins observed on the adaxial surfaces of *klv* leaves were also rescued by the *KLV* gene (see Fig. S3E in the supplementary material).

Based on these complementation experiments, we conclude that *KLV* mediates not only the systemic negative regulation of nodulation, but also diverse aspects of plant development, including regulation of SAM maintenance, vascular development and induction of flowering.

KLV has the highest similarity to RPK2 (TOAD2) of *Arabidopsis*

KLV is a single exon of 3414 nucleotides that encodes a 1137 amino acid protein with several distinct motifs: a putative extracellular domain comprising 22 LRRs, followed by a transmembrane domain and a serine/threonine protein kinase domain (Fig. 3A). Between the fourteenth and fifteenth LRR unit there is a 71 amino acid stretch called the island region.

The intracellular domain of KLV is highly conserved among other plant receptor kinases and comprises 12 motifs that constitute the kinase catalytic domain (Hanks and Quinn, 1991). Owing to the frameshift and the resulting premature stop codon, the kinase domain of the *klv* mutant completely lacks motifs VII-XI, which contain the activation loop that is important for kinase activation.

Among more than 200 *Arabidopsis* LRR-RLKs, KLV shares the highest identity with At3g02130, which has been reported as RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) in regulating anther development (Mizuno et al., 2007) and as TOADSTOOL 2 (TOAD2) in mediating embryonic pattern formation (Nodine et al., 2007). The amino acid sequence of KLV shares 62.5% identity with that of RPK2. KLV also shares high levels of identity with the rice (*Oryza sativa*) proteins Os07g0602700 (57.6%) and Os03g0756200 (53.5%). The putative orthologs of KLV that are present in *G. max* share overall protein identities of 72.6% (Gm13g06210), 71.4% (Gm19g03710), 52.1% (Gm08g26996) and 65.5% (Gm18g50200) with *Lotus* KLV (Fig. 3B and see Fig. S4 in the supplementary material). However, the functions of these genes in *G. max* and *Oryza sativa* remain unknown.

KLV and *HAR1* are expressed in the vascular tissues

The expression levels of *KLV* and *HAR1* were analyzed by real-time RT-PCR. *KLV* was expressed in all organs tested, including the shoot apex, and there were only small differences in expression levels between the organs (Fig. 3C). *M. loti* inoculation did not affect the steady-state levels of *KLV* mRNA. By contrast, the expression level of *HAR1* was relatively low in the root, nodule, young leaf, flower and shoot apex, compared with most other organs examined (Fig. 3D). These observations are consistent with the fact that *KLV*, but not *HAR1*, is involved in the maintenance of proper SAM structure.

To investigate the tissue-specific expression of *KLV*, further analysis was performed using laser microdissection (LMD). The vascular tissues and the mesophyll tissues were dissected and isolated separately from the first true leaves of wild-type plants 14 DAI with *M. loti* (Fig. 3E), and total RNA was extracted from each sample. Real-time RT-PCR revealed that both *KLV* and *HAR1* were expressed strongly in the vascular tissues (Fig. 3F). In particular, *KLV* expression was detected almost exclusively in the vascular tissues (Fig. 3G).

The *klv har1* double mutant shows no additive effect on nodulation

To test whether *KLV* and *HAR1* are involved in a common regulatory pathway, we generated the *klv har1-7* double mutant by crossing. The *har1-7* mutant is a possible null allele isolated from Miyakojima MG-20 by EMS mutagenesis (Magori et al., 2009). The F_1 plants that were heterozygous for both of the mutations showed wild-type nodulation (data not shown).

The *klv har1* double mutants were not significantly different from *klv* single mutants in terms of nodule number (Fig. 4A,C) and non-symbiotic shoot growth (Fig. 4B,D), indicating that *HAR1* is

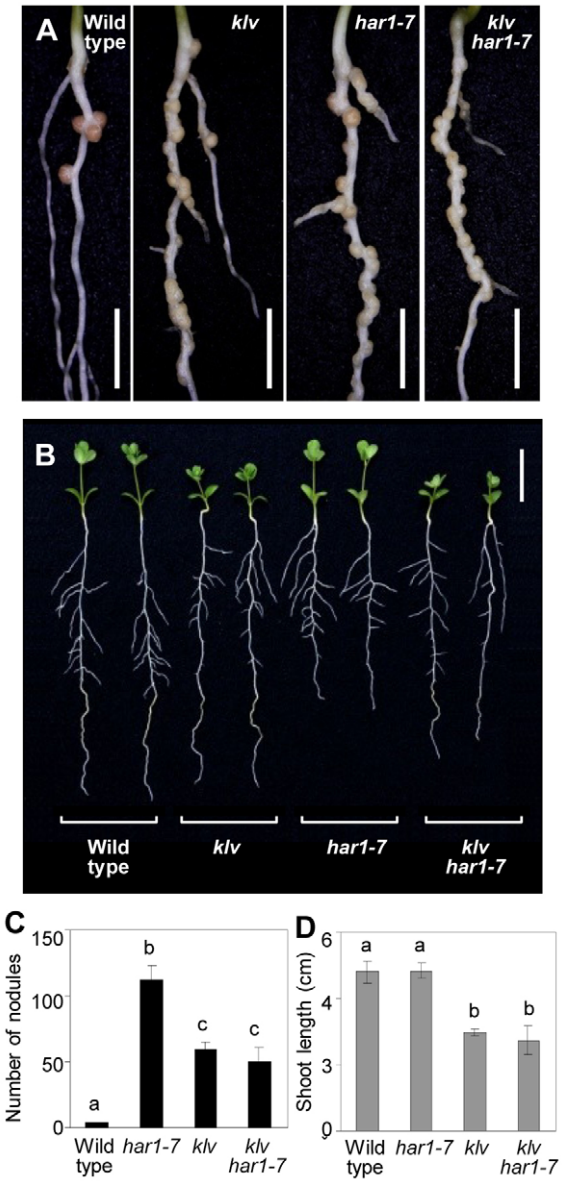

Fig. 4. Genetic analysis with *KLV* and *HAR1*. (**A**) Magnified images of nodulating *L. japonicus* roots at 14 DAI with *M. loti*. (**B**) Uninoculated plants at 14 DAG. (**C**) Plants were inoculated with *M. loti* at 21 DAG and the numbers of nodules and nodule primordia larger than 0.2 mm in diameter were determined at 21 DAI. (**D**) Shoot lengths of uninoculated plants at 21 DAG. For C and D, 6 *klv har1* double mutants and 13 each of wild-type, *har1-7* and *klv* single mutant plants were analyzed. For a, b and c, $P<0.02$ (*t*-test). Error bars indicate mean ± s.e.m. Scale bars: 1 cm.

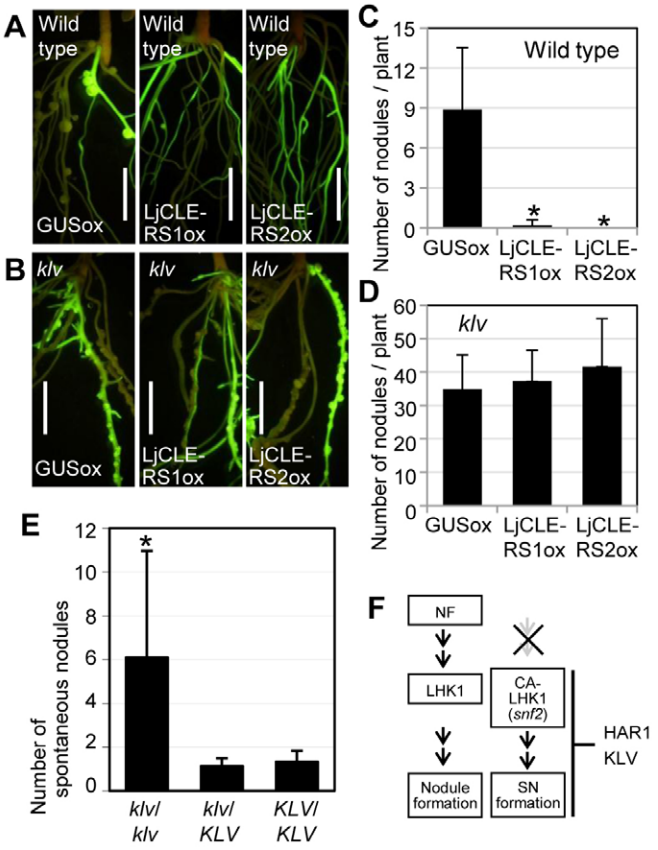

Fig. 5. Genetic analysis with *KLV*, *LjCLE-RS1*, *LjCLE-RS2* and *LHK1*. (**A**,**B**) Wild-type (A) and *klv* (B) transgenic *L. japonicus* hairy roots overexpressing *GUS* (GUSox), *LjCLE-RS1* (LjCLE-RS1ox), or *LjCLE-RS2* (LjCLE-RS2ox). The *GFP* gene was used as a transformation marker. Transformed hairy roots were inoculated with *M. loti* for 14 days. Scale bars: 5 mm. (**C**,**D**) Total numbers of nodules per individual transformed plant were determined at 14 DAI with *M. loti* (n=7-12). Error bars indicate mean + s.d. *$P<0.02$, compared with the GUS overexpressing control plants (*t*-test). (**E**) The numbers of spontaneous nodules on *klv snf2* F_2 plants. These plants were either homozygous or heterozygous for the dominant *snf2* mutation, which causes spontaneous nodule formation. Plants homozygous for *klv* (*klv/klv*), heterozygous (*klv/KLV*), or homozygous for wild-type *KLV* (*KLV/KLV*) were analyzed (n=9-15). Error bars indicate mean + s.d. *$P<0.02$, compared with *KLV/KLV* plants (*t*-test). (**F**) Model of spontaneous nodule (SN) formation. In *snf2*, the constitutively active LHK1 (CA-LHK1) induces downstream signaling to form spontaneous nodules without rhizobial inoculation or nodulation (Nod) factors (NF).

not epistatic to *KLV* in nodulation or shoot development. Thus, the double mutations have no additive effect on nodulation, suggesting that *KLV* and *HAR1* function in the same genetic pathway.

KLV is epistatic to *LjCLE-RS1*- and *LjCLE-RS2*-induced nodule suppression

Overexpression of either of the two *CLE* peptide genes, *LjCLE-RS1* and *LjCLE-RS2*, suppresses nodulation systemically via the HAR1 RLK (Okamoto et al., 2009). To examine whether KLV is required for the *LjCLE-RS1*- and *LjCLE-RS2*-induced suppression of nodulation, we introduced overexpression constructs of each gene into the *klv* hypernodulating mutant by hairy root transformation. Overexpression of either *LjCLE-RS1* or *LjCLE-RS2* significantly suppressed nodulation in wild-type plants (Fig. 5A,C), as reported previously (Okamoto et al., 2009). By contrast, the *klv* transgenic hairy roots overexpressing *LjCLE-RS1* or *LjCLE-RS2* still exhibited typical hypernodulating phenotypes (Fig. 5B,D). These results indicate that KLV, like HAR1, is also required for the *LjCLE-RS1*- and *LjCLE-RS2*-induced negative regulation of nodulation.

Cytokinin signaling is necessary and sufficient for root nodule development (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007). A gain-of-function mutation (*snf2*) in the cytokinin receptor LHK1 results in the development of nodules

even in the absence of rhizobia (i.e. spontaneous nodules) (Tirichine et al., 2007). To investigate whether KLV affects this spontaneous nodule formation, we crossed *klv* with a *snf2* mutant. Five weeks after germination, the number of spontaneous nodules in the *klv snf2* double mutants that were homozygous for *klv* (*klv*/*klv*) was significantly higher than in the *snf2* single mutants (*KLV*/*KLV*) or in the *snf2* mutants that were heterozygous for *klv* (*KLV*/*klv*) (Fig. 5E), suggesting that KLV negatively regulates the spontaneous nodule formation caused by constitutively active LHK1. Thus, we hypothesize that KLV acts on LHK1, or downstream of LHK1, as is the case with HAR1 (Fig. 5F) (Tirichine et al., 2007).

KLV interacts with HAR1 and itself in *N. benthamiana*

The genetic interaction and the vascular-specific expression of *KLV* and *HAR1* prompted us to examine whether KLV can form a receptor complex with HAR1. To examine the physical interactions between these RLKs, we performed transient co-expression of epitope-tagged KLV and HAR1 in *N. benthamiana* leaves and the resulting protein extracts were subject to co-immunoprecipitation. First, we confirmed the presence of KLV-Myc and HAR1-Myc in total protein extracts (Fig. 6A,B, lower left). Although HA-fused proteins were hardly detected in total protein samples (Fig. 6A,B, upper left), we could detect KLV-HA and HAR1-HA in the immunoprecipitates using anti-HA antibody (Fig. 6A,B, upper right). These data validate co-expression of epitope tag-fused RLKs in *N. benthamiana*.

By co-immunoprecipitation using anti-HA antibody, we detected the interaction of HAR1-HA with KLV-Myc (Fig. 6A, lower left, fourth lane). Similarly, we observed interaction of KLV-HA with HAR1-Myc by a reciprocal co-immunoprecipitation using anti-HA antibody (Fig. 6B, lower left, third lane). Interactions between KLV-HA and KLV-Myc (Fig. 6A, lower left, third lane) and between HAR1-HA and HAR-Myc (Fig. 6B, lower left, fourth lane) were also detected. These biochemical data indicate that KLV has the ability to form a heterodimer (or oligomer) with HAR1 and forms a homodimer. It is also possible that HAR1 might form a homodimer, at least in *N. benthamiana*.

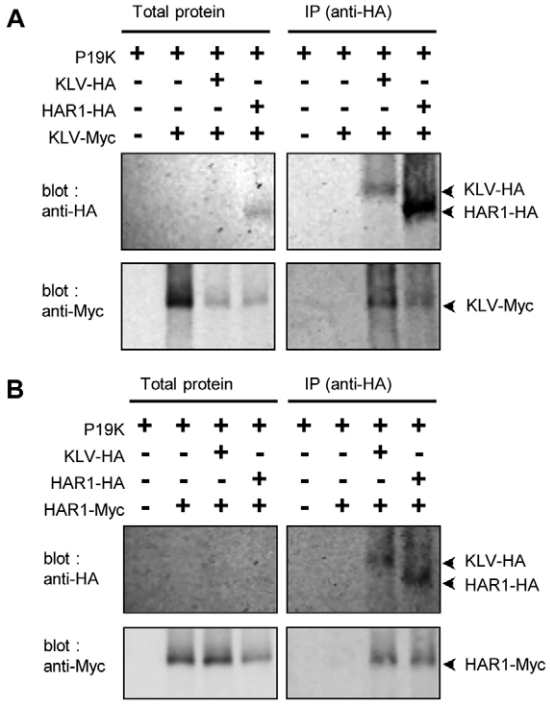

Fig. 6. Physical interactions of KLV with HAR1. (**A**,**B**) KLV-Myc (A) and HAR1-Myc (B) were co-expressed with KLV-HA or HAR1-HA in *N. benthamiana*. Total protein extracts were immunoprecipitated using anti-HA antibody. Total protein extracts and immunocomplexes were analyzed by western blotting. KLV-HA, HAR1-HA, KLV-Myc and HAR1-Myc were detected by anti-HA or anti-Myc antibody. The experiment was repeated at least twice with a similar result.

DISCUSSION

KLV and *HAR1* negatively regulate nodulation in the same genetic pathway

In the present study, we demonstrated that *KLV* encodes an LRR-RLK that mediates the long-distance negative regulation of nodulation. The functional requirement of KLV and HAR1 in the shoot suggests that the shoot is a crucial compartment that monitors and regulates root nodule development. The *HAR1* expression pattern is consistent with a recent report that the *HAR1* promoter is active in the vascular bundle, especially in the phloem (Nontachaiyapoom et al., 2007). It has been proposed that the putative root-derived signal molecule(s) are transported through the vascular tissues to the shoot (Magori and Kawaguchi, 2009). Thus, the expression of *KLV* and *HAR1* in the vascular tissues (Fig. 3E-G) makes biological sense. These receptors could potentially receive such root-derived mobile ligand(s) in the shoot vasculature and trigger the downstream signaling pathways, leading to the generation and transportation of these shoot-derived signals to the root, controlling nodulation.

The nodule number in *klv har1* double mutants was neither additive nor intermediate, indicating that *KLV* and *HAR1* function in the same genetic pathway to suppress nodulation (Fig. 4A,C). In terms of nodule number, the *klv* mutation seemed to have an epistatic effect on nodulation in the *har1-7* background. This is likely to be because the *klv*-like growth inhibition in the double mutant (Fig. 4B,D) has a negative influence on nodule number independently of any epistatic interaction with *HAR1*. For example, nodulation in the double mutant might be reduced because of a limitation in photosynthates needed for nodule formation.

Further experiments were carried out to investigate downstream and upstream of KLV in the signal transduction pathway. Little is known about the pathway downstream of HAR1. A root-controlled hypernodulating mutant, *too much love* (*tml*), has been isolated (Magori et al., 2009). Double-mutant analysis and grafting experiments indicate that *TML* functions downstream of *HAR1*, but the molecular properties of TML remain to be elucidated. In addition, the *har1 snf2* double mutant exhibited an excessive number of spontaneous nodules, suggesting that HAR1 acts downstream of LHK1, negatively regulating root nodule formation (Tirichine et al., 2007). The analysis of the double mutant *klv snf2* indicated that KLV also acts on LHK1 and/or downstream of LHK1 in the nodulation signaling pathway (Fig. 4G). The small peptides derived from the *LjCLE-RS1* and *LjCLE-RS2* genes are potential candidates for the root-derived mobile signals that function upstream of HAR1, as these genes are upregulated in roots in response to rhizobial infection, and overexpression of these genes negatively regulates nodulation via HAR1 (Okamoto et al., 2009). The overexpression of *LjCLE-RS1* or *LjCLE-RS2* in the roots of the *klv* mutant did not affect its hypernodulating phenotype (Fig. 5B,D). Therefore, functional KLV is required for *LjCLE-RS1*-

or *LjCLE-RS2*-mediated suppression of nodulation. Furthermore, if any pathway other than *HAR1-KLV* is present, then *CLE* overexpression should have reduced the hypernodulation of *har1* and *klv*. The lack of any such effect suggests that both KLV and HAR1 are completely indispensable for the *LjCLE-RS1*- and *LjCLE-RS2*-induced systemic regulation of nodulation. These results also support the conclusion that HAR1 and KLV act in the same pathway and that no other pathway has a major function downstream of LjCLE-RS1 and LjCLE-RS2.

KLV mediates multiple aspects of shoot development in *Lotus*

It has been reported that the *klv* mutant shows not only hypernodulation, but also pleiotropic non-symbiotic phenotypes, such as late flowering, aberrant leaf vein morphology, dwarfed shoots and fasciated stems. However, it was not clear whether only one gene could be responsible for all these phenotypes as *klv* was isolated by ion beam irradiation, which has the ability to induce large deletions (Shikazono et al., 2005). Our complementation study with the *KLV* gene indicated that KLV functions in several non-symbiotic signaling pathways that control SAM maintenance, leaf vascular development and flowering time. Expression of *KLV* was confirmed in various organs of *Lotus* plants with or without *M. loti* inoculation (Fig. 3C). Interestingly, *KLV*, but not *HAR1*, is expressed in the shoot apex, a compartment that is crucial for proper shoot development. This expression pattern also supports the hypothesis that KLV is involved in pleiotropic organ development.

A model of the molecular mechanisms of KLV signaling

Consistent with our genetic studies, the biochemical analyses demonstrate that KLV physically associates with HAR1 in *N. benthamiana*. This suggests that KLV and HAR1 can form a receptor complex in *Lotus*. Taken together, we propose the following model for the mechanisms of the systemic regulation of nodulation. First, rhizobial infection and the subsequent nodulation signaling in the root trigger the generation of LjCLE-RS1 and LjCLE-RS2 peptides. These peptides, or their downstream signaling molecules, might travel from the root to the shoot. In the shoot, the KLV-HAR1 receptor complex perceives the root-derived signals. Then, the activation of downstream signaling leads to the production of as yet unidentified shoot-derived signals, which are transported to the root and suppress further nodule formation (see Fig. S5A in the supplementary material).

Because our biochemical data were obtained from a heterologous system using *N. benthamiana*, further investigations might be necessary to understand the native mode of action of the KLV-HAR1 receptor complex in *Lotus* in non-symbiotic and symbiotic conditions.

Previous studies showed that the kinase domain of NARK has the capacity for intermolecular autophosphorylation in vitro (Miyahara et al., 2008), suggesting that HAR1, the *Lotus* ortholog of NARK, also might undergo homodimerization. The ability of KLV and HAR1 to form a homodimer implies the presence of a large receptor complex (for example, a heterotetramer) comprising KLV and HAR1. However, it is also possible that KLV and HAR1 homodimers control different aspects of developmental signaling. KLV possesses non-symbiotic functions in the regulation of flowering time, vascular development and SAM homeostasis. In these non-symbiotic regulations, the KLV-HAR1 complex seems to have no function because the *har1* single mutant does not exhibit these phenotypes. The KLV homodimer (and, in addition, other heteroreceptor complexes containing KLV) could mediate multiple signal transduction pathways that regulate shoot development in legumes (see Fig. S5B in the supplementary material).

The *Arabidopsis* gene with the highest similarity to *KLV* is *RPK2* (*TOAD2*), which has been reported to play roles in *Arabidopsis* anther development (Mizuno et al., 2007) and embryonic pattern formation (Nodine et al., 2007). The *rpk2* mutant displays multiple phenotypes, including male sterility, increased inflorescence branching and spindly shoots. By contrast, the *klv* mutant does not show any male sterility, although the isolation of other alleles of *klv* would be necessary to revisit this issue.

In *Arabidopsis*, the regulation of meristem maintenance has been intensively studied. It has been shown that CLV1 forms homodimers (Bleckmann et al., 2010; Guo et al., 2010). In addition, CLV2, an LRR receptor-like protein that lacks a kinase domain, forms a complex with CORYNE [CRN; also known as SUPPRESSOR OF LLP1 2 (SOL2)], a membrane-associated kinase that regulates the maintenance of meristem (Bleckmann et al., 2010; Guo et al., 2010; Zhu et al., 2010). These receptor complexes have been proposed to perceive CLV3, a small signaling peptide containing the CLV3/ESR (CLE) domain, in parallel. Recent studies have demonstrated that RPK2 regulates SAM homeostasis in parallel with the CLV1 and CLV2-CRN pathways (Kinoshita et al., 2010). RPK2 forms a homoreceptor complex, but does not interact with CLV1. This is in contrast to the finding that KLV and HAR1 function in the same genetic pathway to regulate nodulation and potentially form a receptor complex. In terms of regulation of the SAM, the ability to homodimerize independently of HAR1 or CLV1 is thought to be conserved between KLV and RPK2.

Further genetic and biochemical studies might identify other signaling components that interact with KLV. Nevertheless, our present data demonstrate that KLV is an essential component that orchestrates diverse aspects of plant development.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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