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

Hikota Miyazawa¹2,*, Erika Oka-Kira²,*, Naoto Sato², Hirokazu Takahashi³, Guo-Jiang Wu²,*, Shusei Sato⁴, Masaki Hayashi⁵, Shigeyuki Betsuyaku², Mikio Nakazono³, Satoshi Tabata⁴, Kyuya Harada⁵, Shinichiro Sawa², Hiroo Fukuda² and Masayoshi Kawaguchi¹67,‡

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; Schauer et al., 1998; Szczyslowski 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, Pism 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 ABERANT 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. Drafting 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

1Division of Symbiotic Systems, National Institute for Basic Biology, Nishigonaka 38, Myodaiji, Okazaki, Aichi 444-8585, Japan. 2Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan. 3Graduate School of Science, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo, 113-8657, Japan. 4Kazusa DNA Research Institute, Kazusa-kamatari 2-6-7, Kisarazu, Chiba 292-0818, Japan. 5National Institute of Agrobiological Sciences, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan. 6Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Honcho 4-1-8, Kawaguchi, Saitama 332-0112, Japan. 7Department of Basic Biology, School of Life Science, Graduate University for Advanced Studies (SOKENDAI), Okazaki, Aichi 444-8585, Japan.

*These authors contributed equally to this work.

1Present address: South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China

2Author for correspondence (masayosi@nibb.ac.jp)

Accepted 15 October 2010
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 HARI 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 HARI 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 nonsymbiotic 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 nodd (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 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 HARI mediate the systemic regulation of nodule function in the same genetic pathway. Furthermore, biochemical data demonstrate that KLV interacts with HARI 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-29 (Kawaguchi, 2000) was used as the wild type. After overnight water absorption, plants were grown with or without Mesorhizobium loti MAFF 30-1099 in autoclaved vermiculite supplied with Broughton and Dilworth (B&D) solution (Broughton and Dilworth, 1971) containing 0.5 mM KNO3, under 16-hour light/8-hour dark cycles at a light intensity of 150 μE/m2/s at 22°C in a Biotron LH-300 incubator (Nikon-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'-TCTTCTCATCATCATTACGTCTC-3'; PCR-Rv1, 5'-ACCCAAA-CGTCAGTACACTGTGACT-3'; Sequence-Fw1, 5'-CTGTGTTG-GTATGCGGCTTTG-3'; Sequence-Fw3, 5'-CTGAATTCCTCTAGAACC-ATGTC-3'; Sequence-Rv1, 5'-TGGCCTGATTCCTGTGGGATC-3'; PCR-Fw2, 5'-TCTGTTCTCATGCTTAATTTGGC-3'; PCR-Rv2, 5'-GCAACTGATGGTGGACCA-3'; and Sequence-Fw4, 5'-ATGATCACTGCGTCAGG-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. A 3.7 kb fragment containing an upstream region of the gene was amplified using a forward primer containing a NotI adaptor sequence (5'-AAGGAAAAGCCGGTCCGCTTGAATGGGCAC-3') and a reverse primer (5'-CCATAGGATATGTCTACAGG-3'). The resulting PCR product was digested and ligated into the NotI and SpeI sites of pART27. This plasmid was digested with SpeI and ligated to the 6.9 kb SpeI 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 hypopcocyl transformation. Transformation was performed as described previously (Stiller et al., 1997), with slight modifications. The T2 plants were used for analyses. A primer set for NPTII (forward, 5'-ATGGCAATTACCTTATCCGC-3'; reverse, 5'-TCAGAACATCCGTCAAGAA-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 shoots 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 RTP-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'-CATGCGTTGCACATACATACCA-3' and 5'-TCCCCACTCCTCAGACATAC-3'; KLV, 5'-CTGCGACTTGGCGTGTGGTTG-3' and 5'-AGCCACGACCGCTTCCATAC-3'; HARI, 5'-TTTGTATGACCGCTTCTGCTC-3' and 5'-CTGACTCTCTCTCG-CCACTTC-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). QuantiT RT 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 F2 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'-CAGATCTGACAAACGCTTACGCGTTGATG7'-reverse primer, 5'-TGAATGGACCTACACTCGCGATGATATG-3'; digest with SmfI (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'-GATGATGATTAAATGCTTATCTATCTTA-3'; reverse primer, 5'-AATCTGGAACGTCTGGAGTCGATGTTG-3) and digestion with *AgeI* (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 (*snf2*) mutant from MG-20 by ethylene sulfonate (EMS) mutagenesis. Sequencing analysis revealed that this *snf2* 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 F2 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::pmPr90.

For transient expression, *A. tumefaciens* strains carrying each construct together with another *Agrobacterium* strain harboring the p19 silencer 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**

*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 (CTTGT 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 stems (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
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 87±3 (line 2). 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
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 a 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 show 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 F1 plants that were heterozygous for both of the mutations were analyzed by real-time RT-PCR. The expression of both KLV and HAR1 was completely restored in the klv har1 double mutant (Fig. 3G).

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.
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 HAR1-Myc (Fig. 6A, lower left, third lane) and between HAR1-HA and KLV-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*.

**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 HARI-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 (Kinoshiita 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.

**Acknowledgements**

We thank Dr David Baulcombe and Plant Bioscience (Norwich) for *Agrobacterium* strain carrying the p19 silencing suppressor; Dr Nono Suganuma (Aichi University of Education, Aichi, Japan) and Saori Tomisawa for providing the *Lotus har1-7* mutant and the *M. loti* background snf2 mutant seeds; and Dr Shimpeii Magori (State University of New York, Stony Brook, NY, USA), Dr Louis Irving and Dr Krzysztof Szczegolowski (Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, Ontario, Canada) for reviewing the text. H.M. was funded by a Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science. This work was supported in part by the Center for the Promotion of Integrated Sciences (CPS) of Sokendai. This work was supported by Grants-in-Aid for Scientific Research for Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (18056004) and for Core Research for Evolutional Science and Technology (CREST) from the Japan Science and Technology Agency.

**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.058891/-/DC1

**References**


Control of nodule and shoot development


