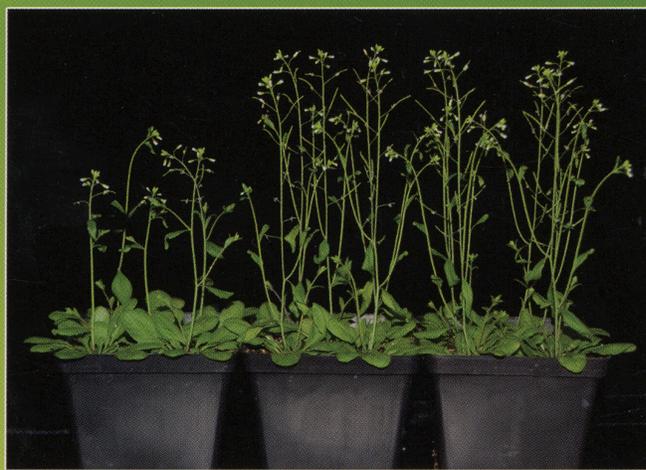


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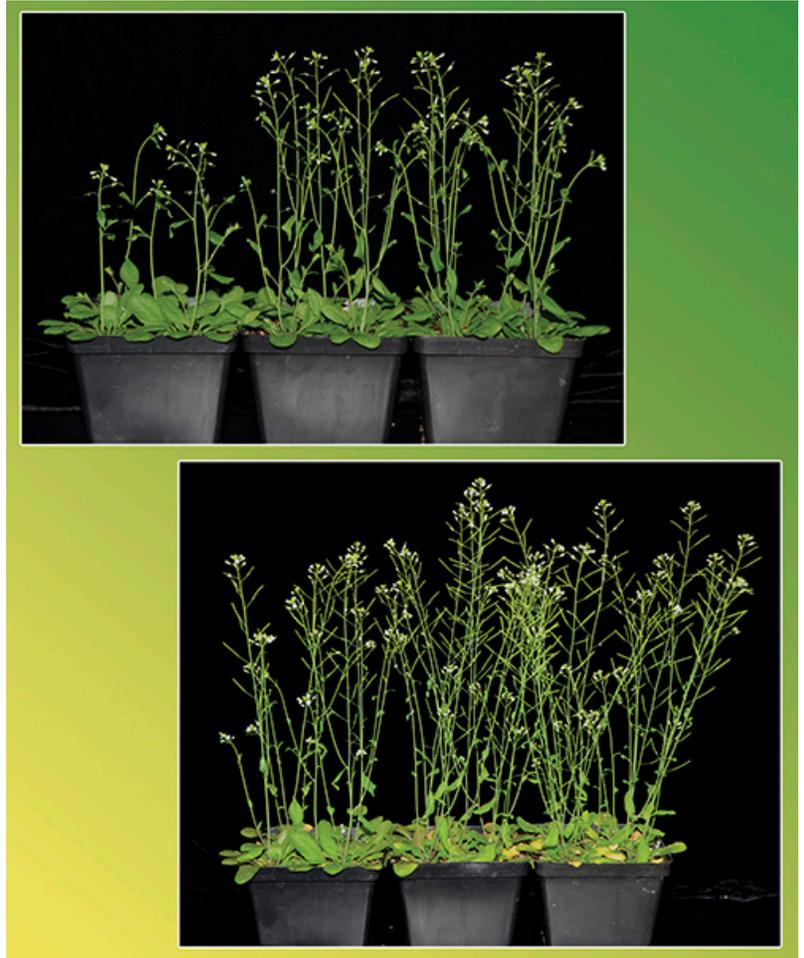
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Plant growth promoted by effector overexpression is seen in the vegetative growth of transgenic *Arabidopsis* plants overexpressing *LjPSK1* or *AtPSKR1*, observed at days 30 (above) and 36 (below) after transplanting. (Wang et al.)

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Phytosulfokine Is Involved in Positive Regulation of *Lotus japonicus* Nodulation

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Phytosulfokine (PSK) is a tyrosine-sulfated peptide that is widely distributed in plants, participating in cell proliferation, differentiation, and innate immunity. The potential role of PSK in nodulation in legumes has not been reported. In this work, five PSK precursor genes were identified in *Lotus japonicus*, designated as *LjPSK1* to *LjPSK5*. Three of them (*LjPSK1*, *LjPSK4*, and *LjPSK5*) were found to be expressed in nitrogen-fixing root nodules. *LjPSK1* and *LjPSK4* were not induced at the early stage of nodulation. Interestingly, while the expression of *LjPSK4* was also found in spontaneous nodules without rhizobial colonization, *LjPSK1* was not induced in these pseudo nodules. Promoter- β -glucuronidase analysis revealed that *LjPSK1* was highly expressed in enlarged symbiotic cells of nodules. Exogenous addition of 1 μ M synthetic PSK peptide resulted in increased nodule numbers per plant. Consistently, the number of mature nodules but not the events of rhizobial infection and nodule initiation was increased by overexpressing *LjPSK1* in transgenic hairy roots, in which the expression of jasmonate-responsive genes was found to be repressed. These results suggest that PSK is a new peptide signal that regulates nodulation in legumes, probably through cross-talking with other phytohormones.

Legumes interact with soil bacteria rhizobia and form a new root organ known as the nodule, in which rhizobia fix atmospheric dinitrogen into reduced nitrogen compounds that are useable by plants. Nodulation (Nod) factors are the most important signal molecule secreted by rhizobia during establishment of the *Rhizobium*-legume symbiosis (Downie 2014; Oldroyd and Downie 2008). At the early stage of symbiosis, the roots of legumes exude specific secondary metabolites, such as flavonoids, that are capable of entering rhizobia and inducing the expression of nodulation genes responsible for Nod factor biosynthesis. Rhizobial Nod factors are lipo-chitoooligosaccharides

Nucleotide sequence data are available in the GenBank database under accession numbers KM065832 for *LjPSK1*, KM065833 for *LjPSK2*, KM065834 for *LjPSK3*, KM065835 for *LjPSK4*, and KM065836 for *LjPSK5*.

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*The e-Xtra logo stands for “electronic extra” and indicates that six supplementary figures are published online.

that are recognized by two lysin-motif (LysM) receptor kinases (NFR1 and NFR5 in *Lotus japonicus* or NFP and LYK3 in *Medicago truncatula*). After perception of the Nod factor signal, the receptors elicit a specific signal cascade, resulting in the reprogrammed expression of downstream genes, such as *NIN* and *ENOD40* (Fang and Hirsch 1998; Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003; Smit et al. 2007). Susceptive root systems allow rhizobia to enter epidermal cells through the development of infection threads (IT). Subsequently, rhizobia are released from the end of IT into de-differentiated host cortical cells. Functional nitrogen-fixing nodules are formed when infected rhizobia undergo differentiation into bacteroids that are enclosed by a plant-derived membrane known as the peribacteroid membrane. Root nodules or nodule-like structures can also be induced spontaneously, which can develop in the absence of rhizobia in legume roots with gain-of-function mutations in Ca^{2+} /calmodulin-dependent protein kinase (CCaMK) or cytokinin histidine kinase receptor (Gleason et al. 2006; Tirichine et al. 2006). Spontaneous nodules exhibit cortical cell division as the wild-type nodules do but do not contain rhizobia and, thus, do not fix atmospheric nitrogen.

The number of nodules developed on a root system is strictly regulated through a complex root-to-shoot-to-root negative feedback loop, known as autoregulation of nodulation (Ferguson et al. 2010). Root-derived signals are transported to the shoot, in which the signals are perceived by a leucine-rich repeat receptor kinase (LjHAR1, MtSUNN, and GmNARK) (Krusell et al. 2002; Schnabel et al. 2005; Searle et al. 2003). Null mutations of these genes result in deregulated hypernodulation after inoculation with rhizobia. Recently, root-derived CLAVATA3/ESR-related (CLE) glycopeptides from *L. japonicus* have been found to serve as the ligands of LjHAR1 and to be capable of suppressing nodulation (Okamoto et al. 2013). CLE are small, secreted peptides that universally exist in plant species, including legumes. At least 39, 25, and 39 CLE genes have been identified in the genomes of *L. japonicus*, *Medicago truncatula*, and *Glycine max*, respectively (Funayama-Noguchi et al. 2011; Mortier et al. 2010, 2011). In *L. japonicus*, *LjCLE-RS1* and *LjCLE-RS2* are strongly upregulated in roots that have been inoculated with rhizobia. Overexpression of either of *LjCLE-RS1* and *LjCLE-RS2* genes in hairy roots can result in a systemic inhibition of nodulation in a HAR1-dependent manner (Okamoto et al. 2009). Conversely, knockdown of *MtCLE12* and *MtCLE13* increases the number of nodules per root system (Mortier et al. 2012). Functionally similar CLE genes have also been found in *Glycine max* (Reid et al. 2011).

Other plant peptides have also been implicated in nodule development. The putative peptide products of *ENOD40* are required for the differentiation of root cortical cells and the initiation of nodule primordia. The expression of *ENOD40* genes is induced in roots at the very early stage of rhizobial infection or Nod factor application. Knockdown of *ENOD40* gene expression leads to significant suppression of nodule formation but has no effect on the early bacterial infection events, indicating a growth-regulating role of *ENOD40* in nodule organogenesis (Kumagai et al. 2006; Wan et al. 2007). Recently, *Medicago truncatula* C-terminally encoded peptide 1 (MtCEP1) has been shown to act as a developmental signal to enhance nodulation. Overexpression of MtCEP1 or application of the MtCEP1 peptide to roots results in inhibition of lateral root formation and enhancement of nodulation (Imin et al. 2013). The molecular mechanism by which CEP1 regulates nodulation is not clear. Nodule-specific cysteine-rich peptides have recently been identified, and their role in bacteroid differentiation during symbiosis has been suggested (Farkas et al. 2014; Penterman et al. 2014; Van de Velde et al. 2010).

Phytosulfokine (PSK)- α represents the first sulfated peptide ($Y_{SO_3H}IY_{SO_3H}TQ$) identified in plants. PSK- α has been shown to regulate cell proliferation in cell cultures of *Asparagus officinalis* L. and rice (*Oryza sativa* L.) (Matsubayashi and Sakagami 1996; Matsubayashi et al. 1997). PSK- α also acts as a growth-promotion factor for hypocotyls, roots, and leaves (Huang et al. 2010; Kutschmar et al. 2009; Stührwohldt et al. 2011). PSK is derived from prepropeptides encoded by five PSK genes in *Arabidopsis thaliana*. It acts as a peptide hormone, whose signal is perceived by the *Arabidopsis* PSK receptor kinase 1 (AtPSKR1) (Matsubayashi et al. 2006), a leucine-rich repeat-containing receptor-like kinase. The PSK signal can also, to a minor extent, be perceived by AtPSKR2 (Kutschmar et al. 2009). PSK precursors are sulfated on two conserved tyrosine residues at their C-termini by a tyrosylprotein sulfotransferase (TPST) (Komori et al. 2009). The sulfated precursors are secreted into the extracellular space or apoplast after cleavage of their N-terminal signal sequence by signal peptidases. A subtilisin serine protease, AtSBT1.1, has been proposed to be required for the processing of AtPSK4 (Srivastava et al. 2008). PSK also participates in wounding responses and immune signal

transduction during plant-microbe interactions. PSK signaling plays an opposite role in the immune response of *Arabidopsis* to biotrophic and necrotrophic pathogens. This antagonistic manner appears to depend on modulated homeostasis between the salicylate and jasmonate signaling pathways (Igarashi et al. 2012; Mosher et al. 2013). Homologous genes of PSK are widely present in higher plants including legumes, but whether PSK regulates legume nodulation is not known.

In this work, we identified five PSK precursor genes in *L. japonicus* and characterized their expression patterns. The potential functions of a nodule-specific PSK precursor gene, *LjPSK1*, and a chemically synthesized PSK peptide on nodulation were examined. Our results suggest that PSK may positively regulate legume nodulation in a manner independent of the Nod factor signaling pathway.

RESULTS

Identification of PSK precursor genes in *L. japonicus*.

To identify PSK precursor genes in *L. japonicus*, *Arabidopsis* PSK precursors were used as queries to search all available *L. japonicus* databases by BLAST. Two genes, annotated as chr5.CM0359.140.r2 and chr2.CM0124.210.r2/partial, were found in the *L. japonicus* genome. Further search of the expressed sequence tag database using the C-terminal regions of chr5.CM0359.140.r2 and chr2.CM0124.210.r2/partial led to identification of five putative PSK-encoding sequences, including GO020953.1, FS350221.1, GO018273.1, GO036043.1, and GO036835.1. Among them, GO020953.1 and FS350221.1 are identical to chr5.CM0359.140.r2 and chr2.CM0124.210.r2, respectively. These PSK-encoding sequences were amplified by polymerase chain reaction (PCR), were confirmed by DNA sequencing, and were named *LjPSK1* to *LjPSK5*. Alignment of deduced amino acid sequences of *LjPSK1* to *LjPSK5* with AtPSK1 to AtPSK5 showed that all PSK precursors contain a cleavable secretory signal sequence at the N-termini and an active peptide sequence close to the C termini, where conserved amino acids (Cys, two Arg or Lys, His, and Asp) can be identified (Fig. 1A). Analysis of the amplified genomic DNAs of *LjPSK1* to *LjPSK5* showed that *LjPSK* genes contain two to three exons (Fig. 1B), which is similar to *AtPSK* and *OsPSK* genes (Yang et al. 2000, 2001).

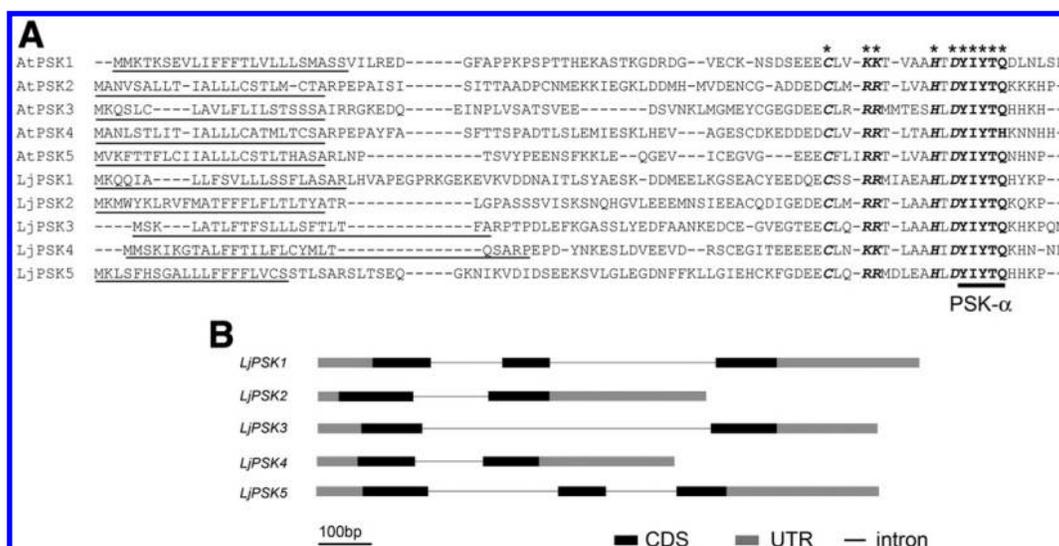


Fig. 1. Amino acid sequence alignment and gene structure of the phytosulfokine (PSK) gene family in *Lotus japonicus*. **A**, Amino acid sequences of the PSK gene family from *L. japonicus* and *Arabidopsis thaliana* were aligned using ClustalX. The conserved residues were marked with asterisks. The predicted secretory signal peptides were underlined. **B**, Gene structures of *LjPSK1* to *LjPSK5* were predicted, using the mRNA-to-genomic alignment program Spidey. *LjPSK* contains one to two introns that are indicated by lines. Black and gray boxes represent the coding sequences (CDS) and untranslated regions (UTR), respectively.

Expression profiles of *LjPSK* genes.

To characterize the expression profiles of *LjPSK* genes, we evaluated the transcript abundance by quantitative reverse transcriptional real-time (qRT)-PCR in roots, stems, leaves, flowers, nodules and pods (Fig. 2A). Transcripts of *LjPSK* genes were detected in all tissues tested, but certain *LjPSK* genes were predominantly expressed in one or two tissues. For example, *LjPSK1* and *LjPSK4* were expressed at much higher levels in nodules than in other tissues. The *LjPSK3* mRNA was most detected in stems and flowers. *LjPSK5* was found mostly in roots, nodules, and pods. Analysis of the transcriptomic data in the other legume model plant *Medicago truncatula* reveals that *MtPSK* genes are highly expressed in nodules, implying a conserved role of PSK in nodulation (Supplementary Fig. S1)

(Roux et al. 2014). The maturation process of a PSK peptide includes sulfation of the peptide by a TPST, encoded by *AtTPST* in *Arabidopsis*. In *Medicago*, a putative *MtTPST* is constitutively expressed in both roots and nodules, with an elevated level in the latter. We also identified the putative ortholog of *AtTPST* in *L. japonicus* (*LjSGA_033116.1*). Expression analysis by qRT-PCR revealed that *LjTPST* is expressed at a higher level in nodules than roots, which is consistent with the expression pattern of *MtTPST* in *Medicago* spp. (Supplementary Fig. S2).

Specific expression of *LjPSK1* and *LjPSK4* in nodules.

Nodules at different development stages were collected for qRT-PCR analysis of the expression of *LjPSK1* and *LjPSK4*.

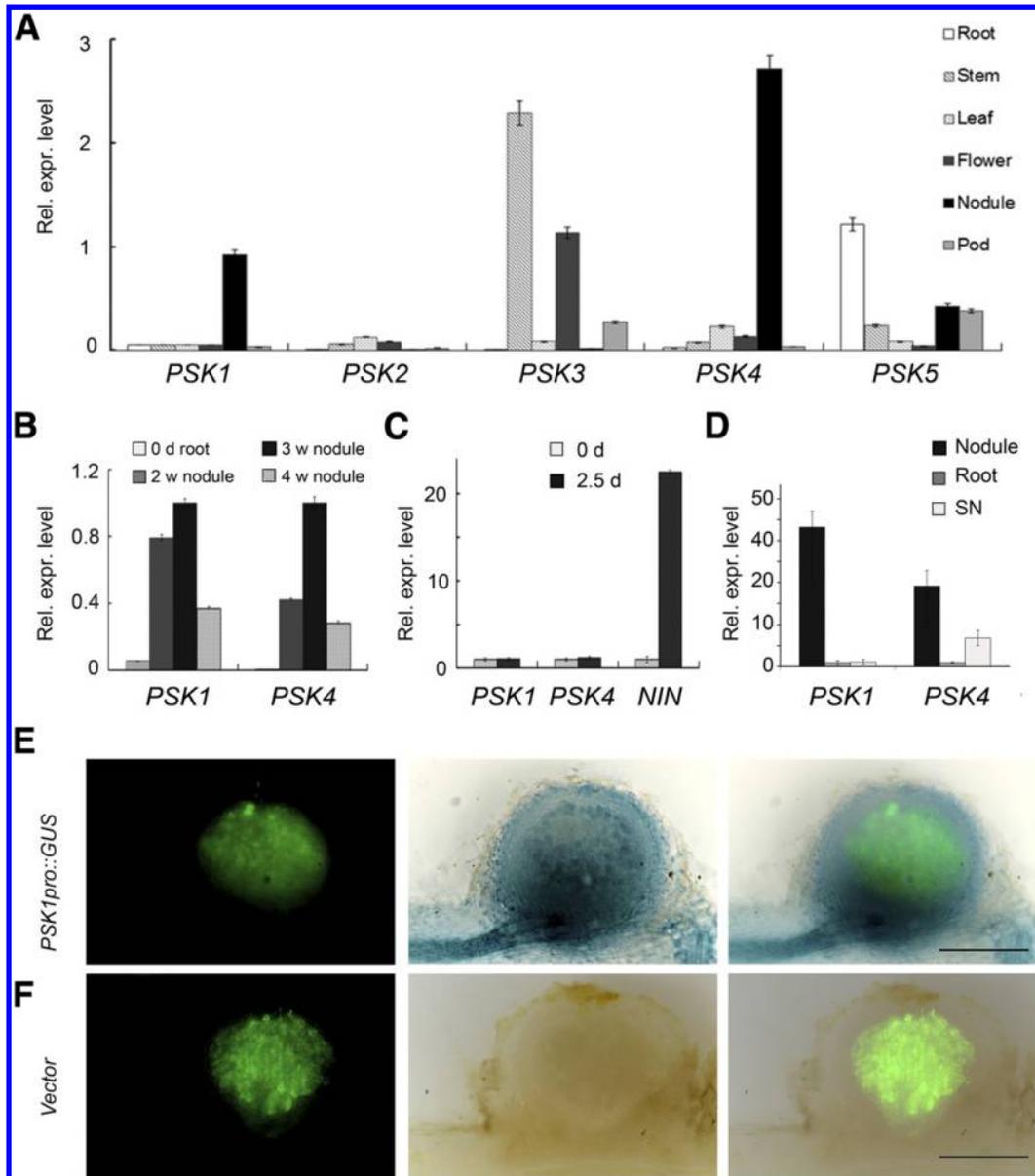


Fig. 2. Expression profiles of *LjPSK1* to *LjPSK5*. **A**, Expression patterns of *LjPSK1* to *LjPSK5* genes assessed by quantitative reverse transcription-polymerase chain reaction. Total RNA was isolated from *Lotus japonicus* uninoculated roots, 3-week-old stems, leaves, nodules, flowers, and pods. Relative expression levels (Rel. expr. level) were normalized against the internal control of *ATPase* gene expression. **B**, RNA samples were obtained from nodules 2 to 4 weeks after inoculation with *Mesorhizobium loti*. Uninoculated roots served as a control. **C**, Expression of *LjPSK1* and *LjPSK4* in roots 2.5 days postinoculation with *M. loti*, as compared with the uninoculated roots (0 d). The expression of *NIN* was used as a marker gene to indicate normal response of an early nodulation gene to rhizobial infection. **D**, Expression of *LjPSK1* and *LjPSK4* in 3-week-old wild-type nodules, nodule-stripped roots, and spontaneous nodules produced on transgenic hairy roots expressing *LjCCaMK^{T265D}* on *nfr5-2* mutant plants. Error bars indicate \pm standard error from three biological replicates. **E**, Transgenic hairy roots expressing *LjPSK1* promoter- β -glucuronidase (GUS) fusion were inoculated with *M. loti* constitutively expressing GFP and nodules were stained for 8 h for GUS activity analysis. **F**, Nodules on transgenic hairy roots expressing an empty vector served as a negative control.

The results showed that the expression of both genes peaked in nodules 3 weeks postinoculation with *Mesorhizobium loti* and decreased drastically in 4-week-old nodules, suggesting that PSK is involved in nodule development and may not be required for nitrogen fixation in mature nodules (Fig. 2B). At the early stage of nodulation, the expression of *LjPSK1* and *LjPSK4* did not appear to be induced by inoculation with *M. loti* (Fig. 2C) as compared with the positive control gene *LjNIN*, which showed normal induction by rhizobial inoculation (Fig. 2C). Similar samples were used to test the expression level of *LjTPST*, and no induction of *LjTPST* at the early stage of nodulation was observed.

In nodules, the expression pattern of *LjPSK1* and *LjPSK4* was further investigated in spontaneous nodules in comparison with the wild-type nodules and nodule-stripped roots 3 weeks postinoculation with *M. loti*. To produce spontaneous nodules, we used *Agrobacterium rhizogenes*-mediated transformation to generate chimeric transgenic plants with transformed hairy roots attached to an untransformed shoot (Kumagai and Kouchi 2003). Spontaneous nodules were developed on transgenic hairy roots constitutively expressing the gain-of-function mutation of *LjCCaMK*, *LjCCaMK^{T265D}*, under the control of a ubiquitin promoter. The *nfr5-2* mutant plants were used to produce transgenic hairy roots. As shown in Figure 2D, the expression level of the nodule-specific *LjPSK1* in spontaneous nodules was similar to that in roots. However, the expression level of *LjPSK4* was elevated in spontaneous nodules. The results suggest that the

induced expression of *LjPSK1* but not *LjPSK4* in wild-type nodules depends on rhizobial colonization in nodules.

Promoter- β -glucuronidase (GUS) analysis of *LjPSK1* in nodules.

To study the expression pattern of *LjPSK1* at a cellular level in nodules, an approximate 4-kb promoter fragment upstream of the start codon was cloned and was used to drive the expression of the *GUS* reporter gene. The *har1* hypermodulation mutant plants were used to produce transgenic hairy roots, which were found to be able to develop more nodules. GUS-staining analysis showed high expression of *LjPSK1* in the whole nodule, including the central zone and surrounding cortical cells (Fig. 2E). Interestingly, at higher magnifications, higher GUS activity was observed in *Rhizobium*-infected cells than uninfected cells in the central zone of a nodule (Fig. 2E). Nodules formed on hairy roots expressing an empty vector served as a control, which showed no GUS activity (Fig. 2F).

Promotion of *L. japonicus* root growth and nodule formation by synthetic PSK- α .

PSK- α has been reported to function as a growth factor to modulate cell proliferation and differentiation. At a concentration range between 10 nM and 10 μ M, synthetic PSK- α peptide promoted *A. thaliana* root growth (Fig. 3A), which is consistent with the observation described previously (Kutschmar et al. 2009). The length of *A. thaliana* roots increased as the concentration of PSK- α in the growth medium was elevated to approximately 1 μ M (Fig. 3A). *L. japonicus* roots were significantly elongated from 3.24 to 4.17 cm after 6 days of growth on plates containing 1 μ M PSK- α (Fig. 3B), suggesting that a common PSK- α response system exists in *A. thaliana* and *L. japonicus*. To estimate a possible effect of PSK- α on nodulation, *L. japonicus* plants were supplied with nitrogen-free liquid medium containing both rhizobia and 1 μ M PSK- α . The nodule number per root system, as counted 12 days after rhizobial inoculation, was higher in roots treated with PSK- α than in the control roots that were either untreated with PSK- α or treated with a mixture of the free amino acids constituting PSK (Fig. 3C). The promotion effect of PSK- α on nodulation was relatively weak, yet statistically significant. These observations indicate that PSK- α could positively regulate primary root growth and nodulation in *L. japonicus*.

Promotion of nodulation on transgenic hairy roots overexpressing *LjPSK1* or *AtPSKR1*.

To confirm the role of PSK signaling in nodulation, the *L. japonicus* PSK- α precursor gene *LjPSK1* and *A. thaliana* PSK- α receptor gene *AtPSKR1* were overexpressed in *L. japonicus* hairy roots under the control of an enhanced *Cauliflower mosaic virus* (CaMV) 35S or a ubiquitin promoter, respectively. The transcript levels of *LjPSK1* and *AtPSKR1* were determined by RT-PCR, confirming that they were overexpressed in transgenic roots as compared with the roots transformed by the empty vector, pC2301, or pUBGFP (Fig. 4C and D). The numbers of white bumps and yellow and red nodules, corresponding to the nodule primordia and developing and mature nodules, respectively, were recorded 12 days postinoculation with *M. loti*. The results show that the nodule numbers per plant, particularly the developing and mature ones, were significantly greater on the hairy roots overexpressing *LjPSK1* and *AtPSKR1* than those on the controls (Fig. 4A and B). This experiment was repeated three times and similar results were obtained, suggesting that the PSK- α peptide and PSK signaling play a positive role in regulation of nodule development. It is possible that the increased nodule number may arise as an accessory consequence of the enhanced hairy-root growth by PSK signaling, because treatment of PSK- α

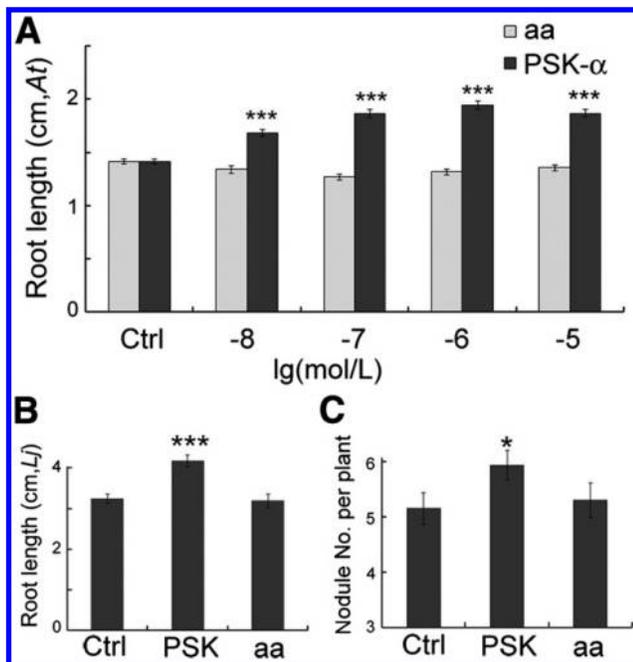


Fig. 3. Effects of phytosulfokine (PSK)- α peptide on primary root growth and nodulation of *Lotus japonicus*. **A**, Sterilized *Arabidopsis* seeds were grown on half-strength Murashige Skoog (MS) medium containing 10^{-8} to 10^{-5} M of PSK- α peptide. Root length (mean \pm standard error [SE]) was determined 6 days after seed germination from 50 to 60 plants per treatment. Treatment with a mixture of the amino acids (aa) comprising PSK served as a control, showing no enhanced root growth. Plants grown on medium without PSK treatments were used as an untreated control (Ctrl). **B**, *L. japonicus* seedlings were transferred onto half-strength MS plates containing 1 μ M PSK- α and were grown for 6 days. Root length was determined (mean \pm SE) with 25 to 40 plants. **C**, *L. japonicus* seedlings were transplanted to growth pots, were inoculated with *Mesorhizobium loti*, and were treated with 1 μ M PSK- α . Nodules per plant were counted from more than 35 plants 12 days after inoculation with *M. loti*. Values that are significantly different from the control, as determined by Student's *t* test, are marked with one ($P < 0.05$) or three ($P < 0.001$) asterisks.

peptide was able to promote the primary root growth (Fig. 3B). However, it is more likely that PSK- α signaling has a primary effect on nodulation, because enhanced nodulation on the hairy roots expressing either *LjPSK1* or *AtPSKR1* could be easily observed as compared with the control hairy roots expressing an empty vector and there was no significant alteration between the two hairy-root samples (Supplementary Fig. S3).

Enhancement of the PSK- α signaling in *Arabidopsis* plants overexpressing *LjPSK1*.

We further examined whether overexpression of *L. japonicus* PSK1 in *Arabidopsis* would have any effect on vegetative growth similar to that triggered by overexpression of *AtPSKR1*. For this purpose, transgenic *Arabidopsis* plants expressing *LjPSK1* or *AtPSKR1* were generated using the same constructs described above. Homozygous T3 transgenic seeds were obtained and two independent lines for *LjPSK1-OX* (overexpressing) or *AtPSKR1-OX* were used for phenotype analysis. Similar to the observation of *AtPSKR1* overexpression (Hartmann et al. 2013; Kutschmar et al. 2009; Matsubayashi et al. 2006), *Arabidopsis* transgenic plants overexpressing *LjPSK1* exhibited phenotypes of leaf enlargement, root elongation (Fig. 4E, F, and G), and enhanced plant growth (Supplementary Fig. S4). Analysis of qRT-PCR revealed an eightfold increase in the *AtPSKR1* mRNA level in the *AtPSKR1-OX* line over the control plant (Fig. 4H). While the *LjPSK1* transcript could not be detected in the wild-type *Arabidopsis* plants, a high level of the *LjPSK1* mRNA could be detected in the *LjPSK1-OX* line (Fig. 4H). We conclude that overexpression of *LjPSK1* or *AtPSKR1* in transgenic *Arabidopsis* plants can lead to enhanced PSK- α signal transduction, promoting vegetative growth.

No observable effect of PSK signaling on rhizobial infection and nodule initiation.

To determine whether PSK signaling modulates *L. japonicus* nodulation at the early stage of *Rhizobium*-legume symbiosis, we examined rhizobial infection and nodule initiation on transgenic hairy roots overexpressing *LjPSK1* or *AtPSKR1*. Microscopic observations showed that the total rhizobial infection events, including numbers of infection foci and IT, were not significantly different between transgenic roots overexpressing *LjPSK1* or *AtPSKR1* and the controls expressing the corresponding empty vectors. The numbers of nodule primordia per plant on transgenic roots were also not significantly changed. We further analyzed the expression of early nodulation genes *NIN* and *ENOD40.1* in the transgenic roots overexpressing *AtPSKR1* and found that the abundance of *NIN* and *ENOD40.1* transcripts was not significantly altered in transgenic roots as compared with those transformed by the empty vector. These results suggest that the effect of PSK on nodulation may not be associated with the rhizobial infection and nodule primordium initiation in the early stage of nodulation (Supplementary Fig. S5).

Repression of jasmonate-responsive genes by overexpression of *LjPSK1* or *AtPSKR1*.

In *Arabidopsis*, PSK signaling has been shown to regulate jasmonic acid (JA)-mediated plant immunity. JA has been reported as a negative regulator for nodulation of legumes with both determinate and indeterminate nodules, such as *L. japonicus* and *Medicago truncatula* (Nakagawa and Kawaguchi 2006; Sun et al. 2006). To test if the effect of PSK on *L. japonicus*

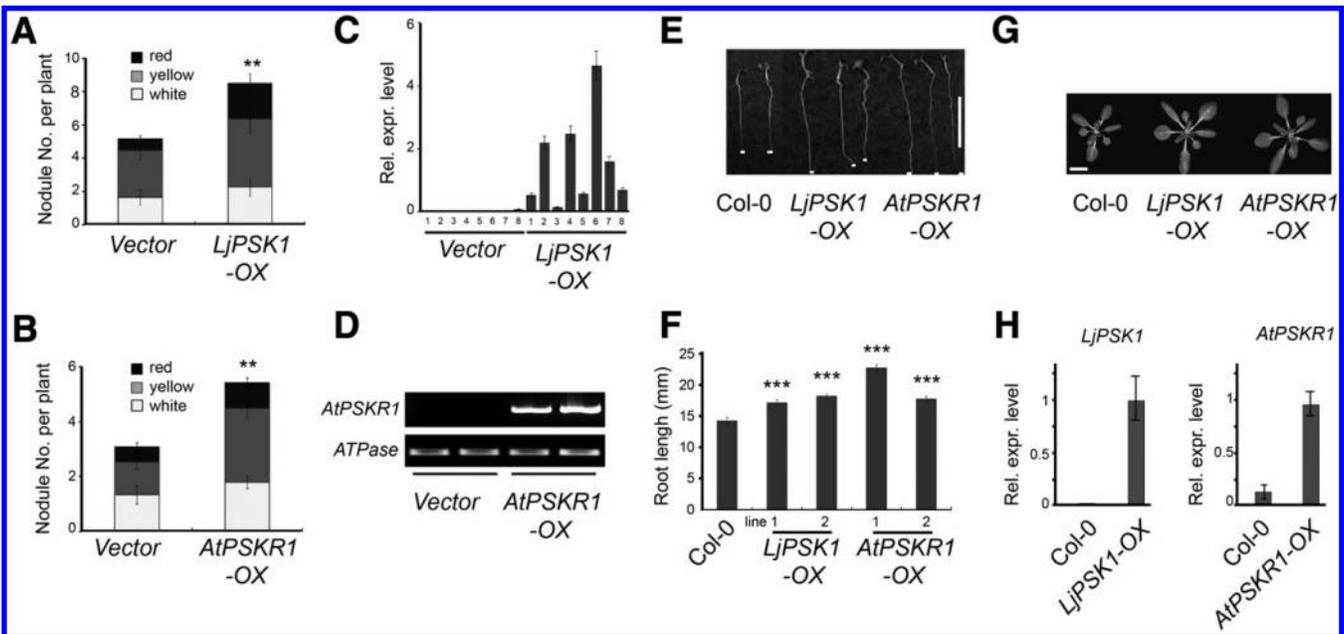


Fig. 4. Effects of *LjPSK1* or *AtPSKR1* overexpression on nodulation and plant growth. Transgenic hairy roots of *Lotus japonicus* expressing *LjPSK1* or *AtPSKR1* were generated via *Agrobacterium rhizogenes*-mediated transformation. Transgenic plants expressing the empty vectors (pC2301 for *LjPSK1-OX* and pUBGFP for *AtPSKR1-OX*) served as controls. Transgenic *Arabidopsis* plants were obtained and T3 homozygous seeds were used for assays. Two representative lines from each group were characterized and compared with wild-type (Col-0) plants. Values in A, B, and F represent the mean \pm standard error (SE) of 15 to 30 plants. **A**, The nodule numbers per plant were increased significantly ($P < 0.01$) in hairy roots expressing *LjPSK1-OX* ($n = 21$) as compared with the control plants ($n = 18$) 12 days postinoculation with *Mesorhizobium loti*. Two additional replications were performed with a total of 51 plants for the control and 65 plants for *LjPSK1-OX* lines, producing a similar result. Nodule primordia (white), young nodules (gray) and mature nodules (black) were recorded separately. **B**, Hairy roots expressing *AtPSKR1-OX* ($n = 53$) formed more nodules as compared with the control ($n = 26$). Statistical significant difference is indicated by two asterisks ($P < 0.01$). **C**, Quantitative and reverse transcription-polymerase chain reaction (RT-PCR) and **D**, RT-PCR were used to assess the expression levels of *LjPSK1* and *AtPSKR1* in hairy roots. **E**, *Arabidopsis* plants grown on half-strength Murashige Skoog (MS) medium for 6 days. *Arabidopsis* root elongation was enhanced significantly by *LjPSK1-OX* or *AtPSKR1-OX*. **F**, Statistical analysis of primary root length (mean \pm SE) of seedlings grown on half-strength MS medium for 6 days. **G**, *Arabidopsis* plants grew in growth cabinets for 2 weeks. Leaf growth was enhanced by expressing *LjPSK1-OX* or *AtPSKR1-OX*. **H**, Expression levels of the *LjPSK1* and *AtPSKR1* transcripts in transgenic *LjPSK1*- and *AtPSKR1*-overexpression lines. Values represent the average levels (\pm SE) of two transgenic lines of either *LjPSK1-OX* or *AtPSKR1-OX*. Bars, 1 cm.

nodulation is correlated with suppression of JA signal transduction, we analyzed the expression of putative JA-responsive genes in three to eight independent transgenic root lines overexpressing *LjPSK1* or *AtPSKR1* in the absence of rhizobia. It has previously been reported that the *L. japonicus* genes encoding proteins homologous to PDF1.2, JAR1, and MYC2 are induced by JA (Suzuki et al. 2011). PDF1.2 is a defensin in the JA-dependent defense response, JAR1 is an enzyme for the production of active JA derivative (e.g., JA-Ile), and MYC2 is a transcription activator of JA responses. Analysis of qRT-PCR results revealed that the expression of the three genes decreased significantly in transgenic roots overexpressing *LjPSK1* or *AtPSKR1* (Fig. 5) with the exception of *JAR1*, whose expression was not apparently changed in *LjPSK1-OX* roots (Fig. 5). These results suggest that the JA signal transduction pathway is downregulated by overexpression of *LjPSK1* or *AtPSKR1*, which may explain why nodulation was increased in *L. japonicus* roots overexpressing *LjPSK1* or *AtPSKR1*.

DISCUSSION

Recent studies have shown that tyrosine-sulfated peptides, including PSK, peptide containing sulfated tyrosine 1 (PSY1), and root-growth factor, are widely present in higher plants (Delay et al. 2013; Matsuzaki et al. 2010; Mosher et al. 2013). They function to control cell differentiation and organ development in plants. Legume root nodules are a unique nitrogen-fixing organ composed of both the rhizobia and host cells. The initiation and development of nodules are tightly regulated by rhizobial Nod factors and plant hormones, including cytokinin, auxin, ethylene, jasmonate, and salicylic acid. PSK has been shown to regulate not only plant development but also plant innate immunity. In this work, we identified five *L. japonicus* PSK precursor genes (*LjPSK1* to *LjPSK5*) and found that the expression of *LjPSK1* and *LjPSK4* were specific in nodules. Our

findings show that PSK is a positive regulator for nodule development and may antagonize the JA signaling, which negatively regulates nodulation in a manner independent of the Nod factor pathway. Therefore, PSK may represent a new player in the regulatory cascade of nodule development.

Search for a receptor of PSK in *L. japonicus*.

Chemically synthesized PSK- α can activate PSK signaling through the receptor kinase *AtPSKR1* to promote cell proliferation and plant growth (Kutschmar et al. 2009; Matsubayashi and Sakagami 1996; Matsubayashi et al. 1997; Stührwohldt et al. 2011). Our data confirmed a positive effect of synthesized PSK- α peptide on root growth in *L. japonicus* (Fig. 3B), suggesting that this peptide could be perceived by an unidentified *LjPSK* receptor, a possible ortholog of *AtPSKR1*. Our search of available databases using *AtPSKR1* has not identified a gene encoding a putative *LjPSK* receptor. As the *L. japonicus* genome sequencing and annotation continues to be improved, an ortholog of *AtPSKR1* may be identified in future studies.

Role of PSK in nodule initiation, development, and maturation.

The promotion effect of PSK- α on nodulation was relatively weak, despite being statistically significant (Fig. 3C). One of the explanations for this is that the active concentrations of PSK- α were difficult to be accurately maintained in pots in a greenhouse in the presence of rhizobia, because stability of the peptide could be affected by growth conditions. Because nodule primordia are initiated from cortical cell division, the perception of PSK by cortical cells is key to the function of PSK. Epidermal cells of roots might also be a natural barrier for the penetration of exogenous PSK. To overcome this, we generated *L. japonicus* transgenic hairy roots with enhanced endogenous PSK signaling by overexpression of the *LjPSK1* precursor gene or *AtPSKR1*. More nodules per plant were produced in these transgenic roots, suggesting that PSK signaling was enhanced (Fig. 4). Nodule numbers per plant are a measurable trait contributed by the events of bacterial infection, number of IT, number of nodule primordia, and rate of nodule development. Our observations revealed that both processes of bacterial infection and nodule initiation did not seem to be affected significantly by overexpressing *LjPSK1* or *AtPSKR1*. The lack of a role of *LjPSK1* and *LjPSK4* in early nodulation coincided with the low levels of *LjPSK1* and *LjPSK4* gene expression in the early stage of nodulation (Fig. 2C). Moreover, transgenic roots overexpressing *LjPSK1* or *AtPSKR1* did not alter the expression of *LjNIN* and *LjENOD40*, two symbiotic genes downstream of the Nod factor signaling, suggesting that PSK signaling may not play a major role in the early stage of nodulation.

The increased nodule numbers per plant in transgenic roots expressing *LjPSK1* and *AtPSKR1* (Fig. 4A and B) appear to result from an increased rate of nodule growth and maturation. We expressed *LjPSK1* or *AtPSKR1* in transgenic *Arabidopsis* plants and were able to confirm the function of *LjPSK1* or *AtPSKR1* overexpression in enhancing PSK signaling (Fig. 4E and F). In *Arabidopsis*, *AtPSK2*, *AtPSK3*, and *AtPSK5* are more strongly expressed in lower mature leaves than in upper young leaves (Matsubayashi et al. 2006). PSK- α treatment promotes root growth by increasing meristem size and cell size (Kutschmar et al. 2009). Both reports imply PSK in the potential regulation of cellular growth rather than cell division and differentiation. It is possible that PSK- α may induce cell enlargement of *L. japonicus* nodules to promote nodule development and maturation.

Redundancy of *LjPSK* genes.

In *Arabidopsis*, five PSK precursor genes are expressed in all plant tissues with different and partly overlapping expression

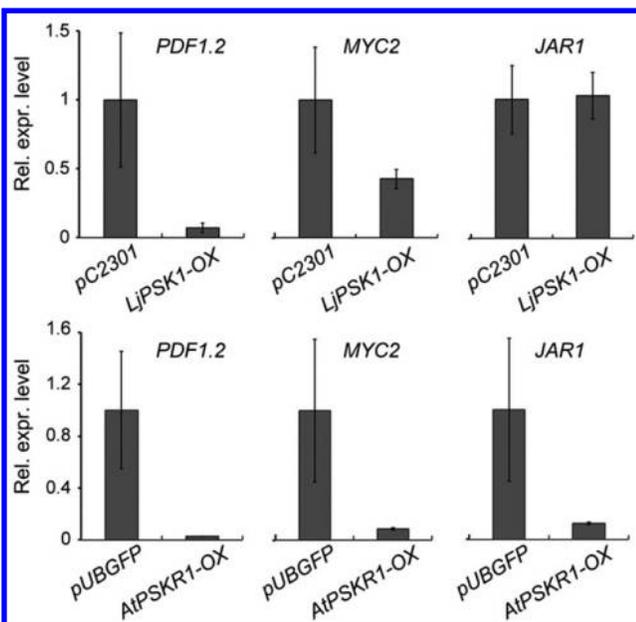


Fig. 5. Downregulation of jasmonic acid (JA)-responsive genes in transgenic roots. Total RNA was isolated from uninoculated hairy roots expressing *LjPSK1-OX* ($n = 8$), *AtPSKR1-OX* ($n = 3$), and the corresponding vector controls pC2301 ($n = 3$) and pUBGFP ($n = 4$). Quantitative reverse transcription-polymerase chain reaction was used to assay JA-responsive genes *PDF1.2*, *MYC2*, and *JAR1*. Values indicated the average expression levels of the individual hairy-root lines. Error bars indicate \pm standard deviation.

patterns (Kutschmar et al. 2009; Matsubayashi et al. 2006). *AtPSK* can also be induced by wounding and microbial interaction, indicating the specific response of PSK signaling to different stresses (Loivamäki et al. 2010; Matsubayashi et al. 2006). We found that *LjPSK1*, *LjPSK4*, and *LjPSK5* were highly expressed in mature nodules in *L. japonicus* (Fig. 2A). The abundant levels of *LjPSK* transcripts in developing nodules suggest that PSK signaling is involved in nodule growth and maturation. At the same time, the redundancy of *LjPSK* genes in *L. japonicus* makes it difficult to study gene function through reverse genetics analysis such as RNA interference or *LORE1* insertion mutants (Urbański et al. 2012). In this study, we identified *L. japonicus psk1* (30030761) and *psk4* (30006132) mutant lines from the *LORE1* mutant collections (Urbański et al. 2012). The homozygous plants of the two mutant lines exhibited normal growth and nodulation as compared with those in the wild-type *L. japonicus*. Although reduced nodulation was observed in the *Ljpsk1* mutant line, the effect was very weak (Supplementary Fig. S6). To overcome the redundancy of *LjPSK* genes, double mutant *psk1/psk4* plants should be generated through crossing of the two mutants. It remains to be tested if defective nodulation phenotypes can be observed in double mutant *psk1/psk4* plants. *LjPSK* genes showed different tissue-specific expression patterns, except *LjPSK2*, which presented low expression levels in all tissues tested (Fig. 2). It is worth noting that refined regulation of *LjPSK* was found in nodule cells, including infected and uninfected cells (Fig. 2D and E). Thus, PSK signaling plays a global role in plant growth, development, and reproduction, including nodule cell development.

Cross-talks of PSK- α with JA signaling.

PSK- α has been reported to modulate salicylic acid/JA hormone homeostasis to coordinate plant defense in *A. thaliana* (Mosher et al. 2013). JA is generally considered a defense hormone and a negative regulator on root-nodule formation in *L. japonicus*, although a positive role of JA on nodulation has also been reported (Suzuki et al. 2011). Most recent observations have indicated that JA biosynthesis and signaling genes are repressed by rhizobial inoculation in the early stage of nodule development (Breakspear et al. 2014). The down-regulation of JA-responsive genes in *L. japonicus* transgenic roots expressing either *LjPSK1* or *AtPSKR1* corresponded to the enhancement of nodulation (Fig. 5). Our results are consistent with the notion that JA may be a negative regulator of nodulation and suggest that PSK may antagonize JA signaling to control nodule development. It is also possible that PSK may suppress host defense responses in nitrogen-fixing nodules. During evolution, legumes might have hijacked a universal signaling pathway to regulate nodule organ development. In summary, the expression of two of the five *LjPSK* genes is highly enhanced during nodule development in *L. japonicus*, and PSK signals may be perceived by a receptor to control nodule growth and maturation.

MATERIALS AND METHODS

Plant growth and bacterial strains.

For nodulation assays, plants of *L. japonicus* MG-20 Miyakojima or Gifu B-129 were grown in a mixed soil medium containing perlite and vermiculite at a 1:1 volume ratio supplied with a half-strength B&D nitrogen-free medium in growth cabinets with a 16-h-light and 8-h-dark cycle at $22 \pm 1^\circ\text{C}$. *Mesorhizobium loti* MAFF303099 was used for inoculation of *L. japonicus* plants. The *M. loti* MAFF303099 strain that constitutively expresses a *lacZ* marker was used for rhizobial infection assays. *A. thaliana* plants were grown on soil supplied with a full-nutrition medium in a growth chamber with a 16-h-light and 8-h-dark cycle at $22 \pm 1^\circ\text{C}$. *A. rhizogenes* LBA1334 was used for hairy-root transformation.

RNA isolation and gene expression assay.

For tissue-specific expression assays, total RNA from roots, stems, and leaves was isolated using Trizol reagent (Invitrogen, Shanghai, China) and total RNA from flowers, nodules, and pods was isolated by Trizol plants reagent (Transgen Biotech, Beijing), which was optimized for total RNA isolation from plant materials containing abundant polysaccharides and polyphenol. Total RNA was treated with DNaseI (Takara, Dalian, China) to eliminate potential genomic DNA contamination. Reverse transcription products were used for subsequent qRT-PCR analysis under the following condition: 5 min at 95°C , followed by 40 cycles of 15 s at 94°C and 1 min at 60°C . Primers used for qRT-PCR included *LjPSK1*-F (GTAGCAC CAGAAGGTCCAAG), *LjPSK1*-R (TAGATGTAATCCAAGT GGGC), *LjPSK2*-F (TGGCAACCTTCTTCTTCTTCTT), *LjPSK2*-R (GCTGTTCATTTCTTCTTCTTCCA), *LjPSK3*-F (CATTTGCTCG TCCTACTCCT), *LjPSK3*-R (ACATTCTTCTGTCCCTTCCA), *LjPSK4*-F (GAATCGCTGGATGTTGAGGA), *LjPSK4*-R (GGG TTGTTATGCTTCTGCGT), *LjPSK5*-F (CTAACCAAGTGAACA AGGGAA), *LjPSK5*-R (TGTAGATGTAGTCCAGGTGAGC), *LjTPST*-F (TGAAGTGCGCCATTGTGTACAG), and *LjTPST*-R (GCTCCTCAGTAAGACCAACATATAACAT). For overexpression assays, total RNA was isolated from several independent hairy-root lines or *Arabidopsis* seedlings and was assigned to real-time RT-PCR or semiquantitative RT-PCR, using qRT-PCR primers and *AtPSKR1*-OX primers, respectively. The *AtPSKR1* transcripts in *Arabidopsis AtPSKR1*-OX lines were quantitatively measured by using qRT-PCR primers *AtPSKR1*-qF (AGTCAAT GAGCCATCTCCTG) and *AtPSKR1*-qR (CCAAACTCCTCC CAAATAGG). The expression of *PDF1.2*, *JAR1*, and *MYC2* was quantified in three to eight independent hairy-root lines, using the following primers: *LjPDF1.2*-F (GTGATCAGAGGTGT AAAGCC), *LjPDF1.2*-R (AGTTATCACTGCACTGGAAG), *LjJAR1*-F (GGTGAAGAGTATGAAATTGTTATGACC), *LjJAR1*-R (CCACATCTCCTAGCCTATACCG), *LjMYC2*-F (CGAAGAACG GCAGCAGTAAC), and *LjMYC2*-R (AGCTCAGAATCTCAC CGGATT). The expression levels of each gene were normalized with the *LjATPase* (AW719841) and *AtEF1-a* reference genes, using the following primers: *ATPase*-F (CAATGTC GCCAAGGCCATGGTG), *ATPase*-R (AACACCACTCTC GATCATTTCTCTG), and *AtEF1-a*-F (GAGGCAGACTGTT GCAGTCG), *AtEF1-a*-R (TCACTTCGCACCCTTCTTGA) in *L. japonicus* and *Arabidopsis*, respectively.

PSK- α treatment.

A. thaliana Col-0 seeds were sterilized with 0.1% active chlorine for 10 min and were washed with sterilized water five times. The germination of seeds was synchronized at 4°C in the dark for 1 day and were then maintained on one-half Murashige Skoog (MS) medium with a 16-h-light and 8-h-dark cycle at $22 \pm 1^\circ\text{C}$. Seedlings were grown on solid medium containing 0.8% agar and different concentrations of PSK- α peptide, which was synthesized, by the Chinese Peptide Company (Hangzhou, China), with purity of 97.6%. Root length was measured after 6 days of growth. *L. japonicus* seeds were sterilized and synchronized. The swollen seeds were spread on plates containing one-half MS medium. Plates were placed upside down for 30 to 40 h at 22°C in the dark for embryonic root elongation (approximately 1 cm). Seeds were then transferred to new one-half MS plates (1.2% agar, pH 6.8, with or without 1 IM PSK- α) for 5 days for root elongation. At least 25 plants were measured for root length. For nodulation assays, *L. japonicus* seedlings were transplanted to growth pots and were inoculated with nitrogen-free liquid medium containing both rhizobia (optical density = 0.03) and 1 IM PSK- α after 3 days of growth. Plants were supplied with half-strength nitrogen-free liquid medium and

1 M PSK- α aqueous solution. Nodule numbers were counted 12 days after inoculation with *M. loti*.

Plasmid construction.

For overexpression, the *LjPSK1* cDNA was placed behind an enhanced CaMV 35S promoter using primers *LjPSK1*-F (AACCATGGAATGAACCAATGAAGCAAC) and *LjPSK1*-R (CCACTAGTTTAGGGCTTATAGTGTGG). The whole expression unit was then cloned to the *KpnI/SacI* site of pCAMBIA2301, generating pC2301-d35S-*LjPSK1*, which contains both GUS and kanamycin selection markers for *A. rhizogenes*-mediated hairy-root transformation and *A. tumefaciens*-mediated stable transformation. The *AtPSKR1* cDNA was amplified using primers *AtPSKR1*-F (AGCTCTAGATGTTCTTGAAATGCGTGTTCATC) and *AtPSKR1*-R (AATGGCGCGCCCTAGACATCATCAAGCCAAG). The PCR fragment was cloned to the *XbaI/AscI* site of pUBGFP or pUBHyg (Maekawa et al. 2008), generating pUBGFP-*AtPSKR1* for hairy-root transformation in *L. japonicus* and pUBHyg-*AtPSKR1* for stable transformation in *Arabidopsis*. *AtPSKR1* was expressed under the control of a *Ubi* gene promoter. For expression of *LjCCaMK*^{T265D}, *LjCCaMK* cDNA was amplified and cloned to the *XbaI/AscI* site of pUBGFP using primers *LjCCaMK*-F (AGCTCTAGAGCCACCATGGGATATGATCAAACCAGAAAG) and *LjCCaMK*-R (AAGGCGCGCCCTATGATGGACGAAGAGAAGAGAG). The T265D site mutation was constructed by primers T265D-mF (GTTTCTATGAGAAGGATTGGAAGGGCATTAC) and T265D-mR (GTAATGCCCTTCCAATCCTTCTCATAGAAAC), using an overlapping PCR method. For promoter-GUS reporter analysis, a 4,144-bp promoter fragment upstream of the translation start site (ATG) of *LjPSK1* was amplified from the MG20 genomic DNA and was inserted upstream of the GUS gene in the binary vector pX2181 at the *Bam*HI/*Sma*I site. The promoter fragment was amplified using primers *LjPSK1p*-F (AAGGATCCATGGCACCATATACTTGGGC) and *LjPSK1p*-R (AACCCGGGTGTTCATTTCAGGGAATAAGGG).

Plant transformation.

Agrobacterium rhizogenes-mediated hairy-root transformation was performed as described previously (Wang et al. 2013). After the hairy roots were generated, positive roots expressing pUBGFP-*AtPSKR* or pUBGFP were selected using the GFP marker under a fluorescence microscope. For selection of positive hairy roots expressing pC2301-d35S-*LjPSK1* or pC2301, root tips of 2 to 3 mm long were excised for GUS staining. Two GFP-positive or GUS-positive hairy roots were preserved on each plant and all the negative roots were removed. Plants were then transplanted to growth cabinets for 6 days of growth before inoculation with *M. loti*. *Arabidopsis* transformation was carried out by the floral-dip method (Clough and Bent 1998). Transgenic seeds were selected on one-half MS plates containing 50 mg of kanamycin or 20 mg of hygromycin per liter.

GUS staining.

Transgenic hairy roots expressing the *GUS* reporter gene under the control of the *LjPSK1* promoter were inoculated with *M. loti* to induce nodulation. Two weeks later, nodules were washed with water and were incubated in GUS staining solution (100 mM Na₃PO₄ buffer, pH 7.0, 0.1% Triton X-100, 0.1% N-laurylsarcosine, 10 mM EDTA, 1 mM K₃Fe(CN)₆, 1 mM K₄Fe(CN)₆, and X-Gluc at 0.5 mg/ml) for 8 h at 37°C, after vacuuming for 5 min.

IT assay.

Transgenic plants were grown in growth cabinets for 6 days before inoculation with *M. loti* MAFF303099 harboring

a plasmid, pPN28, for constitutive expression of β -galactosidase (*lacZ*). IT in hairy roots were visualized after staining with X-gal solution, as described previously (Wang et al. 2013).

Identification of *L. japonicus* LORE1 mutants.

Genomic DNAs were isolated from individual mutant plants and were used for PCR amplification of 5 min at 95°C and 35 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C, followed by 6 min at 72°C. Primers for the identification of *Ljpsk1* and *Ljpsk4* mutants were *LjPSK1*-mF (TGCTCTGCATTTGA ACTAAGGATGCAC), *LjPSK1*-mR (TGCTCGTCTCCATGTAG CACCAGAAGG), *LjPSK4*-mF (TCTGGAGCGCTGGGCCAAG TAACC), and *LjPSK4*-mR (CCACTTCCAAGTTTCACACCT CCTCC). Primer P2 (CCATGGCGGTTCCGTGAATCTTAGG) coupled with *LjPSK1*-mF or *LjPSK4*-mF were used for the identification of *LORE1* insertions as described (Urbański et al. 2012).

DNA sequence analysis.

Multiple sequence alignment of PSK was performed using ClustalX. The gene structure of *LjPSK* was predicted by the Spidey program. Sequence data from this article have been deposited to GenBank with accession numbers of KM065832 for *LjPSK1*, KM065833 for *LjPSK2*, KM065834 for *LjPSK3*, KM065835 for *LjPSK4*, and KM065836 for *LjPSK5*.

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