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An MAP kinase interacts with LHK1 and regulates nodule organogenesis in *Lotus japonicus*

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Symbiosis receptor-like kinase (SymRK) is a key protein mediating the legume-*Rhizobium* symbiosis. Our previous work has identified an MAP kinase kinase, SIP2, as a SymRK-interacting protein to positively regulate nodule organogenesis in *Lotus japonicus*, suggesting that an MAPK cascade might be involved in *Rhizobium*-legume symbiosis. In this study, LjMPK6 was identified as a phosphorylation target of SIP2. Stable transgenic *L. japonicus* with RNAi silencing of *LjMPK6* decreased the numbers of nodule primordia (NP) and nodule, while plants overexpressing *LjMPK6* increased the numbers of nodule, infection threads (ITs), and NP, indicating that LjMPK6 plays a positive role in nodulation. LjMPK6 could interact with a cytokinin receptor, LHK1 both *in vivo* and *in vitro*. LjMPK6 was shown to compete with LHP1 to bind to the receiver domain (RD) of LHK1and to downregulate the expression of two *LjACS* (1-aminocyclopropane-1-carboxylic acid synthase) genes and ethylene levels during nodulation. This study demonstrated an important role of LjMPK6 in regulation of nodule organogenesis and ethylene production in *L. japonicus*.

cytokinin, ethylene biosynthesis, LHK1, *Lotus japonicus*, MAPK cascade, root nodule symbiosis

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INTRODUCTION

During the *Rhizobium*-legume symbiosis, the host plants gain fixed nitrogen, while the rhizobia obtain a steady carbon supply in a protected cellular environment (Cao et al., 2017; Gresshoff, 2003). To establish this mutualistic relationship, both the hosts and the symbionts have to continually communicate with each other by responding to molecular signals secreted from each partner. Compatible rhizobia recognize host flavonoids and synthesize Nod factors (NFs), which are then perceived by plant Lysin-motif receptor kinases (Madsen et al., 2003; Radutoiu et al., 2003). After perception of NFs, the hosts initiate multiple physiological responses including root hair deformation, calcium influx, calcium spiking and oscillation, cortical cell division, and expression of *nodulin* genes to allow rhizobial infection and nodule development (Heidstra et al., 1994; Miller et al., 2013; Morieri et al., 2013; Vijn et al., 1993).

The puzzle for cytokinin being involved in nodule morphogenesis was solved with identification of two alleles of cytokinin receptors, CRE1 (CYTOKININ RESPONSE 1) in *Medicago truncatula* and LHK1 (Lotus HISTIDINE KINASE 1) in *Lotus japonicus* (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007). *L. japonicus*

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carrying a loss-of-function *LHK1* allele (known as *hit1*) develops only a limited number of nodules in the later stage of inoculation. These data pinpoint to the critical role of LHK1 in nodule organogenesis (Gonzalez-Rizzo et al., 2006; Miri et al., 2016; Tirichine et al., 2007). LHK1 is the most homologous protein to *Arabidopsis* AHK4 (*Arabidopsis* histidine kinase 4) with 64% identities based on their amino acid sequences, and the functional similarity was confirmed by the report that *Arabidopsis* AHK4 could rescue the phenotype of Lotus *hit1-1* mutants (Held et al., 2014; Murray et al., 2007). As a typical two-component system, the histidine kinase transfers the phosphoryl group from the receiver Asp onto a His in HISTIDINE PHOSPHOTRANSFER proteins (HPs) (Punwani et al., 2010). The phosphoryl group is then transferred from HPs onto RESPONSE REGULATOR proteins (RRs) via direct interaction (Dortay et al., 2007; Tanaka et al., 2004; Verma et al., 2015).

Ethylene plays a negative role in nodule organogenesis. In *M. truncatula*, the ethylene-insensitive mutant *sickle* (*SICKLE* encodes the ortholog of EIN2, ETHYLENE INSENSITIVE 2) is hyperinfected by rhizobia, resulting in the formation of a drastically increased number of nodules (Oldroyd et al., 2001; Penmetsa and Cook, 1997). Recently, *L. japonicus EIN2a/EIN2b* double mutant was shown to be hyperinfected by rhizobia resulting in hypernodulation (Miyata et al., 2013), indicating the negative role of ethylene in symbiosis. In addition to this, ethylene is also known to coordinate with cytokinin to regulate plant growth and development. Interestingly, a recent study concluded that NFs treatment could induce cytokinin accumulation and the expression of *ACS* genes involved in ethylene biosynthesis in *M. truncatula* (van Zeijl et al., 2015). In *L. japonicus*, LHK1-dependent root growth could be completely suppressed by inhibition of the ethylene biosynthesis (Held et al., 2014; Murray et al., 2007; Wopereis et al., 2000). Recent study showed that ethylene production is significantly elevated in the gain-of-function mutant of *LHK1*, *snf2* in *L. japonicus* (spontaneous nodule formation), which confirmed the crosstalk between cytokinin and ethylene (Reid et al., 2018).

The mitogen-activated protein kinase (MAPK) cascades play critical roles in plant growth and development, hormone biosynthesis and signaling, and biotic and abiotic stress responses. The *Arabidopsis* genome encodes 20 MAPKs, 10 MAPKKs, and more than 80 MAPKKKs. The leguminous plants including *L. japonicus*, *M. truncatula*, and *Phaseolus vulgaris* encode 19, 18 and 15 MAPKs, and 7, 4 and 9 MAPKKs, respectively (Neupane et al., 2013). Among the MAPKs, only three MAPK proteins (MPK3, MPK4, and MPK6) in *Arabidopsis* have been well-studied (Gao et al., 2008; Gudesblat et al., 2007; Jia et al., 2016; Nie and Xu, 2016). AtMPK3 and AtMPK6 were shown to play positive roles in ethylene biosynthesis and plant defense response by inducing second metabolite biosynthesis, stomatal immunity, and defense-related genes expression, while AtMPK4 seems to play a negative role in plant response to pathogens (Moussatche and Klee, 2004; Xu et al., 2016). Although increasing evidence has indicated the critical role of MAPKs in plant development and responses to the environmental stimuli, only a few publications have reported the involvement of MAPKs in symbiosis. Our previous report has shown that SIP2, a *L. japonicus* MAPKK, interacts with symbiosis receptor-like kinase (SymRK) which is required for nodulation and plays a positive role in nodule organogenesis (Chen et al., 2012; Endre et al., 2002). Medicago MtMAPKK4, the ortholog of LjSIP2, has been shown to play a positive role in mediating root nodule symbiosis (Chen et al., 2017), whereas MtMKK5 seems to play a negative role in root nodule symbiosis (Ryu et al., 2017). Interestingly, both MtMAPKK4 and MtMKK5 were shown to interact with MtMPK3 and MtMPK6 (Chen et al., 2017; Ryu et al., 2017). All these data indicate that MAPK cascade might be involved in root nodule symbiosis.

In this study, we demonstrated that LjMPK6 acts as a phosphorylation target of SIP2 to positively regulate nodule formation and negatively regulate ethylene content in *L. japonicus*. In addition, LjMPK6 might be involved in regulation of cytokinin signaling through direct interaction with LHK1 and competition with LHP1 to bind to LHK1 receiver domain.

RESULTS

Identification of LjMPK6 as a mitogen-activated protein kinase in *L. japonicus*

Our previous work has shown that an MAP kinase kinase, SIP2, could phosphorylate *Arabidopsis* AtMPK6 *in vitro* (Chen et al., 2012), suggesting that LjMPK6, the orhtolog protein of AtMPK6 in *L. japonicas*, might be a phosphorylation target of SIP2. To test this hypothesis, an *in vitro* kinase assay was carried out to test the trans-phosphorylation between SIP2 and kinase-dead version of LjMPK6^{K95R} (the conserved lysine required for ATP binding was substituted to arginine). LjMPK6^{K95R} was shown to be phosphorylated by both wild-type SIP2 and constitutively activated SIP2^{DD} (Figure 1C), indicating that LjMPK6 is a phosphorylation target of SIP2. Amino acid sequence analysis showed that LjMPK6 contains an ATP binding site and a typical TEY activation loop and shares the highest similarity with *M. truncatula* MtMPK6 (Figure 1A and B). To determine whether LjMPK6 is a functional kinase, a constitutively active mutant LjMPK6^{Y147C} (the conserved amino acid tyrosine-147 was replaced with cysteine) was created (Berriri et al., 2012; Kiegerl et al., 2000; Zhou et al., 2009). All the wild-type and mutant versions of LjMPK6 were expressed in *E. coli* and used in an *in vitro* kinase assay in the presence of

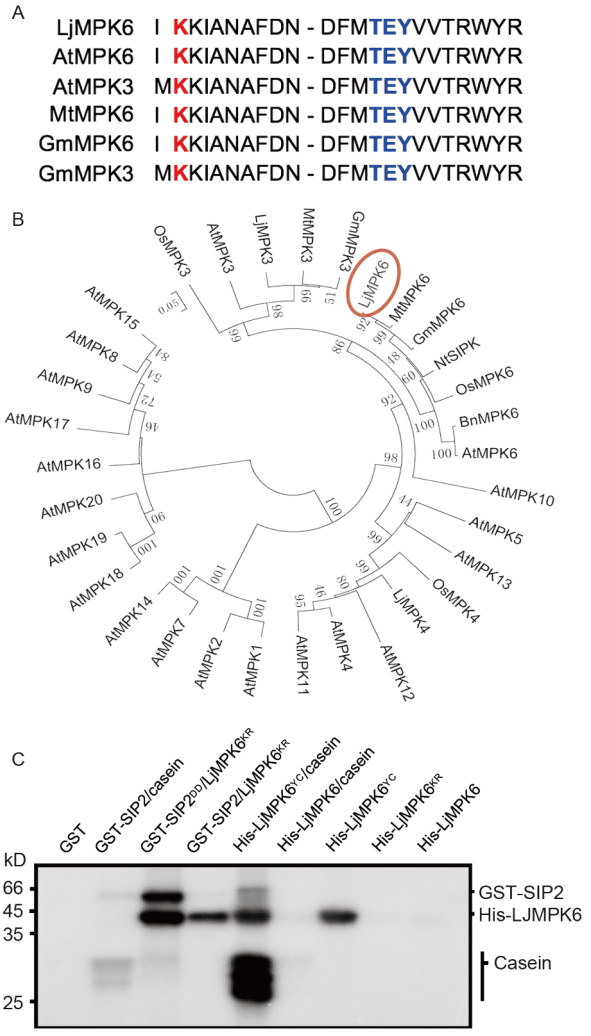

Figure 1 (Color online) Phylogenetic tree and *in vitro* MAP kinase activity assays of LjMPK6. A, Sequence alignment of the MAPK phosphorylation sites of MPK6 and MPK3 homologs from *L. japonicus* (Lj), *M. truncatula* (Mt), *Arabidopsis thaliana* (At) and *Glycine max* (Gm). B, Phylogenetic tree of MAPKs from *L. japonicus* (Lj), *M.truncatula* (Mt), *Glycine max* (Gm), *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Nicotiana tabacum* (Nt), and *Brassica napus* (Bn). The rooted phylogenetic tree was generated using MEG5.0. C, LjMPK6 is a phosphorylation substrate of SIP2. Wild-type LjMPK6, the constitutively activated version LjMPK6^{YC} (Y147C), and kinase-dead version LjMPK6^{KR} (K95R) were expressed and purified as His-tag fusion proteins from *E. coli*. SIP2 and the constitutively activated SIP2, SIP2^{DD} (S242D, T246D) were purified as GST-tag fusion proteins from *E. coli*. Purified proteins were inoculated with or without casein in the presence of [γ-^{32}P]-ATP before analyzed on SDS-PAGE gel. The gel was subjected to autoradiography. Molecular mass standard (kD) was shown on the left side.

[γ-^{32}P]-ATP. The data showed that the constitutively activated LjMPK6^{Y147C} had strong kinase activities showing as both autophosphorylation of itself and transphosphorylation on casein (Figure 1C), which is a universal target for Ser/Thr kinases (Wang and Zhu, 2016). However, both the autophosphorylation and transphosphorylation activities of the wild-type LjMPK6 could not be detected in our experiment (Figure 1C), possibly due to an extremely low kinase activity *in vitro*. These data suggest that LjMPK6 is a mitogen-activated protein kinase functioning as a phosphorylation target of SIP2.

Expression pattern of *LjMPK6*

To study the expression pattern of *LjMPK6* during plant growth and nodulation, total RNA was extracted from leaves, nodules, flowers, pods, and root tissues inoculated with *M. loti* MAFF303099 or treated with crude NF extract (Wang et al., 2015). Quantitative PCR (qPCR) analysis revealed that the expression of *LjMPK6* was detected in all the tissues (Figure 2A). In the plants inoculated with rhizobia or treated with crude NF extract, the expressions of both *LjMPK6* and the nodulation marker gene *NIN* were significantly induced compared to the control (Figure 2B and C, Figure S1 in Supporting Information), indicating that the mRNA transcript of *LjMPK6* is induced by rhizobial infection in *L. japonicus*.

Stable transgenic *L. japonicus* plants expressing the reporter *GUS* gene under the control of the native *LjMPK6* promoter were generated to confirm the expression pattern of LjMPK6. Histochemical GUS staining showed that *LjMPK6* was expressed in different tissues including pod, flower, anther with pollen, root, leaf, stem, and nodule (Figure 2D–G, Figure S2A–D in Supporting Information). Promoter activity of *LjMPK6* was detected in susceptible region of epidermal cells, including root hairs and nodule primordium 5 days post inoculation (dpi) with *M. loti* (Figure 2H and I) as well as in the nodule primordium when the ITs reach to the cortex cells (Figure S2E–H in Supporting Information). In addition, *LjMPK6* was also detected in all cells of young nodules (Figure 2J), parenchyma, and vascular bundles in the mature nodules (Figure 2K).

To investigate the cellular localization of LjMPK6 during nodulation, LjMPK6-YFP fused to a nuclear localization signal (NLS) under the control of its native promoter was expressed in *L. japonicus* hairy roots. LjMPK6 was detected in the susceptible region of epidermal cells, including root hairs and proliferative cortical cells 7 days after inoculation with *M. loti* (Figure 2L and M). In the hairy roots 10 dpi with rhizobia, LjMPK6 was detected in the cortical cells of nodule primordium (Figure 2N). LjMPK6 was also observed in the parenchyma cells and vascular bundles of mature nodules (Figure 2O). The expression pattern and protein localization suggest that LjMPK6 plays a role in nodulation in *L. japonicus*.

LjMPK6 is involved in nodule organogenesis

An *L. japonicus* mutant line (ID: 30004627) containing a transposon insertion in the third exon of *LjMPK6* was obtained from the *L. japonicus LORE1* retrotransposon inser-

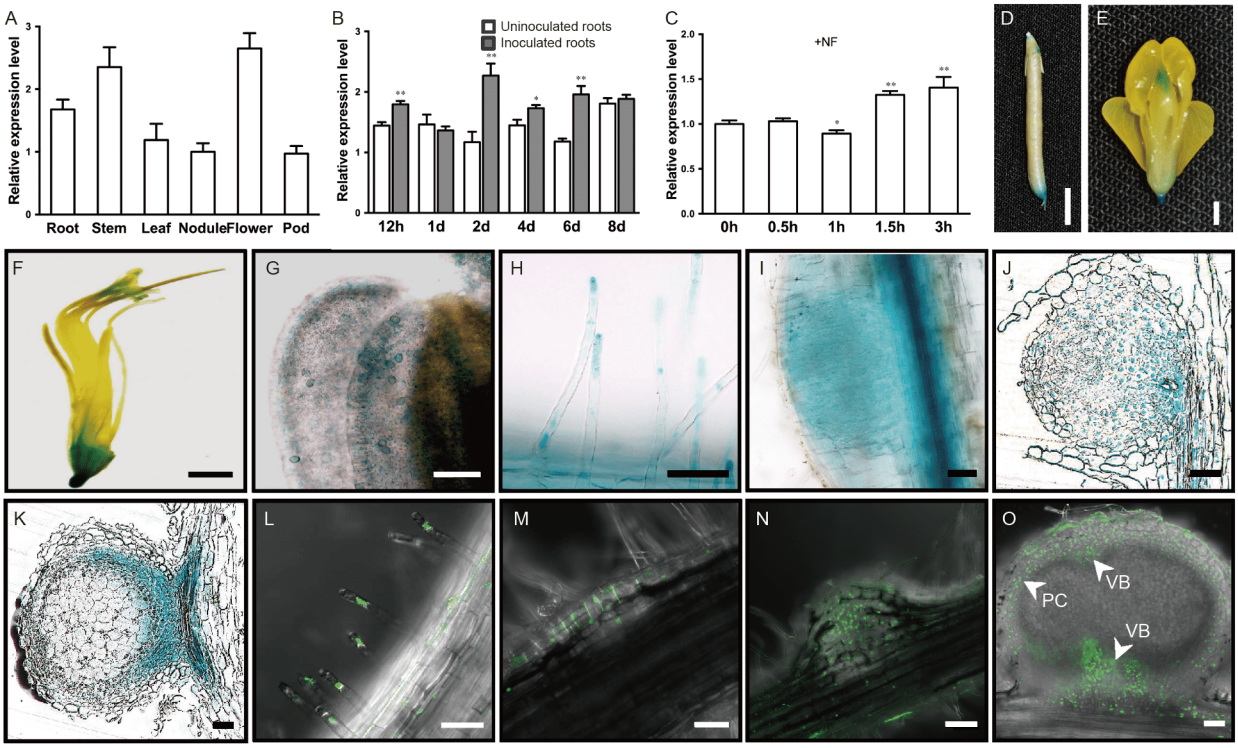

Figure 2 Expression profile of *LjMPK6* in *L. japonicus*. A, Expression levels of *LjMPK6* in different tissues. Roots, stems, leaves, and nodules were harvested 2 weeks post inoculation (wpi) with *M. loti*, flowers and pods were collected from two-month-old plants. B, Samples were taken from roots inoculated with *M. loti* for 12 h, 1, 2, 4, 6 and 8 d and from the uninoculated roots. C, Root tissues of 7-day-old seedlings were treated with crude nod factor (NF) extract for 0, 0.5, 1, 1.5 and 3 h. Total RNA was isolated from wild-type *L. japonicus* MG20 and used for qRT-PCR to measure the expression of the *LjMPK6*. *ATPase* gene was used as an internal control. Error bars represent SE of the experimental values obtained from three biological replicates. Asterisks above the bars indicate significant differences (**P<0.01, *0.01<P<0.05). D–G, Activity of *LjMPK6* promoter determined by GUS staining. Pod (D), flower (E, F), and pollen (G) were incubated in GUS staining solution for 2 h at 37°C. H–K, Histochemical analysis of GUS activity in stable transgenic *L. japonicus* roots carrying *LjMPK6pro*:*GUS*. Root hairs and nodule primordia 5 days post inoculation (dpi) (H and I) and root nodules 7 and 14 dpi (J and K) with *M. loti* were stained for GUS for 2 h at 37°C. L–O, LjMPK6 fused with YFP was expressed under the control of its native promoter in transgenic hairy roots of *L. japonicus* plants carrying *LjMPK6pro*:*LjMPK6*::*YFP-NLS*. Green fluorescence indicates the localization of LjMPK6-YFP fusion protein in transgenic hairy roots 7 dpi with *M. loti* (L and M), in a nodule primordium 10 dpi with *M. loti* (N), and in a mature nodule 2 wpi with *M. loti* (O). PC, parenchyma cells; VB, vascular bundles. Scale bar, 250 μm (D–F, H) and 50 μm (G, I–O).

tion collection (Figure S3A in Supporting Information) (Fukai et al., 2012; Urbański et al., 2012). Heterozygous *LjMPK6*$^{+/-}$ plants had normal vegetative growth and nodulation phenotype, but with a defect in reproduction. The pods of *LjMPK6*$^{+/-}$ mutant plants were shorter than those of the wild type plants and produced much fewer seeds than the wild type plants (Figure S3B in Supporting Information). However, homozygous mutant seedlings were unable to be identified from 68 progenies of *LjMPK6*$^{+/-}$ heterozygous plants (Figure S3C in Supporting Information). We then tried to use the CRISPR/Cas9 approach to knock out *LjMPK6* in *L. japonicus*, but no *LjMPK6* knock-out mutant plants could be obtained (data not shown). These observations suggest that LjMPK6 might play an essential role in plant development and whose deletion might cause a lethal phenotype in *L. japonicus*.

We switched to knock down the expression of *LjMPK6* using RNAi technology under the control of the nodule-specific *LjNAD1* promoter and stable transgenic *L. japonicus* plants were generated (Wang et al., 2016) (Figure 3A, Figure S4A and B in Supporting Information). Compared with the expression level in the wild-type plants, transcription of *LjMPK6* in two independent T1 lines of stable transgenic *LjMPK6* RNAi plants were reduced by 20%–35% as measured by qRT-PCR (Figure 3D). The nodule number in the *LjMPK6* RNAi plants was significantly reduced as compared to that in the control plants 2 weeks post inoculation (wpi) with *M. loti* (Figure 3B, C and E). The number of NP in the RNAi plants was also significantly reduced 1 wpi with *M. loti* (Figure S5A in Supporting Information). While the nodule structure was not different between control plants and *LjMPK6* RNAi plants (Figure S5C in Supporting Information).

To further confirm the positive role of LjMPK6 in symbiosis, we generated stable transgenic plants with overexpression of *LjMPK6* under the control of *Ljubiquitin* promoter (Maekawa et al., 2008). However, the stable transgenic plants overexpressing *LjMPK6* were unable to be obtained (Figure S6A and B in Supporting Information). We then asked if the excessive expression of *LjMPK6* causes cell

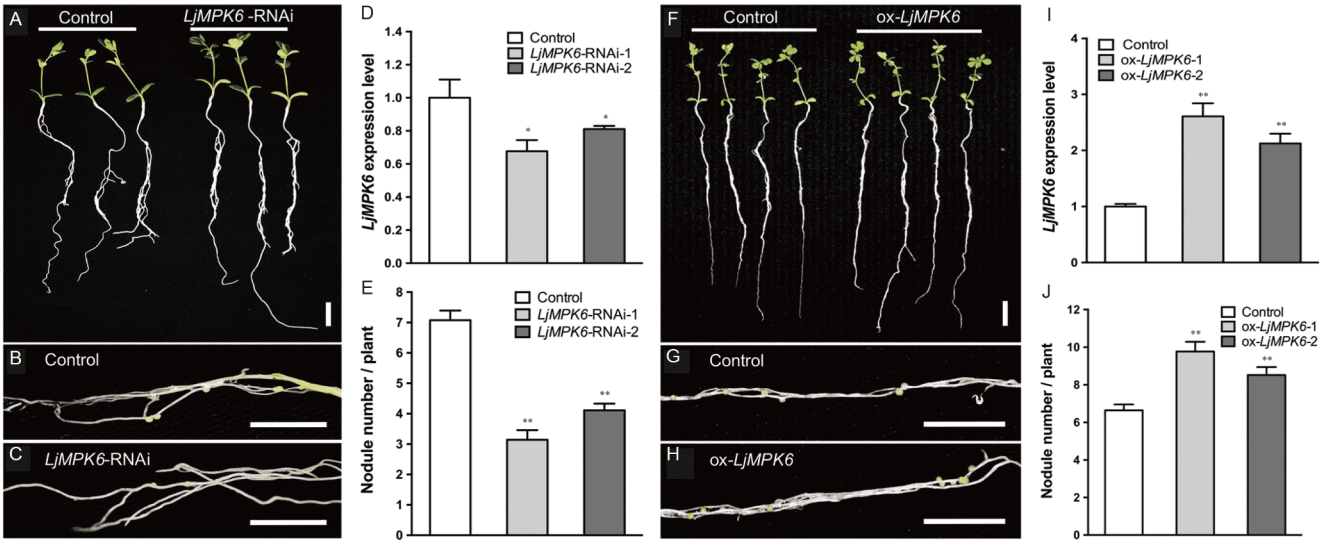

Figure 3 (Color online) Phenotypes of transgenic Lotus plants carrying *LjMPK6* RNAi and *LjMPK6*-ox. A–C, Nodulation phenotypes of *LjMPK6*-RNAi stable transgenic line 1 in comparison with the control plants.Stable transgenic plants expressing *LjMPK6* RNAi under the control of the nodule-specific promoter of *LjNAD1*. D, Expression levels of *LjMPK6* in the nodules of *LjMPK6*-RNAi line 1 and line 2. E, Nodule numbers of *LjMPK6*-RNAi line 1 and line 2.25, 21, and 28 independent plants 14 dpi with *M. loti* for *LjMPK6* RNAi line 1, line 2, and control were used for statistical analysis of nodule density, respectively. F–H, Nodulation phenotypes of *LjMPK6*-ox line 1 in comparison with the control plants. Stable transgenic plants *LjMPK6*-ox under the control of the maize *ubiquitin* promoter. I, Expression levels of *LjMPK6* in the roots of *LjMPK6*-ox line 1 and line 2. J, Nodule numbers of *LjMPK6*-ox line 1 and line 2. 30, 28, and 20 plants 14 dpi with *M. loti* for *LjMPK6*-ox line 1, line 2 and control were used for statistical analysis of nodule density, respectively. Expression levels of genes were quantified by qRT-PCR using total RNA from nodules (D) and roots (I) 2 wpi. Wild-type *L. japonicus* MG20 served as a control. Images show the wild-type control and stable transgenic plants growing under nitrogen fertilizer-free conditions 2 wpi with *M. loti*. Error bars represent SE of the experimental values from three biological replicates, asterisks above the bars indicate significant differences (**P<0.01, *0.01<P<0.05). Scale bar, 1 cm.

death. To test this, different promoters were used to drive the expression of *LjMPK6* in *Nicotiana benthamiana* leaves, the cell death phenotype was observed when the expression of LjMPK6 was under the control of the *Ljubiquitin* promoter (Figure S6C in Supporting Information). Thus, we used maize *ubiquitin* promoter which has a weaker promoter activity than *Ljubiquitin* promoter does to generate stable transgenic plants with *LjMPK6* overexpressed (Figure S4C–E in Supporting Information). Two independent T2 stable transgenic lines with a 2- to 3-fold mRNA increase were used for nodulation assays (Figure 3F and I). The results showed that nodule number in *LjMPK6-ox* line 1 and line 2 was significantly larger than that in the control plants 2 wpi with *M. loti* (Figure 3G, H and J). The numbers of ITs and NP were examined in two *LjMPK6-ox* transgenic lines 7 dpi with the *M. loti* strain that was labeled with constitutive expression of *LacZ*. In *LjMPK6-ox* line 1, both the numbers of ITs and NP were significantly increased, whereas in line 2, only the number of ITs, but not NP, was significantly increased, compared with control plants (Figure S5B in Supporting Information). These data indicate that LjMPK6 plays a positive role in nodule organogenesis in *L. japonicus*.

LjMPK6 interacts with LHK1

Arabidopsis MPK6 has been implicated to be involved in multiple physiological responses (Han et al., 2010; Li et al., 2012; Meng et al., 2013; Meng et al., 2014; Rasmussen et al., 2012). To identify possible downstream targets of LjMPK6, a yeast two hybrid screen was carried out in which LjMPK6 was used as a bait. Approximately two million yeast colonies from the *L. japonicus* cDNA library were screened on a selective medium (SD/-Leu-Trp-His-Ade) for interaction with LjMPK6. Plasmids were isolated from positive interacting clones and used to retransform yeast cells followed by selection on the selective medium. One clone that encodes the C-terminal 215 amino acids of LHK1 was confirmed as an interacting protein of LjMPK6. In *L. japonicus*, LHK1 has previously been shown to promote nodule organogenesis, as well as to restrict the IT numbers (Miri et al., 2016; Murray et al., 2007; Tirichine et al., 2007).

LHK1 contains an extracellular CHASE domain, an intracellular kinase domain (KD), and a receiver domain (RD) (Murray et al. 2007; Tirichine et al. 2007). We asked which domain of LHK1 mediates the interaction with LjMPK6. The result showed that LjMPK6 interacted with LHK1-RD (receiver domain) but not LHK1-KD (kinase domain) in yeast two hybrid system (Figure 4A). In *Arabidopsis*, AtMPK6 and AtMPK3 have overlapping functions involved in various physiological responses. We thus asked whether LHK1 interacts with other *L. japonicus* MAPK members. Yeast two hybrid result showed that LHK1-RD interacted with

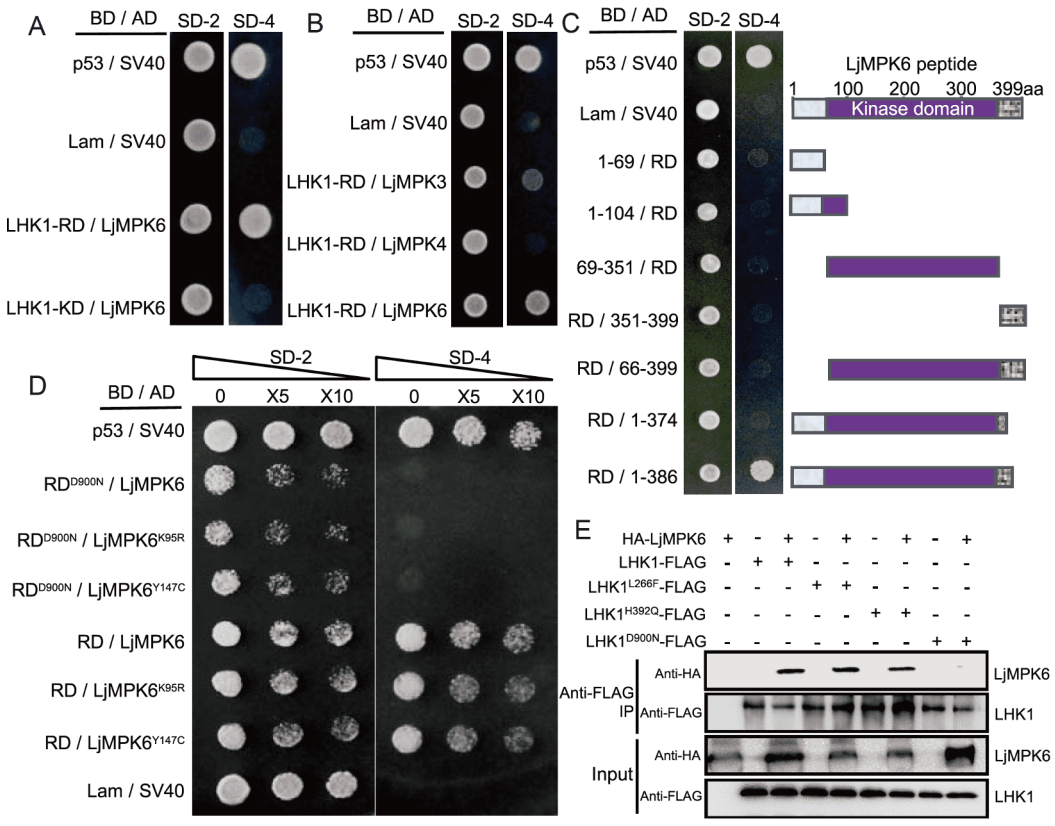

Figure 4 (Color online) Interaction between LjMPK6 and LHK1. A, Interaction between LjMPK6 and LHK1 in yeast cells. B, Interaction between LHK1 and *L. japonicus* MAPKs in yeast cells. C, Interaction between truncated versions of LjMPK6 and LHK1. D, Interactions between LHK1-RD mutants and LjMPK6 mutants. Yeast strain Y187 carrying the GAL4 DNA binding domain plasmid (BD) was mated with yeast strain AH109 harboring the GAL4 activation domain plasmid (AD). Mated yeast cells were grown on SD media lacking Leu and Trp (SD-2) and selected for interaction on SD-Leu-Trp-His-Ade (SD-4) selection medium for 3 days. The interaction between mammalian p53 and SV40 served as a positive control, whereas co-expression of lamin (Lam) and SV40 served as a negative control. E, Interaction between LjMPK6 and LHK1 *in planta*. Full length LHK1 and its mutants were expressed as FLAG-tagged fusion proteins, while LjMPK6 was expressed as HA-tagged fusion protein. Proteins were co-expressed in *N. benthamiana*. Coimmunoprecipitation assay was carried out using anti-FLAG M2 Affinity Gel (Sigma). Both input samples and immunoprecipitated samples were resolved on 10% SDS-PAGE gels and subjected to Western blot analysis.

LjMPK6 and LjMPK3, but not with LjMPK4 (Figure 4B). To better understand the mechanism of interaction between LjMPK6 and LHK1, truncated versions of LjMPK6 were constructed and tested in yeast cells for interaction with LHK1-RD. The result showed that truncated LjMPK6 lacking either N- or C-terminus could not interact with LHK1-RD, suggesting that both the N- and C-termini of LjMPK6 contribute to its interaction with LHK1 (Figure 4C). Since both LjMPK6 and LHK1 are protein kinases, we sought to test whether the kinase activities of both proteins were required for their interactions. According to the report of AHK4, the closest homolog of LHK1 in *Arabidopsis* (Mähönen et al., 2006), a phosphorelay-inactive version of LHK1-RD was made by replacing Asp-900, a conserved amino acid required for transphosphorylation on HPs, with Asn and used for testing the interaction with LjMPK6. The results showed that only the native RD but not the mutant RD^{D900N} of LHK1-RD could interact with the wild-type LjMPK6, kinase-inactive $LjMPK6^{K95R}$, and kinase-constitutively active $LjMPK6^{Y147C}$. These results demonstrated that the phosphor-relay activity of LHK1-RD is required while the kinase activity of LjMPK6 is not necessary for their interactions in yeast cells (Figure 4D). All the expression of the fusion proteins in yeast cells was examined using immunoblot with specific antibodies (Figure S7 in Supporting Information).

To further verify the interaction between LjMPK6 and LHK1 *in planta*, HA-tagged LjMPK6 and FLAG-tagged LHK1 were transiently co-expressed in *N. benthamiana*. Co-immunoprecipitation assay was performed using anti-FLAG antibody and the precipitated proteins were detected using anti-HA antibody. To test whether the kinase activity of LHK1 is required for its interaction with LjMPK6, the constitutively active $LHK1^{L266F}$ (a gain-of-function mutant by replacing Leu-266 with Phe) (Tirichine et al., 2007), the autophosphorylation-inactive $LHK1^{H392Q}$ (a substitution of the autophosphorylation site His-392 by Gln), and the phosphorelay-inactive $LHK1^{D900N}$ (a substitution of the transphosphorylation site Asp-900 by Asn) were generated according to the report of AHK4, the closest homolog of

LHK1 in *Arabidopsis* (Mähönen et al., 2006; Tirichine et al., 2007). The results showed that LjMPK6 could be pulled down with LHK1, LHK1^{L266F}, or LHK1^{H392Q}, but not LHK1^{D900N} by the anti-FLAG affinity gel (Figure 4E). These data confirmed that LjMPK6 could interact with LHK1 regardless of its kinase status, while the transphosphorylation activity of LHK1 receiver domain is required for its interaction with LjMPK6.

LjMPK6 inhibits the binding of LHP1to the receiver domain of LHK1

The two-component system of cytokinin sensing in plants contains three major protein components, including a cytokinin receptor, a histidine phosphotransfer protein (HP), and a response regulator protein (RR). During phosphorylation relay, HP transfers phosphoryl group from the receiver domain of cytokinin receptor to RR through direct interaction (Hutchison and Kieber, 2002; Tanaka et al., 2004). Since the transphosphorylation activity of LHK1 receiver domain is required for interaction with LjMPK6, we tested whether LjMPK6 could affect the interaction between LHK1 and LHPs. To test this, LHP1 (AHP1 homologous in *L. japonicus*) tagged with MBP was used to interact with GST-tagged LHK1-RD in the presence or absence of His-tagged LjMPK6. The result showed that LHP1 could be pulled down by glutathione sepharose resins containing GST-LHK1-RD, indicating that LHP1 physically interacts with the receiver domain of LHK1. The glutathione sepharose resins containing GST-LHK1-RD and LHP1 were then incubated with different concentrations of LjMPK6. As the concentration of LjMPK6 increased, the signal representing LHP1 protein pulled down by glutathione sepharose decreased (Figure 5). In contrast, no competition effect of BSA on the interaction of LHK1-RD and LHP1 was detected in the control experiment (Figure S8 in Supporting Information). Interestingly, both the kinase-constitutively active version of LjMPK6^{Y147C} and the kinase-inactive version of LjMPK6^{K95R} showed an inhibition on the interaction between LHP1 and LHK1-RD (Figure 5). These data suggest that regardless of kinase status, the presence of LjMPK6 could inhibit the interaction between LHK1-RD and LHP1.

LjMPK6 regulates ethylene production during nodulation

It has been shown that ethylene production is significantly elevated in the gain-of-function mutant of *LHK1* (Reid et al., 2018). MPK6 could directly phosphorylate ACS6 to increase ethylene level in *Arabidopsis* (Han et al., 2010; Liu and Zhang, 2004). Based on the interaction of LjMPK6 and LHK1, we asked whether LjMPK6 could regulate ethylene production during nodulation. The stable transgenic plants with both *LjMPK6* RNAi and overexpression were used to measure the ethylene contents 7 dpi with rhizobia. In the control experiment, no significant differences of ethylene production among the wild-type control, *LjMPK6*-ox, and *LjMPK6*-RNAi plants uninoculated with *M. loti* were detected (Figure 6A). However, ethylene production was significantly decreased in *LjMPK6*-ox-1 plants and increased in *LjMPK6*-RNAi-1 plants 7 dpi with *M. loti* (Figure 6A). The data indicate that the ethylene production is negatively regulated by LjMPK6 during nodulation in *L. japonicus*. Since the ACS enzyme is the key regulator in ethylene biosynthesis, the expression levels of *ACS* genes in *LjMPK6* RNAi and overexpressed stable transgenic plants were measured by qPCR. The results showed that the expression levels of *LjACS1* and *LjACS2* were significantly increased and decreased in *LjMPK6*-RNAi and *LjMPK6*-overexpression stable transgenic plants, respectively (Figure 6B

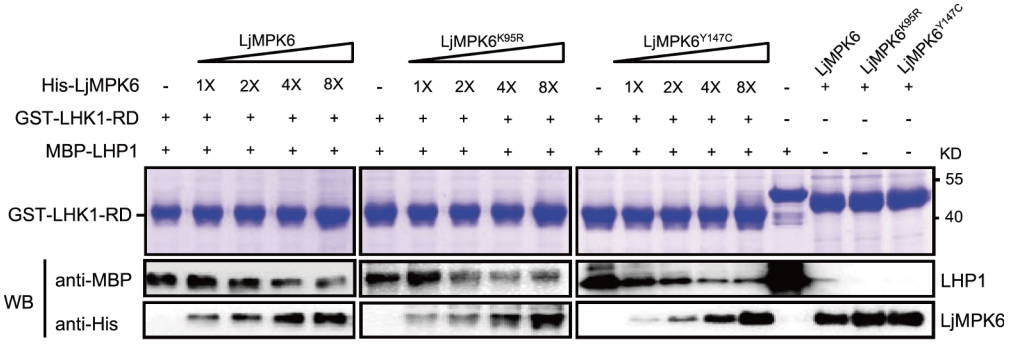

Figure 5 (Color online) Effect of LjMPK6 on the interaction between LHK1 and LHP1. Competition of LjMPK6 with LHP1 for binding to LHK1. LHK1-RD was expressed in *E. coli* and purified as a GST fusion protein. LjMPK6 and its mutants were purified as His-tag fusion proteins. LHP1 was purified as a MBP fusion protein. Protein-protein interaction between LHK1 and LHP1 was measured in the presence of increased concentrations of LjMPK6 using GST pull down. MBP-LHP1 was mixed with glutathione sepharose resins containing GST-LHK1-RD, after stringent washing, different concentrations of His-LjMPK6 or its mutant versions were added to compete with MBP-LHP1 for binding to GST-LHK1-RD. After stringent washing, proteins bound on resins were eluted with SDS loading buffer and analyzed by Commassie blue staining or immunoblotting with specific antibodies. Upper panels in the left 15 lanes show GST-LHK1-RD protein levels on Commassie blue staining gels. Middle panels in the left 15 lanes show the protein levels of MBP-LHP1 retained on GST-LHK1-RD resins and detected by immunoblot with anti-MBP antibody. Lower panels in the left 15 lanes show LjMPK6 or its mutants retained on GST-LHK1-RD resins and detected by immunoblot with anti-His antibody. Panels in the right 4 lanes show input of purified MBP-LHP1 and His-LjMPK6.

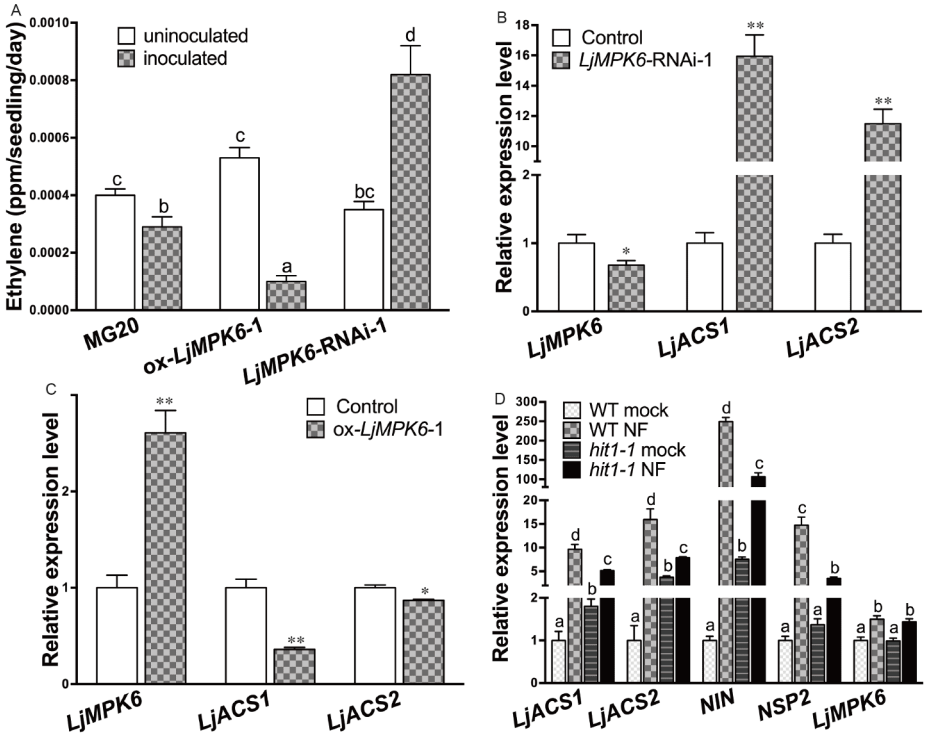

Figure 6 The regulation of ethylene production by LjMPK6 in *L. japonicus*. A, Ethylene evolution by *LjMPK6*-ox-1 and *LjMPK6*-RNAi-1 stable transgenic plants. Seedlings grown on MS medium with phytagel were inoculated with *M. loti* for 7 days, then the vials were capped for 24 h before measurement, and the levels of ethylene in the vials were measured by gas chromatography analysis. Error bars represent SE of the experimental values from three biological replicates. Different letters above the bars indicate a significant difference ($P<0.05$). B, Expression of *ACS* genes in the wild-type control and *LjMPK6*-RNAi-1 plants during nodulation. Plant roots were harvested 2 wpi with *M. loti*. C, Expression of *ACS* genes in the wild-type control and ox-*LjMPK6*-1 plants during nodulation. Plant roots were harvested 2 wpi with *M. loti*. D, Expression of *ACS* genes and *LjMPK6* in the wild-type control and *hit1-1* mutant plants (Gifu) 3 h after NF or mock treatment. Total RNA was isolated for qRT-PCR quantification of gene expression, and the *ATPase* gene was used as an internal control. Wild-type *L. japonicus* MG20 served as the control. Asterisks indicate significant differences between comparisons (**$P<0.01$, *$0.01<P<0.05$). Different letters above the bars indicate a significant difference ($P<0.01$).

and C). These data indicate that LjMPK6 negatively regulates the transcription of *LjACS1* and *LjACS2*, which might cause the changes of ethylene production during nodulation in *L. ljaponicus*. A recent study concluded that nod factor treatment could induce cytokinin accumulation and the expression of *ACS* genes in *M. truncatula* (van Zeijl et al., 2015), suggesting that there might be a crosstalk between cytokinin and ethylene during the legume-*rhizobium* symbiosis. Based on the finding that LjMPK6 associates with LHK1, we asked whether LHK1 is involved in regulation of the expression *of ACS* genes in *L. japonicus*. To test this hypothesis, crude NF extract was used to treat both the wild-type *L. japonicus* Gifu and the *LHK1* loss-of-function mutant *hit1-1* to monitor the expression of *ACS* genes. NF treatment significantly induced the expression of *LjACS1*, *LjACS2*, *LjMPK6* and marker genes *NSP2* and *NIN* in the wild-type plants (Figure 6D), indicating that the two ethylene biosynthesis genes were inducible by NF. However, in the *hit1-1* mutant plants, NF-induced mRNA accumulation of *LjACS1*, *LjACS2* and marker genes *NSP2* and *NIN* was significantly diminished (Figure 6D), indicating that LHK1 is at least partially required for NF-induced expression of these two *LjACS* genes but not for *LjMPK6* in *L. japonicus*.

DISCUSSION

MAPK cascades are signal modules highly conserved in all eukaryotes and regulate various aspects of plant development and response to stresses (Brodersen et al., 2006; Cardinale et al., 2002; Chai et al., 2014; Kim et al., 2003; Lu et al., 2002; Meldau et al., 2012; Singh et al., 2015; Takahashi et al., 2007). In this study, we showed that LjMPK6 works as a MAPK cascade component with SIP2 to regulate nodule organogenesis and ethylene production (Figure 7). In addition, LjMPK6 interacts with LHK1 and competes with LHP1 binding to LHK1 receiver domain (Figure 7), suggesting that the association of LjMPK6 with LHK1 is involved in the regulation of root nodule symbiosis.

The MAPK cascades are involved in the regulation of various biotic and abiotic stress responses, hormone responses, cell division, and developmental processes (de Zelicourt et al., 2016; Hettenhausen et al., 2015; Liu et al., 2014; Nakagami et al., 2005; Stanko et al., 2015; Xu and

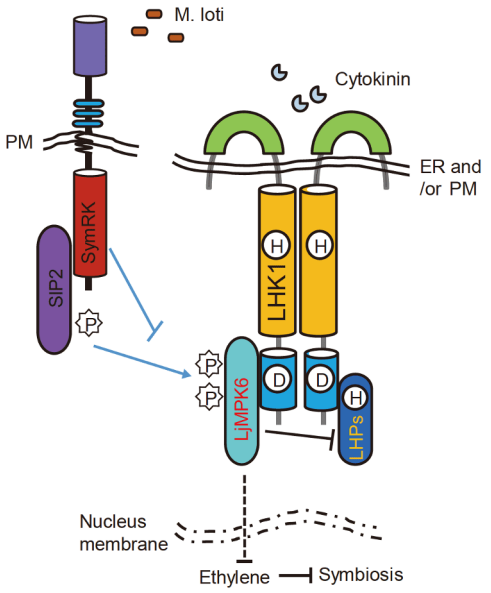

Figure 7 A proposed model for the role of LjMPK6 in modulating root nodule symbiosis. SIP2 is an MAP kinase kinase, which interacts with SymRK to regulate nodule organogenesis in *L. japonicus* (Chen et al., 2012). LjMPK6 is an MAP kinase that can be directly phosphorylated by SIP2. Activated SymRK may inhibit phosphorylation of LjMPK6 by SIP2 (blue line) (Chen et al., 2012). LjMPK6 interacts with LHK1 and competes with LHP1 binding to LHK1 receiver domain (black line). Thus, we proposed that LjMPK6 negatively regulates ethylene biosynthesis and positively regulates nodule formation (black line). LHK1 contains an extracellular CHASE domain (green), an intracellular kinase domain (yellow, H represents the autophosphorylation site of amino acid His-392), and a receiver domain (blue, D represents the phosphorelay site of amino acid Asp-900). The letter "H" in LHPs protein represents a phosphorylation site by LHK1.

Zhang, 2015). In *Arabidopsis*, AtMPK6 is shown to be involved in the regulation of various physiological processes, including development, abscission, ethylene biosynthesis, and responses to pathogens. The phosphorylation of MPK6 in *L. japonicus* has been shown to be activated by rhizobial treatment, suggesting that rhizobia might have uncharacterized microbe-associated molecular patterns (MAMPs) to trigger the immune response (Lopez-Gomez et al., 2012). However, the knowledge about the involvement of MAPK cascade in symbiotic signaling pathway is limited. The present data showed that LjMPK6 is a phosphorylation target of SIP2 and positively regulates nodule development, which is consistent with the role of SIP2 in *L. japonicus* (Figures 1C and 3) (Chen et al., 2012). These conclusions confirmed that SIP2-LjMPK6 cascade is involved in symbiotic signaling pathway as a direct downstream component of SymRK. However, whether an MAP kinase kinase kinase is involved in regulation of SIP2-LjMPK6 is of great interest. Moreover, the present study showed that both LjMPK6 and LjMPK3 could interact with LHK1 (Figure 4B), suggesting that LjMPK3 might also contribute to this early symbiosis signaling, which remains to be explored in future work. Furthermore, the ethylene production was significantly reduced in *LjMPK6* overexpression plants and induced in *LjMPK6* RNAi plants during nodulation (Figure 6A), further indicating that LjMPK6 plays an important role in this regulation.

It should be noted that besides the function in symbiosis (Figure 3), LjMPK6 plays a role in physiological and developmental regulation. This conclusion was supported by two lines of evidence: (i) a homozygous line of the *LORE1* insertional mutant of *mpk6* could not be isolated from the self-fertilizing progeny of the heterozygous *mpk6*$^{+/-}$ plants (Figure S3 in Supporting Information); (ii) we were not able to generate stable transgenic plants knocking-out *LjMPK6* using CRISPR-Cas9 technology. The data support the possibility that the absence of LjMPK6 is lethal in *L. japonicas*, which is consistent with a recent observation in rice in which homologous *OsMPK6* knock-out plants cannot be obtained from T-DNA insertion mutant pool or from heterozygous lines generated using CRISPR-CAS9 due to the possible embryo-lethal defects (Minkenberg et al., 2017; Yi et al., 2016). However, the critical role of LjMPK6 in plant development in *L. japonicus* might be different from that in *Arabidopsis*, since the lethal phenotype was exhibited only in the *Arabidopsis mpk3/mpk6* double knock-out mutants but not in either single mutant plants (Wang et al., 2007). Furthermore, LjMPK6 also plays a role in the cell death. We generated stable transgenic plants under the control of *L. japonicus ubiquitin* promoter and 16 stable transgenic plants were obtained based on GUS staining. However, the overexpression levels of *LjMPK6* could not be detected via qPCR (Figure S6B in Supporting Information). One possible reason is that *L. japonicus ubiquitin* promoter is too strong to induce excessive expression of LjMPK6 which may cause cell death, which was observed in *N. benthamiana* when *LjMPK6* was overexpressed under the control of the *L. japonicus ubiquitin* promoter (Figure S6C in Supporting Information).

The activity of *LHK1* promoter was detectable in the root epidermis, including root hairs, nodule emergence, mature nodule parenchyma, and nodule vasculature (Held et al., 2014). Our data showed that *LjMPK6* was detected in the susceptible region of epidermal cells, including root hairs, young nodule cells, and mature nodule parenchyma nodule vasculature (Figure 2H–O). The overlap in gene expression suggests that the two proteins have temporal opportunity to interact with each other *in planta*. In a typical two-component system, phosphor-relay from histidine kinase to downstream target protein is essential for signaling transduction. Thus, mutation at the key amino acid could lose the ability of histidine kinase to transfer the phosphoryl group from the receiver domain onto HP proteins, such as AHK4^{D973N} (Hwang and Sheen, 2001). The interaction between LHK1 and LjMPK6 depends on the phosphorylation status of LHK1 (Figure 4D and E), suggesting that LjMPK6 might be

involved in phosphorelay between LHPs and LHK1.

MAPK cascade is involved in many different physiological responses via phosphorylation of multiple downstream target proteins (Pitzschke, 2015). In *Arabidopsis*, ERFs (ethylene response factors), ACSs, and WRKY transcription factors were shown as direct phosphorylation targets of AtMPK3 and/or AtMPK6 (Joo et al., 2008; Mao et al., 2011; Meng et al., 2013). The interaction between LjMPK6 and LHK1 revealed that a novel regulation mechanism was identified in symbiotic signaling pathway. The expression of genes downstream LHK1-mediating cytokinin signaling, including *NIN* and *NSP2*, is positively regulated by the presence of LjMPK6, further supporting the conclusion that LjMPK6 positively regulates root nodule symbiosis. However, the kinase activity of LHK1 and LHP1 binding to LHK1 were inhibited by LjMPK6 (Figures 4 and 5). Therefore, it is possible that the inhibition on LHP1 binding to LHK1 by LjMPK6 may be separately regulated, which is not related to the common symbiotic signaling pathway.

Different phytohormones were shown to regulate nodule development, and thus the host plants must maintain well-balanced levels of different phytohormones to allow the right number of nodules to be developed. Our data showed that the ethylene production is negatively regulated by LjMPK6 during symbiosis, while in *Arabidopsis*, AtMPK6 positively regulates ethylene levels in plant response to pathogens. The data indicate that MPK6 plays a positive role in both legume-*rhizobium* symbiosis and plant pathogenesis based on the regulation of ethylene levels. Cytokinin could play a double role both positively and negatively in regulating nodule organogenesis, whereas ethylene was known to play a negative role in nodule formation. However, it has been shown that cytokinin could increase ethylene production through regulating the stability of ACS proteins (Frankowski et al., 2007; Hansen et al., 2009). In a recent study, ethylene production is significantly elevated in a gain-of-function mutant of *LHK1* (Miri et al., 2016). In addition to the positive role in nodulation, LjMPK6 might negatively regulate cytokinin signaling and ethylene levels. It was believed that ethylene plays a negative role in nodule organogenesis, thus it is possible that the positive regulation on nodule organogenesis in *L. japonicus* is due to a balance between cytokinin and ethylene signaling regulated by LjMPK6.

The root nodule symbiosis involves a complicated network regulated by both hosts and rhizobia. It was shown that different phytohormones including cytokinin, GA, ethylene, and auxin were indicated to tightly control nodule organogenesis. Due to the fact that nodulation happens at different cellular types at different time processes, hormones such as cytokinin and GA might play dual roles (positive and/or negative) in mediating root nodule symbiosis (Gamas et al., 2017). The positive role of cytokinin is to initiate nodule organogenesis, while the negative role of cytokinin might be involved in autoregulation of nodulation, for example, by promoting ethylene production, to tightly control nodule numbers. Indeed, the whole process of symbiotic interaction between leguminous plants and rhizobia is tightly controlled by both partners. Formation of too many or too less nodules on the roots is not beneficial to the host plant (Mortier et al., 2012; Sasaki et al., 2014). Thus, the levels of both cytokinin and ethylene hormones need to be balanced *in planta* to control the numbers of infection threads and nodules. ACS proteins are classified into three subfamilies according to the C-terminal phosphorylation motif. Type-1 ACSs have the C terminus containing three predicted MAPK phosphorylation sites, but the other two types of ACSs do not have (Chae and Kieber, 2005). Cytokinin elevates ethylene levels in plants through inducing the transcription of type-2 *ACS* genes and stabilizing ACS proteins (Frankowski et al., 2007; Hansen et al., 2009). Our data showed that LjMPK6 could regulate the transcriptional levels of *LjACS1* and *LjACS2* (Figure 6B and C), and the expressions of these two *LjACS* genes at least partially depend on LHK1 in *L. japonicus* (Figure 6D). Whether the two ACSs are phosphorylation substrates of LjMPK6, and whether the interaction between LjMPK6 and LHK1 is involved in the expression of ACS genes and/or stabilizing proteins remain to be explored in future work.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *L. japonicus* wild-type (MG20 and Gifu) and mutants were gently rubbed by sandpaper and frozen in liquid nitrogen for 1 min. Surface sterilization was performed by soaking seeds in 75% ethanol for 10 min, followed by 4% sodium hypochlorite for 5–10 min. After washing with sterile water for at least 6 times, seeds were kept in a refrigerator overnight for stratification, and planted on Murashige and Skoog (MS) solid medium, followed by dark treatment for 2 days in a growth chamber maintained at 22°C with a 16 h light/8 h dark cycle. Four-day-old seedlings were used for infection with *Agrobacterium tumefaciens* AGL1 and LBA1334. Five-day-old seedlings were used for inoculation with *M. loti* MAFF303099 or NZP2335:*LacZ* (A_{600}=0.02). Seven-day-old seedlings were incubated in liquid MS containing crude NF extract which was isolated as previously described (Wang et al., 2015) for different times, seedling roots were harvested and frozen in liquid nitrogen for RNA extraction. *N. benthamiana* plants were grown in a growth chamber at 25°C with a 16 h light/8 h dark cycle. *A. tumefaciens* strain EHA105 containing different binary vectors were mixed in an infiltration buffer (10 mmol L^{-1} $MgCl_2$ with 200 μmol L^{-1} acetosyringone) with a final A_{600} at 1.0 and hand-infiltrated into fully-expanded leaves of 4-week-old *N. benthamiana*.

RNA isolation and quantitative real-time PCR assay

Total RNA was extracted from stems, leaves, roots, nodules, unexpanded flowers, and young siliques (1–1.5 cm in length) at least 0.02 g fresh materials for each sample using TRIZOL reagent (Invitrogen), and cDNA was synthesized from 500 ng of total RNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara). qRT-PCR was set up in a 10 μL reaction system with SYBR Select Master Mix (ABI). PCR (50°C for 2 min, 95°C for 2 min, 40 cycles at 95°C for 15 s, 60°C for 1 min) was performed with Vii7 Real-time System (Applied Biosystems). *Adenyl pyrophosphatase* (*ATPase*) gene (AW719841) which is constitutively expressed in *L. japonicus* was used as reference for quantitative analysis (Chen et al., 2012). Melting curve analysis was used to determine the identity of the amplified PCR products. Experiments were performed with three technical replicates. Primers used in this work are listed in Table S1 in Supporting Information.

Statistical analysis

Statistical significance was determined based on Student's *t*-test or nonparametric Kruskal-Wallis analysis according to experimental data.

Yeast two-hybrid screen

The *LjMPK6* cDNA was cloned into pGBKT7 BD (Clontech) and transferred into the yeast strain AH109 for a *L. japonicus* cDNA library screening (Zhu et al., 2008). Yeast colonies grown on synthetic media containing 10 mmol L^{-1} 3-amino-1,2,4-triazole but lacking leucine, tryptophan, histidine, and adenine for 5 days were selected as positive candidates for further sequence analysis. The plasmids purified from yeast colonies were re-transformed into yeast to confirm the interaction with LjMPK6.

Coimmunoprecipitation assay

Full-length cDNA and mutant versions of *LHK1* were amplified by PCR and cloned into pUB-GFP-3xFLAG containing the *L. japonicus ubiquitin* promoter to obtain LHK1:3xFLAG. The *LjMPK6* coding region was ligated into pSPYCE (MR) containing the CaMV 35S promoter to obtain 3xHA:LjMPK6. *A. tumefaciens* strain EHA105 containing different binary vectors were mixed in an infiltration buffer (10 mmol L^{-1} $MgCl_2$ with 200 μmol L^{-1} acetosyringone) with a final A_{600} at 1.0 and hand-infiltrated into fully-expanded leaves of 4-week-old *N. benthamiana*. Three days after infection, leaves were collected and frozen in liquid nitrogen, and crude proteins were extracted with a buffer (500 mmol L^{-1} sucrose, 50 mmol L^{-1} Tris-HCl, pH 8.0, 150 mmol L^{-1} NaCl, 1 mmol L^{-1} $MgCl_2$, and 10 mmol L^{-1} EDTA, 1× cocktail protease inhibitor (Roche), and 75 mmol L^{-1} MG132). After centrifugation in a microcentrifuge at the maximum speed for three times at 4°C, the supernatant was incubated with ANTI-FLAG M2 affinity gel (Sigma) with end-to-end shaking for 5 h at 4°C. Beads were washed three times with Co-IP buffer. Samples were eluted with 5× SDS loading buffer with boiling for 10 min and subjected to SDS-PAGE. Proteins were transferred to the PVDF membrane (Bio-Rad) and probed with anti-HA/FLAG antibody (Promoter Biotechnology Ltd.). All the constructs were verified by DNA sequencing.

Plasmid construction for protein interactions in yeast

Wild-type *LjMPK6*, *LjMPK6* mutants and truncated derivatives of *LjMPK6* cDNAs were amplified by PCR using primers *LjMPK6-Eco*RI-F and *LjMPK6-Bam*HI-R and inserted into pGBKT7 or pGADT7. The *LHK1-RD* and *LHK1-RD*^{DN} cDNAs were amplified by PCR and inserted into pGBKT7 or pGADT7. *LHK1-KD* cDNA was cloned into pGBKT7.

Generation of stable transgenic *L. japonicus* and GUS staining

A 2-kb fragment of the *LjMPK6* promoter was amplified from the *L. japonicus* (MG20) genomic DNA using primers *LjMPK6pro*-F and *LjMPK6pro*-R. The promoter was inserted into binary vector pDX2181 to fuse with the GUS gene. To generate the stable transgenic plants overexpressing *LjMPK6*, *LjMPK6* cDNA fragment was amplified using primers *LjMPK6*-F and *LjMPK6*-R and inserted into pCAMBIA1301U containing a maize *ubiquitin* promoter (Zong et al., 2016) or pUB500 containing a *L. japonicus ubiquitin* promoter (Maekawa et al., 2008). A 341-bp fragment from the 3′-end of the *LjMPK6* cDNA (169 bp 3′-UTR plus 172 bp CDS sequence) was amplified from *L. japonicus* using primers LjMPK6-RNAi-F and LjMPK6-RNAi-R and inserted into vector pCAMBIA1301-*LjNAD1promoter*-intT7 in inverse orientations with an *Actin* intron between them. The promoter of *NAD1* from *L. japonicus* was used to drive the nodule-specific expression of RNAi.

AGL1-carrying plasmid was used to infect *L. japonicus* MG20 as described (Schauser et al., 1998). Transgenic plants were selected on 1/2 MS agar medium supplemented with hygromycin.

For GUS staining, tissues were incubated in a GUS staining solution for 2–5 h at 37°C or room temperature overnight after vacuum infiltration for 10 min as described previously (Wang et al., 2015). The GUS staining tissues were examined using a stereo microscope.

Rhizobial inoculation assay

For rhizobial inoculation assay, five-day-old seedlings were used for inoculation with *M. loti* strain NZP2335 constitutively expressing the *LacZ* reporter gene (A_{600}=0.02) and grown in pots containing sand:vermiculite=1:1 (volume). The number of ITs was counted 5–7 days post inoculation after GUS staining.

Protein extraction from yeast cells and immunoblot analysis

Protein extracts for immunoblot was described previously (Chen et al., 2012). In brief, yeast cells were suspended in lysis buffer and followed by vortexing vigorously with glass beads for 1 min. After centrifugation, the supernatant was transferred to a fresh tube and put in a 100°C bath for 5 min. Samples were immediately loaded on SDS-PAGE gel for immunoblot. Proteins were transferred to PVDF membrane and probed with monoclonal antibodies with either anti-HA (Sigma-Aldrich) or anti-Myc (Invitrogen). Primary antibodies were detected with a peroxidase-conjugated goat anti-mouse IgG secondary antibody (Abmart), followed by signal detection using Super Signal West Pico Chemiluminescence Substrate (Sigma).

Induction of hairy roots and detection

Wild-type MG20 was used to generate hairy roots. LBA1334 carrying pUB-*LjMPK6-YFP-NLS* containing the *LjMPK6* promoter or DX2181-maize ubiquitin promoter-GUS were grown for 3 days at 28°C on fresh selection plates in a growth chamber. Induction and selection of hairy roots were described previously (Chen et al., 2012). Briefly, 4-day-old seedlings were cut at the base of hypocotyls and placed in the *A. rhizogenes* suspension for 30 min, then transferred onto MS agar plates without sucrose and co-cultured for 5 days in a growth chamber. Plants were transferred onto HRE medium (SH medium) plates containing 300 mg mL^{-1} cefotaxime and grown for 10 more days until hairy roots were developed from hypocotyls. Positive hairy roots were examined using a fluorescence microscope or GUS staining.

Preparation and purification of recombinant proteins

LjMPK6, *LjMPK6*^{KR} and *LjMPK6*^{YC} cDNAs were amplified and ligated into pET28a(+) (Novagen) for His-tag fusion. The cDNA sequence of *LHK1-RD* was cloned into pGEX-4T-1 (Amersham Pharmacia Biotech) for GST tag fusion. The coding sequence for LHP1 was cloned into pMAL-c2x (New England Biolabs) for maltose-binding protein (MBP) fusion. *Escherichia coli* strain BL21 (DE3) cells expressing recombinant proteins were induced with 0.4 mmol L^{-1} isopropylthio-β-galactoside (IPTG) for 4 h at 30°C. His-tagged proteins were purified using nickel-agarose beads (Qiagen), GST fusion proteins were purified using glutathione sepharose 4B columns (Sigma-Aldrich) and MBP fusion proteins were purified using amylose resin (New England Biolabs). Samples were purified at 4°C and stored at –80°C with 5% glycerol.

In vitro kinase assay

In vitro kinase assay was performed as previously described (Chen et al., 2012). In brief, for MAP kinase protein assay, about 1 μg kinase protein and 2 μg substrate protein were incubated in 40 μL of 20 mmol L^{-1} HEPES, pH 7.4, 15 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} EGTA, 1 mmol L^{-1} DTT and 2 μCi of [γ-^{32}P] ATP at 28°C for 30 min. Reactions were terminated by adding 5× SDS loading buffer. Samples were analyzed directly on 10% SDS-PAGE gel. The gel was exposed to BAS2500 (Fuji Film, Tokyo, Japan) for autoradiography.

In vitro protein competition analysis

The protein competition assay was described previously (Wang et al., 2015). Glutathione sepharose 4B beads bound with GST-RD were incubated with 15 μg of MBP-LHP1 protein in 600 μL of interaction buffer (20 mmol L^{-1} Tris-HCl, 100 mmol L^{-1} KCl, 2 mmol L^{-1} $MgCl_2$, 5% glycerol, pH 8.0) with gentle shaking for 30 min. After washing three times, five aliquots of glutathione resins containing the GST-RD/LHP1 complex were incubated with different concentrations (0, 10, 20, 40, 80 μg) of LjMPK6 protein or BSA in 600 μL of interaction buffer for 1 h. After washing six times, retained proteins on glutathione resins were eluted in SDS loading buffer and separated on SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue, and another identical gel was used for Western blot analysis using either anti-His or anti-MBP antibodies (Promoter Biotechnology Ltd.). All experiments were performed at least three times.

Measurements of the ethylene level

Surface-sterilized seeds were germinated by dark treatment for 2 days in a growth chamber maintained at 22°C. For ethylene measurements, five seedlings were moved and planted in 25-mL gas chromatography vials containing filter paper cut to fit on the 4 mL MS with 1% sucrose and 0.2% phytagel. Following 5 days at 22°C with a 16 h light/8 h dark cycle, seedlings were inoculated with *M. loti* MAFF3030399 (A_{600}=0.02) for a week, then the vials were capped for 24 h before measurements. The accumulated ethylene was measured by gas chromatography using a Shimadzu GC2010 Plus capillary gas chromatograph system. All genotypes and treatments were measured for at least three vials each.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Expression levels of NIN in *L. japonicus*.

Figure S2 GUS activity analysis in stable transgenic *L. japonicus* plants expressing LjMPK6pro:GUS.

Figure S3 Characterization of an LjMPK6 LORE1 mutant.

Figure S4 Gus staining in transgenic plants expressing GUS reporter under the control of LjNAD1 promoter or maize ubiquitin promoter.

Figure S5 Infection events in LjMPK6 RNAi and LjMPK6-ox plants.

Figure S6 Expression of LjMPK6 in LjMPK6-ox stable transgenic plants under the control of *L. japonicus* ubiquitin promoter.

Figure S7 Immunoblot analysis of protein expression in yeast cells.

Figure S8 Competition of BSA with LHP1 for binding to LHK1.

Figure S9 Expression of NIN and NSP2 in LjMPK6-ox and LjMPK6-RNAi transgenic plants during nodulation.

Table S1 Primers used in this study

Table S2 Accession numbers of genes used in this study

The supporting information is available online at http://life.scichina.com and http://link.springer.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.