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Suppression of LjBAK1-mediated immunity by SymRK promotes rhizobial infection in *Lotus japonicus*

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ABSTRACT

An important question in biology is how organisms can associate with different microbes that pose no threat (commensals), pose a severe threat (pathogens), and those that are beneficial (symbionts). The root nodule symbiosis serves as an important model system for addressing such questions in the context of plant-microbe interactions. It is now generally accepted that rhizobia can actively suppress host immune responses during the infection process, analogous to the way in which plant pathogens can evade immune recognition. However, much remains to be learned about the mechanisms by which the host recognizes the rhizobia as pathogens and how, subsequently, these pathways are suppressed to allow establishment of the nitrogen-fixing symbiosis. In this study, we found that SymRK (Symbiosis Receptor-like Kinase) is required for rhizobial suppression of plant innate immunity in *Lotus japonicus*. SymRK associates with LjBAK1 (BRASSINOSTEROID INSENSITIVE 1-Associated receptor Kinase 1), a well-characterized positive regulator of plant innate immunity, and directly inhibits LjBAK1 kinase activity. Rhizobial inoculation enhances the association between SymRK and LjBAK1 *in planta*. LjBAK1 is required for the regulation of plant innate immunity and plays a negative role in rhizobial infection in *L. japonicus*. The data indicate that the SymRK-LjBAK1 protein complex serves as an intersection point between rhizobial symbiotic signaling pathways and innate immunity pathways, and support that rhizobia may actively suppress the host's ability to mount a defense response during the legume-rhizobium symbiosis.

Key words: legume-rhizobial symbiosis, SymRK, LjBAK1, plant innate immunity, protein phosphorylation

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INTRODUCTION

The nitrogen-fixing legume-rhizobial symbiosis is considered mutualistic because both the plant host and the bacterial symbiont derive benefit; that is, host-derived carbon nutrients are transferred to the bacteria in exchange for nitrogen fixed by the rhizobia. Although exceptions are now known (Giraud et al., 2007), it is well established that a successful legume-rhizobial symbiosis begins with flavonoid-induced synthesis and secretion of a lipo-chitooligosaccharide (LCO) signal molecule (i.e., Nod factor, NF) by the rhizobia, which is in turn recognized by specific lysin-motif receptor kinases (LYKs) in the host plants to initiate symbiotic signaling cascades and nodule development (Liang et al., 2014). In the natural environment, legumes are also confronted by other microbes, many of which are pathogenic. Plants have developed a set of defense reactions to defend against pathogen infection that also involve the recognition of pathogen-derived molecules (Hacquard et al., 2017). For example, chitooligosaccharides

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(COs) include long-chain molecules that activate plant defense responses and short-chain molecules (similar to NFs and referred to as Myc-factors) that mediate symbiotic signaling in a variety of plant species. The receptors that mediate these recognitions are also LYKs (Miya et al., 2007; Wan et al., 2008; Cao et al., 2014; Hayafune et al., 2014; Zipfel and Oldroyd, 2017; He et al., 2019). The receptor for perception of NF, Myc-COs, or COs (long-chain molecules) is a heteromer of two LYK proteins, one with kinase activity and the other without (Miya et al., 2007; Wan et al., 2008; Cao et al., 2014; Hayafune et al., 2014; He et al., 2019; Miyata et al., 2016). Interestingly, rice OsCERK1 was shown to have a dual function, mediating both the arbuscular mycorrhizal symbiosis and the chitin-triggered immune response (Zhang et al., 2014; Miyata et al., 2014), suggesting that these receptors are evolutionarily related. Root nodules that host a massive proliferation of rhizobia develop a weak immunity against the bacterial pathogen *Ralstonia solanacearum* (Benezech et al., 2020). Therefore, two intriguing questions arise. Given the nature of these signals and their corresponding receptors, how do legume hosts distinguish between their compatible rhizobial partners and other noncompatible rhizobia? And how do these same plants distinguish beneficial rhizobia from invading pathogens against which they must mount a defense response?

Nod factors, chitooligosaccharides, and other molecules such as flagellin are referred to as microbe-associated molecular patterns (MAMPs) (Ausubel, 2005). It is the recognition of these molecules that allows plants to detect infections from different microorganisms (Schwessinger and Ronald, 2012; Hacquard et al., 2017). For almost all the legume-rhizobial symbioses, perception of rhizobia-derived NF by NFR1 and NFR5 induces physiological changes essential for subsequent nodule development (Radutoiu et al., 2003; Smit et al., 2007). In addition to these two NFRs, another receptor kinase, SymRK (Symbiosis receptor-like kinase), is required for the mediation of NF signaling and has the potential to form a receptor complex with LjNFR5 in *Lotus japonicus* (Stracke et al., 2002; Antolín-Llovera et al., 2014b). SymRK also plays an essential role in arbuscular mycorrhizal symbioses (AMS) (Stracke et al., 2002) and is therefore a component of the common symbiotic pathway (CSP) that is conserved between the rhizobial and mycorrhizal symbioses. The essential role of SymRK in symbiosis signaling was further revealed by the characterization of several SymRK-interacting proteins. SINA4 (SEVEN IN ABSENTIA), SIP1 (SymRK-Interacting Protein 1), SIP2, and SIE3 (SymRK-Interacting E3 ligase) were shown to associate with SymRK and to regulate root nodule symbiosis (RNS). MtHMGR1 (3-hydroxy-3-methylglutaryl CoA reductase 1) from *Medicago truncatula* was shown to interact with DMI2 (Does not Make Infection 2), the homologous protein of SymRK in *L. japonicus*, to regulate both RNS and AMS (reviewed in Antolín-Llovera et al., 2014a). However, beyond the identification of several SymRK-interacting proteins, the mechanistic role played by SymRK and the identification of its phosphorylation target proteins involved in symbiosis remain to be elucidated.

The pathways that mediate MAMP-triggered immunity (MTI) in plants have been well characterized in *Arabidopsis thaliana* (Ranf, 2017). For example, *Arabidopsis* BAK1 (BRASSINOSTEROID INSENSITIVE 1[BRI1]-Associated Receptor Kinase) plays a central role in plant pathogenesis because it functions as a coreceptor for multiple MAMP receptors, such as the bacterial flagellin receptor FLS2 (FLAGELLIN SENSING 2) (Chinchilla et al., 2007; Heese et al., 2007). BAK1 also serves as a coreceptor for the brassinosteroid receptor BRI1, which is involved in plant development (Li et al., 2002; Nam and Li, 2002). Thus, BAK1 is a key protein that modulates plant innate immunity as well as development.

In the case of the legume symbiosis, the data suggest that the legume host initially recognizes the invading rhizobium as a potential pathogen, inducing a transient defense response (Gourion et al., 2015; Cao et al., 2017). For example, inoculation of *L. japonicus* roots with *Mesorhizobium loti* was shown to elicit phosphorylation of the mitogen-activated protein (MAP) kinases MPK3 and MPK6, which is a typical plant defense response (Lopez-Gomez et al., 2011). Increased production of reactive oxygen species (ROS) and induction of defense-related genes were also observed in *L. japonicus* and soybean upon rhizobial inoculation (Stacey et al., 2006; Libault et al., 2010; Lopez-Gomez et al., 2011). One explanation for these results is that rhizobia may initially trigger weak immune responses in host cells but can suppress these responses during the early stages of infection. Direct evidence in support of this notion came from the finding that rhizobia-derived NF could partially inhibit MTI in different legumes triggered by the addition of different elicitors, including flg22, chitooctaose (CO8), peptidoglycan (PGN), oligogalacturonide heptamers, and even pathogenic culture filters (Feng et al., 2019; Liang et al., 2013; Rey et al., 2019; Shaw and Long, 2003). Interestingly, in addition to various legumes, NF also suppressed immune responses in non-leguminous plants, suggesting the wide conservation of this trait (Liang et al., 2013). In the roots of *M. truncatula*, suppression of immunity mediated by NF is dependent on the presence of MtNFP (Nod factor Perception) but not MtLYK3 or DMI2 (Feng et al., 2019; Rey et al., 2019; Shaw and Long, 2003); however, neither GmNFR1a nor GmNFR5a was required for the suppression of immunity by NF in soybean (Liang et al., 2013). It is possible that these different observations may reflect the existence of homologous proteins in different plant species. However, it remains unclear how weak immunity is activated at the beginning of rhizobial infection and whether other rhizobial molecules are involved in the suppression of this immunity.

In legumes, NF signaling during nodulation is mediated initially by NFR1 and NFR5, as well as SymRK, which then activates the CSP (Kistner et al., 2005; Gherbi et al., 2008). Key components in the CSP from legumes also shape their interactions with other microbes, such as commensal communities (Thiergart et al., 2019), suggesting a broad role for the symbiotic pathway in plant-microbe interactions. Hence, given the ability of rhizobia to suppress plant immunity and evidence for the involvement of the symbiotic pathway in interactions with other microbes, we sought to examine whether any of the key symbiotic components is required for suppression of the host immune response. This avenue of research led us to the discovery that SymRK is required for the suppression of immunity by rhizobial inoculation. SymRK interacts directly with and inhibits the kinase activity of LjBAK1, and LjBAK1 plays a negative role in rhizobial infection. Our data clearly support a model

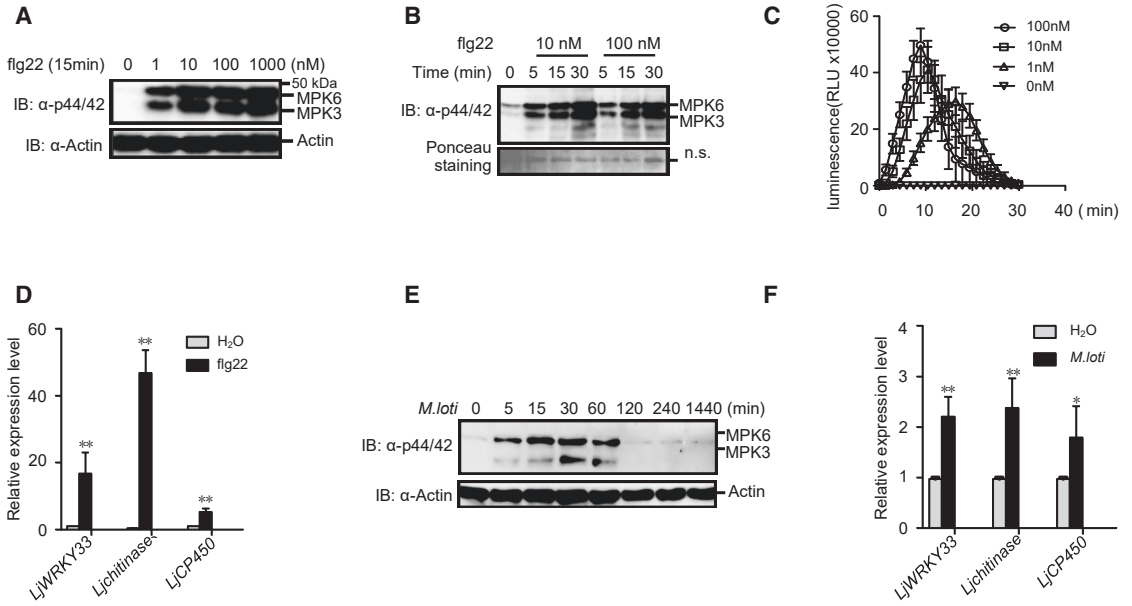

Figure 1. Flg22 and *M. loti* treatments trigger immune responses in the roots of *L. japonicus*.

(A) MPK phosphorylation in roots of *L. japonicus* treated with different concentrations of flg22 for 15 min, determined by immunoblotting using anti-P44/P42 antibody. The lower panel indicates similar loading for each lane represented by immunoblotting using anti-actin antibody.

(B) MPK phosphorylation in roots of *L. japonicus* after flg22 treatment at the indicated time points, determined by immunoblotting with anti-P44/P42 antibody. The lower panel indicates similar loading for each lane represented by a nonspecific band stained with Ponceau S stain.

(C) ROS were monitored in roots of *L. japonicus* for 30 min after treatment with different concentrations of flg22. RLU, Relative Luminescence Units.

(D) and **(F)** Transcript levels of *LjWRKY33* (Lj2g3v2365800), *Ljchitinase* (Lj5g3v1961260), and *LjCP450* (Lj4g3v0189840) in the roots of *L. japonicus* 1 h after 100 nM flg22 or *M. loti* treatment, determined using qPCR. Error bars represent ±SE (n = 3). *$p < 0.05$ or **$p < 0.01$ (Student's t test, significant difference compared with water-treated control).

(E) MPK phosphorylation in roots of *L. japonicus* treated with *M. loti* at the indicated time points, determined by immunoblotting using anti-P44/P42 antibody. The lower panel indicates similar loading for each lane represented by immunoblotting using anti-actin antibody.

in which inhibition of LjBAK1 by SymRK is required for the suppression of immunity during rhizobial infection in *L. japonicus*.

RESULTS

Bacterial flagellin triggers immune responses in the roots of *L. japonicus*

Among currently identified MAMPs, by far the most researched and therefore the best understood is flagellin, the protein that comprises flagella that enable bacterial motility (Ranf, 2017). Rhizobial flagellin appears to lack active flg22, the conserved 22–amino acid epitope that is the most active component of flagellin. However, for the purposes of our studies, flg22 is still a useful general reagent with which to induce MTI in *L. japonicus*. Therefore, to study the interplay between symbiosis and immunity in *L. japonicus*, we used flg22 to induce immune responses in *L. japonicus* roots. As shown in Figures 1A and 1B, the phosphorylation of MAP kinases 3 and 6 (MPK3/6) was detected in *L. japonicus* roots upon treatment with different concentrations of flg22. As indicated, 1 nM flg22 was sufficient to activate phosphorylation of MPK3/6 15 min after treatment. This amount of flg22 is similar to that used in studies of *Arabidopsis* (Smith et al., 2014), indicating that flg22 is a potent MAMP in *L. japonicus*. In addition, an ROS burst and the expression of multiple immune-responsive genes were detected upon treatment with flg22. Flg22 induced a strong ROS burst 10 to 15 min after treatment of *L. japonicus* roots (Figure 1C). Expression of the defense-related genes *LjWRKY33*, *Ljchitinase*, and *LjCP450*was significantly induced 60 min after flg22 treatment (Figure 1D).

Previously, rhizobia were shown to induce weak defense responses, including immune-responsive gene expression, during the very early stages of infection, and these responses were suppressed within approximately 24 h after inoculation (Lohar et al., 2006). Consistent with these findings, weak but measurable MPK3/6 phosphorylation was seen 30 min after rhizobial inoculation in *L. japonicus* roots (Figure 1E). NFR1 and NFR5 are two receptors required for NF-mediated signaling transduction. We next examined whether rhizobial inoculation induced weak MPK3/6 phosphorylation in *nfr1* and *nfr5* mutant plants. As shown in Supplemental Figure 1A, weak MPK3/6 phosphorylation was still observed in *nfr1* and *nfr5* mutant plants. However, in multiple experiments, MPK6 phosphorylation after rhizobial inoculation was much more easily induced than MPK3 phosphorylation compared with flg22-induced MPK3/6 phosphorylation (Figure 1E, Supplemental Figures 1F and 2I). Quantitative PCR revealed an approximately 2-fold increase in the transcript levels of *LjWRKY33*, *LjCP450*, and *LjWRKY53* in *L. japonicus* roots inoculated with rhizobia (Figure 1F).

SymRK is required for suppression of plant defense responses during symbiosis

To study the intersection between symbiosis and pathogenesis, we inoculated *L. japonicus* roots with both flg22 and *M. loti*

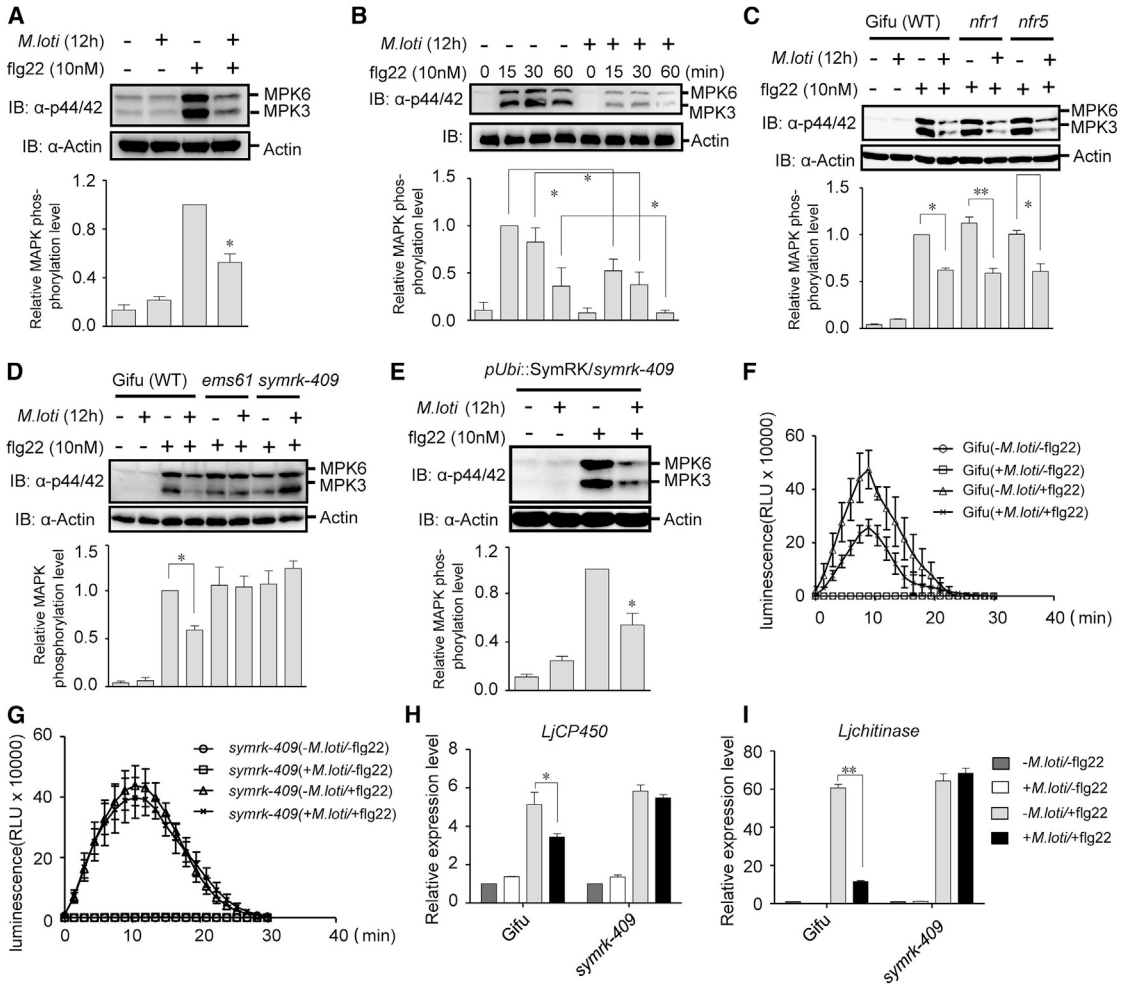

Figure 2. SymRK is required for suppression of immune responses by rhizobial treatment in *L. japonicus*.

(A–E) MPK phosphorylation in the roots of *L. japonicus* determined by immunoblotting using anti-P44/P42 antibody. The upper panel shows the phosphorylation of MPK3/6 under different treatments. The middle panel indicates similar loading for each lane represented by immunoblotting using anti-actin antibody. The lower panel shows the relative MAPK phosphorylation level for each lane quantified from three biological replicates using ImageJ software. The value of the control band in each figure was set to one for comparison. **(A)** Wild-type Gifu pretreated with (+) or without (−) *M. loti* MAFF303099 for 12 h, followed by treatment with 10 nM flg22 for 15 min. **(B)** Wild-type Gifu pretreated with (+) or without (−) MAFF303099 for 12 h, followed by treatment with 10 nM flg22 at different time points. **(C)** and **(D)** Wild-type Gifu, *nfr1*, *nfr5, ems61*, and *symrk-409* mutant roots pretreated (+) with or (−) without MAFF303099 for 12 h, followed by treatment with (+) 10 nM flg22 or (−) H_2O for 15 min. **(E)** Transgenic *symrk-409* mutant plants expressing *SymRK* under the control of the *L. japonicus ubiquitin (pUb)* promoter pretreated with (+) or without (−) MAFF303099 for 12 h, followed by treatment with 10 nM flg22.

(F–G) ROS generation was monitored in roots of wild-type *L. japonicus* (Gifu) pretreated with or without MAFF303099 for 12 h, followed by treatment with 10 nM flg22. RLU, Relative Luminescence Units.

(H–I) Roots of wild-type Gifu and *symrk-409* mutant *L. japonicus* pretreated with or without MAFF303099 for 12 h, followed by treatment with 10 nM flg22 for 60 min. Transcript levels of *LjWRKY33*, *Ljchitinase*, and *LjCP450* were determined by qPCR. Error bars represent ±SE (n = 3). *$p < 0.05$ or **$p < 0.01$ (Student's t test, significant difference between MAFF303099 pretreatment and the unpretreated control). All experiments shown in this figure were performed with at least three biological replicates.

MAFF303099, the specific symbiont of *L. japonicus*. Pretreatment with *M. loti* for 12 h significantly reduced MPK3/6 phosphorylation triggered by flg22 treatment at different time points (Figures 2A and 2B, Supplemental Figure 2A and 2B). These data further support the notion that rhizobia can suppress host immunity during the process of symbiotic interaction. However, pretreatment with rhizobium *Sinorhizobium meliloti*, the specific strain for *Medicago truncatula,* which is unable to colonize *L. japonicus*, did not suppress MPK3/6 phosphorylation induced by flg22 in *L. japonicus* (Supplemental Figure 2C). To ascertain the importance of NF signaling for the suppression of rhizobia-induced defense responses, we examined *Lotus* mutants deficient in *NFR1*, *NFR5*, or *SymRK*. In both *L. japonicus nfr1* and *nfr5* mutant plants, reduction of flg22-induced MPK3/6 phosphorylation after rhizobial inoculation was similar to that seen in wild-type control plants (Figure 2C). By contrast, in the *symrk-409* (a *symrk* knockout mutant line with a retrotransposon inserted at the LRR domain) and *ems61* (another *symrk* knockout mutant line with a nonsense mutation at Trp-808) plants (Supplemental Figure 2D and 2E) (Stracke et al., 2002; Li et al., 2018),

attenuation of flg22-induced MPK3/6 phosphorylation was not detected relative to controls (Figure 2D, Supplemental Figure 2F and 2G). To confirm the involvement of SymRK in the suppression of immune responses, *SymRK* was transgenically expressed in the *symrk-409* mutant plants(Supplemental Figure 2H–2J). Flg22-induced MPK3/6 phosphorylation was rescued in the *symrk-409* plants complemented by the expression of SymRK (Figure 2E). Rhizobial inoculation-induced MPK3/6 phosphorylation was detected at slightly increased levels in the *symrk-409* plants 30 min and 60 min after treatment compared with that in wild-type plants (Supplemental Figure 2K). In SymRK-overexpressing *symrk-409* plants that expressed SymRK under the control of the ubiquitin promoter, a significant reduction in MPK6 phosphorylation level was observed compared with control plants (Supplemental Figure 2L); however, the phosphorylation levels of MPK3 were much lower than the reduced MPK6 phosphorylation levels (Supplemental Figure 2L). The exact function of LjMPK6 and LjMPK3 in RNS remains to be elucidated. In addition to MPK3/6 phosphorylation, suppression of flg22-triggered ROS production and the expression of *LjCP450*, *Ljchitinase*, and *LjWRKY33* genes after rhizobial treatment were attenuated in the *symrk-409* mutant plants (Figures 2F–2I and Supplemental Figure 2M). Collectively, these data suggest that SymRK is required for the suppression of host defense responses activated at the early stage of rhizobial infection or by flg22.

It is well established that rhizobial NF can suppress ROS production and immunity-related gene expression triggered by different elicitors, and this may be important for rhizobial entry into plants (Feng et al., 2019; Liang et al., 2013; Rey et al., 2019; Shaw and Long, 2003). Indeed, pretreatment with NFs suppressed MPK3/6 phosphorylation levels in wild-type *L. japonicus* induced by flg22 (Supplemental Figure 2N). In *M. truncatula*, it has been observed that the SymRK homolog DMI2 is not required for the suppression of immunity by NFs (Feng et al., 2019; Shaw and Long, 2003). We next asked whether SymRK is required for NF-mediated suppression of immunity in *L. japonicus*. An *M. loti* strain with a *nodC* mutation that is therefore unable to produce NF was used to inoculate *L. japonicus* roots. As shown in Supplemental Figure 2O and 2P, pretreatment with *M. loti* (*nodC*$^{-}$) suppressed flg22-induced MPK3/6 phosphorylation in wild-type plants but not in the *symrk* mutant plants, similar to the suppression of flg22-triggered MPK3/6 phosphorylation by wild-type *M. loti* in in *L. japonicus* roots (Figures 2A, 2B, and 2D, Supplemental Figure 2A, 2B, 2F, and 2H). *M. loti* (*nodC*$^{-}$) inoculation also induced weak MPK3/6 phosphorylation in both *nfr1* and *nfr5* mutant plants (Supplemental Figure 2Q and 2R), suggesting that there may be other molecules that suppress plant immunity. Next, we assessed the potential suppression of plant immunity by supernatants obtained from wild-type and *nodC*$^{-}$ mutant rhizobial strains after sonication. As shown in Supplemental Figure 2S, the supernatants from both wild-type and *nodC*$^{-}$ strains suppressed MPK3/6 phosphorylation triggered by flg22 compared with the control. In *symrk-409* mutant roots, flg22-triggered MPK3/6 phosphorylation was still observed at reduced levels after pretreatment with NFs (Supplemental Figure 2T). In a separate experiment, root tissues were pretreated with bacterial peptidoglycan for 6 h, followed by flg22 treatment. No suppression of the immune response was observed compared with the control (Supplemental Figure 2U). These data confirmed that SymRK is not involved in NF-mediated suppression of immunity in *L. japonicus* and that rhizobial molecules other than NF may be required for the suppression of immunity.

SymRK associates with LjBAK1

Given the results presented above, we next asked how SymRK is involved in suppressing the host defense response. To answer this question, the kinase domain of SymRK was used as a bait to screen a yeast two-hybrid library for SymRK-interacting candidate proteins. Among the candidates identified was a protein that shares high homology with the *Arabidopsis* leucine-rich repeat receptor kinase BAK1. We cloned the full-length gene from *L. japonicus* and refer to it as *LjBAK1*hereafter. LjBAK1 contains a signal peptide, a leucine zipper, five leucine-rich repeats (LRR1–LRR5), a Ser-Pro-Pro motif, a hydrophobic transmembrane domain, and a kinase domain.

The interaction between LjBAK1 and SymRK was further tested in yeast cells. As shown in Figure 3A and Supplemental Figure 3A and 3B, yeast cells expressing the cytoplasmic domain (CD) of LjBAK1 and SymRK-CD or a kinase-dead variant of SymRK-CD^{km}, but not LjNFR1-CD or LjNFR5-CD, could grow on quadruple dropout medium compared with negative controls (Figure 3A). These results suggested that LjBAK1 interacts with SymRK but not LjNFR1 or LjNFR5 in yeast cells. In addition, yeast cells expressing the CD of BAK1 homologs from *M. truncatula* or rice and the CD of SymRK from *M. truncatula* or rice could grow on quadruple dropout medium, suggesting that the interaction between BAK1-CD and SymRK-CD may be conserved among different plant species (Figure 3A). To confirm the interaction between SymRK and LjBAK1, coimmunoprecipitation was performed in *Nicotiana benthamiana* transiently expressing LjBAK1 and SymRK and in transgenic roots of *L. japonicus*. In *N. benthamiana* leaves expressing LjBAK1, both HA-tagged SymRK and the kinase-dead version of SymRK-km were pulled down at similar levels with LjBAK1-FLAG by anti-FLAG agarose beads (Supplemental Figure 3C), suggesting that the kinase activity of SymRK is not required for its interaction with LjBAK1. These data are consistent with the results from yeast two hybrid assays. Coimmunoprecipitation assay was performed in the roots of *L. japonicus* expressing FLAG-tagged LjBAK1 and HA-tagged versions of SymRK, LjNFR1, or LjNFR5. As shown in Figure 3B, SymRK-HA but not LjNFR1-HA or LjNFR5-HA could be pulled down using anti-FLAG antibody, indicating that SymRK but not LjNFR1 or LjNFR5 associated with LjBAK1 *in vivo*. We then asked whether rhizobial treatment could regulate the interaction between SymRK and LjBAK1. Transgenic roots expressing both SymRK-HA and LjBAK1-FLAG were inoculated with *M. loti* MAFF303099 for 24 h before coimmunoprecipitation assay. As shown in Figure 3C, the protein level of SymRK-HA immunoprecipitated in the sample inoculated with rhizobia was much higher than that in the sample without rhizobial treatment, indicating that rhizobial treatment enhanced the interaction between SymRK and LjBAK1 *in planta*.

SymRK inhibits the kinase activity of LjBAK1

Both SymRK and LjBAK1 are protein kinases known to have strong auto- and transphosphorylation activities (Yoshida and Parniske, 2005; Saha et al., 2016; Karlova et al., 2009; Yun et al., 2009). We performed an *in vitro* kinase assay using purified recombinant

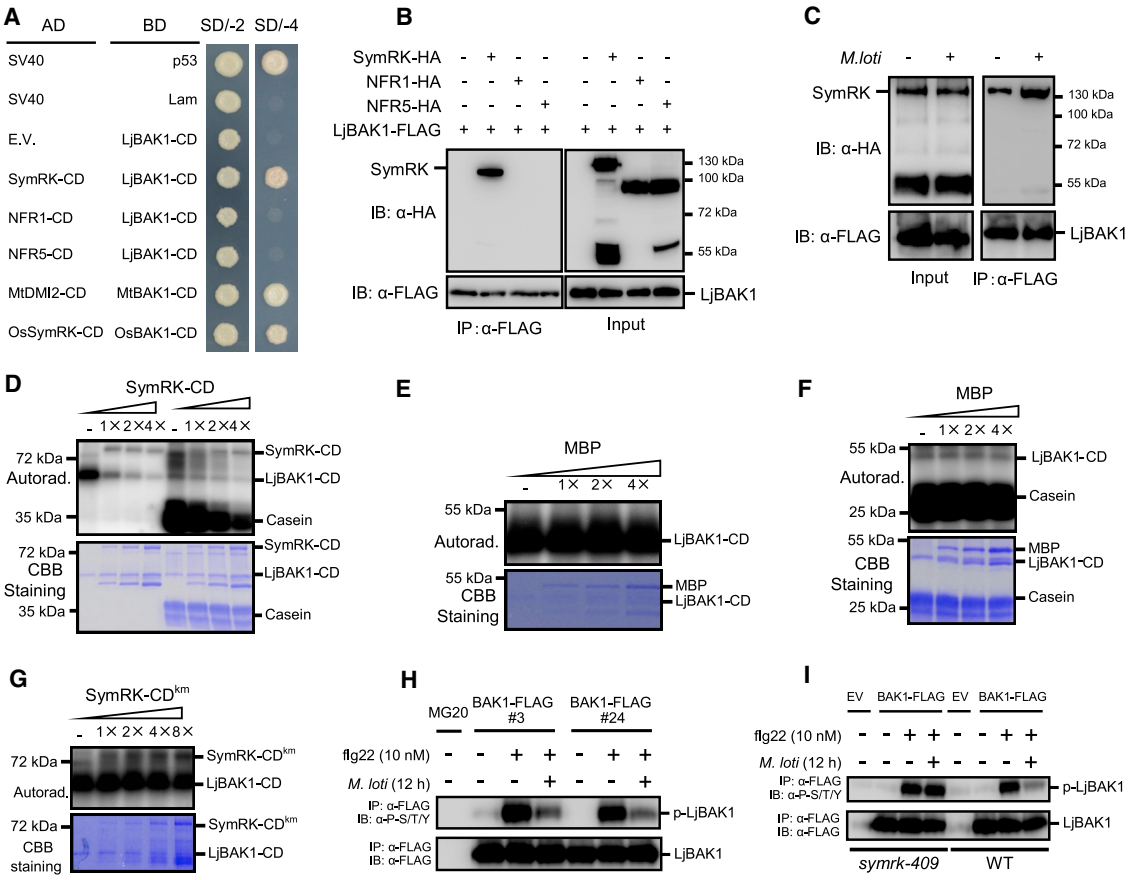

Figure 3. SymRK associates with the kinase domain and inhibits the kinase activity of LjBAK1.

(A) Specificity of the interaction between the kinase domains of SymRK and LjBAK1 in yeast two hybrids. The kinase domains of LjNFR1, LjNFR5, and SymRK were tested for interactions with that of LjBAK1. Homologs of SymRK and LjBAK1 from *M. truncatula* and *Oryza sativa* were tested for interactions in yeast cells. Combinations of p53/SV40 and Lam/SV40 served as positive and negative controls, respectively.

(B) Coimmunoprecipitation assay of SymRK and LjBAK1 in *L. japonicus*. HA-tagged SymRK, LjNFR1, or LjNFR5 was expressed in *L. japonicus* roots expressing SymRK-FLAG by hairy root transformation. Crude proteins were affinity-purified with anti-FLAG M2 Affinity Gel. Solubilized proteins (input) and immunopurified proteins (IP) were analyzed by immunoblotting (IB) using anti-HA or anti-FLAG antibodies.

(C) Rhizobial inoculation enhances the interaction between SymRK and LjBAK1, as determined by a coimmunoprecipitation assay. SymRK-HA was expressed in the roots of *L. japonicus* plants expressing LjBAK1-FLAG. An immunoprecipitation assay was performed with or without rhizobial inoculation using anti-FLAG agarose and anti-HA and anti-FLAG antibodies.

(D–G) SymRK inhibits both auto- and transphosphorylation activities of the kinase domain of LjBAK1. The kinase domain of SymRK (SymRK-CD) fused with an MBP tag and the kinase domain of LjBAK1 (BAK1-CD) fused with a His tag were expressed and affinity-purified from *E. coli*. MBP-SymRK-CD and His-LjBAK1-CD were incubated with or without casein in the presence of [γ-^{32}P]ATP before separation on an SDS-PAGE gel. The gel was stained with Coomassie blue (bottom) and subjected to autoradiography (top). The molecular mass standard (kDa) is shown on the left side. An increasing amount of MBP, MBP-SymRK-CD, or MBP-SymRK-CD^{km} was added to the reaction mix. The 1× amount of protein (MBP, MBP-SymRK-CD, or MBP-SymRK-CD^{km}) was about 0.25 μg per reaction.

(H) Root tissues from *Lotus* wild-type plants (MG20) and two stable transgenic lines (#3 and #24) expressing LjBAK1-FLAG were pretreated with *M. loti* for 12 h, followed by flg22 treatment for 15 min. Crude protein extracted from roots was immunoprecipitated with anti-FLAG agarose followed by detection with anti-phosphoserine/threonine/tyrosine and anti-FLAG antibodies.

(I) Transgenic roots in Gifu wild-type and *symrk-409* mutant plants expressing empty vector (EV) or LjBAK1-FLAG were pretreated with *M. loti* for 12 h, followed by flg22 treatment for 15 min. Crude protein extracted from roots was immunoprecipitated with anti-FLAG agarose followed by detection with anti-phosphoserine/threonine/tyrosine and anti-FLAG antibodies. The immunoblot analyses and Y2H assay were performed with at least three biological replicates.

MBP-SymRK-CD and His-LjBAK1-CD proteins. As shown in Figure 3D, LjBAK1 has strong autophosphorylation activity, as shown by a strong band of LjBAK1-CD in the presence of [^{32}P]-ATP. Casein, the universal target of Ser/Thr kinases, was also detected as a strong band in kinase buffer containing LjBAK1-CD, indicating that LjBAK1 has strong transphosphorylation activity. As shown in Figure 3D, the phosphorylation band intensities of LjBAK1-CD and casein were significantly reduced in kinase buffer with increasing amounts of SymRK-CD. In control experiments, increasing amounts of MBP (maltose-binding protein) did not reduce the phosphorylation band intensities of LjBAK1-CD and casein (Figures 3E and 3F). These data suggest that SymRK can inhibit the kinase activity of LjBAK1 *in vitro*. To test whether the kinase activity of SymRK is required for its inhibitory effects on

LjBAK1, a kinase-dead variant of SymRK-CD^{km} was created and used in a kinase assay. No reduction in the autophosphorylation band intensity of LjBAK1-CD was detected in the presence of SymRK-CD^{km} (Figure 3G). The interphosphorylation between SymRK-CD and LjBAK1-CD was also examined in an *in vitro* kinase assay. As shown in Supplemental Figure 3D, SymRK-CD could phosphorylate LjBAK1-CD^{km}, and LjBAK1-CD could phosphorylate SymRK-CD^{km}; however, both phosphorylation events were detected at very low levels. In the sample that included both SymRK-CD and LjBAK1-CD, the LjBAK1-CD phosphorylation levels decreased significantly, whereas the SymRK-CD phosphorylation levels did not change (Supplemental Figure 3D). These data suggest that the kinase activity of SymRK is required for its inhibitory effects on LjBAK1 kinase activity *in vitro*.

To confirm that the suppression of LjBAK1-CD kinase activity in the *in vitro* kinase assay was not due to the competitive usage of [^{32}P]-ATP by the increased amount of SymRK-CD in the reaction system, we took advantage of *Arabidopsis* BIK1, a known kinase protein that strongly phosphorylates AtBAK1 (Lu et al., 2010). We then tested the interphosphorylation between LjBAK1 and *Arabidopsis* BIK1. In the *in vitro* kinase assay, strong phosphorylation of AtBIK1-km by LjBAK1-CD but not by the kinase-dead version of LjBAK1-CD-km was observed, and AtBIK1 could also strongly phosphorylate LjBAK1-CD-km (Supplemental Figure 3E). These data are consistent with previous reports that AtBAK1 can phosphorylate AtBIK1 and *vice versa* (Lu et al., 2010). In the sample that included both the kinase-active version of LjBAK1-CD and AtBIK1, strong kinase activities of both LjBAK1-CD and AtBIK1 were detected in the *in vitro* kinase assay (Supplemental Figure 3E). The phosphorylation levels of LjBAK1-CD did not change in the sample supplemented with AtBIK1, with strong phosphorylation levels detected compared with the sample that lacked added AtBIK1 (Supplemental Figure 3E). These data suggest that the competitive usage of [^{32}P]-ATP in an *in vitro* assay did not reduce the kinase activity of LjBAK1-CD. *Arabidopsis* BIK1 is a key component that works together with AtBAK1 and PRRs (Pattern Recognition Receptors) to mediate innate immunity. We next tested whether SymRK-CD could regulate the interphosphorylation between LjBAK1 and BIK1 and found that the addition of SymRK-CD decreased the phosphorylation levels of both BIK1 and LjBAK1-CD (Supplemental Figure 3F). These data suggest that additional components may be targeted and suppressed directly or indirectly by SymRK, providing further evidence that suppression of the innate immune pathway is important for regulating rhizobial infection during the legume-rhizobial symbiosis. However, the function of *L. japonicus* BIK1 homologs in regulation of the legume-rhizobial symbiosis is of great interest and remains to be characterized.

To test whether the phosphorylation status of LjBAK1 is suppressed by SymRK *in vivo* after inoculation with rhizobia, stable transgenic *L. japonicus* plants expressing LjBAK1 fused with a FLAG tag were generated. After immunoprecipitation with anti-FLAG antibody, phosphorylation bands were detected using anti-phospo Ser/Thr/Tyr antibody. In two independent transgenic lines, strongly phosphorylated LjBAK1 was detected in the transgenic roots treated with flg22 (Figure 3H). However, the intensity of the band representing phosphorylated LjBAK1 decreased significantly in the samples pretreated with *M. loti* (Figure 3H), indicating that flg22-induced phosphorylation of LjBAK1 was attenuated by pretreatment with rhizobia. To better understand whether the decreased phosphorylation of LjBAK1 is related to SymRK, we transgenically expressed *LjBAK1*-FLAG in both wild-type and *symrk-409* knockout mutant plants using hairy root transformation. As shown in Figure 3I, suppression of phosphorylated LjBAK1 was detected in the roots of wild-type plants but not those of *symrk-409* mutant plants, suggesting that the phosphorylation of LjBAK1 may be suppressed by the presence of SymRK *in planta*.

LjBAK1 is an ortholog of *Arabidopsis* BAK1

The function of *Arabidopsis* BAK1 in both plant development and plant immunity has been extensively studied in recent years (Yasuda et al., 2017). To test whether LjBAK1 is an ortholog of *Arabidopsis* AtBAK1 (Figure 4A), *LjBAK1* was overexpressed in the *bri1-5* mutant, a weak mutant allele of *bri1*. Ectopic expression of *LjBAK1* partially rescued the defective phenotypes of *bri1-5*: the rescued plants showed about 30% to 40% more elongated hypocotyls than *bri1-5* control plants under dark-grown conditions (Supplemental Figure 4A and 4B). This was similar to results reported when *Arabidopsis* AtBAK1 was used to rescue the defects in *bri1-5* mutant plants (Li et al., 2002). *LjBAK1* was also transgenically expressed in the *Arabidopsis bak1-4* knockout mutant. Rosette leaves were restored to normal size in the transgenic plants compared with wild-type *Arabidopsis* (Supplemental Figure 4C and 4D). As a coreceptor for FLS2, *Arabidopsis* AtBAK1 is critically important for mediating flagellin-induced defense responses. The defect in flg22-triggered MPK3/6 phosphorylation in *bak1-4* mutant plants was rescued by the expression of *LjBAK1* compared with wild-type plants (Supplemental Figure 4E). These data indicate that LjBAK1 is an ortholog of *Arabidopsis* AtBAK1 and has similar biological functions in the induction of innate immunity and plant development.

LjBAK1 positively regulates immunity and negatively regulates symbiosis in *L. japonicus*

To dissect the function of LjBAK1 in the symbiotic signaling pathway, we generated *ljbak1* mutant plants using CRISPR-Cas9 editing technology with two guide RNAs (gRNAs) that targeted the 5′-end of *LjBAK1*. After sequencing, two independent mutant lines were identified and named *ljbak1-1* and *ljbak1-2* (Figure 4B). The *ljbak1-1* mutant plant has a cytosine insertion between 555 and 556 base pairs (bp), and the *ljbak1-2* mutant plant has a 5-bp deletion between 410 and 414 bp (Figure 4B). Both *ljbak1-1* and *ljbak1-2* plants make truncated proteins with a frameshift mutation within the *LjBAK1* gene (Figure 4B). One of the gRNAs used to edit LjBAK1 was very close to an uncharacterized gene (Lj1g3v3689960.1) in *L. japonicus*; to test whether this gene was edited, we sequenced it in the *ljbak1-1* and *ljbak1-2* mutant plants and found that it contained no mutations (Supplemental Figure 4F). Similar to the smaller size of *Arabidopsis bak1-4* mutant plants, both *ljbak1-1* and *ljbak1-2* mutants showed a semi-dwarfed phenotype, including shorter root and shoot lengths compared with wild-type MG20 (Figures 4C and 4D). In response to rhizobial treatment, the number of infection pockets (IPs) and infection threads (ITs) per plant were significantly increased (Figure 4E). The densities of IPs and ITs were significantly higher in *ljbak1-1* and *ljbak1-2* mutant plants than in the wild-type control (Figure 4F).

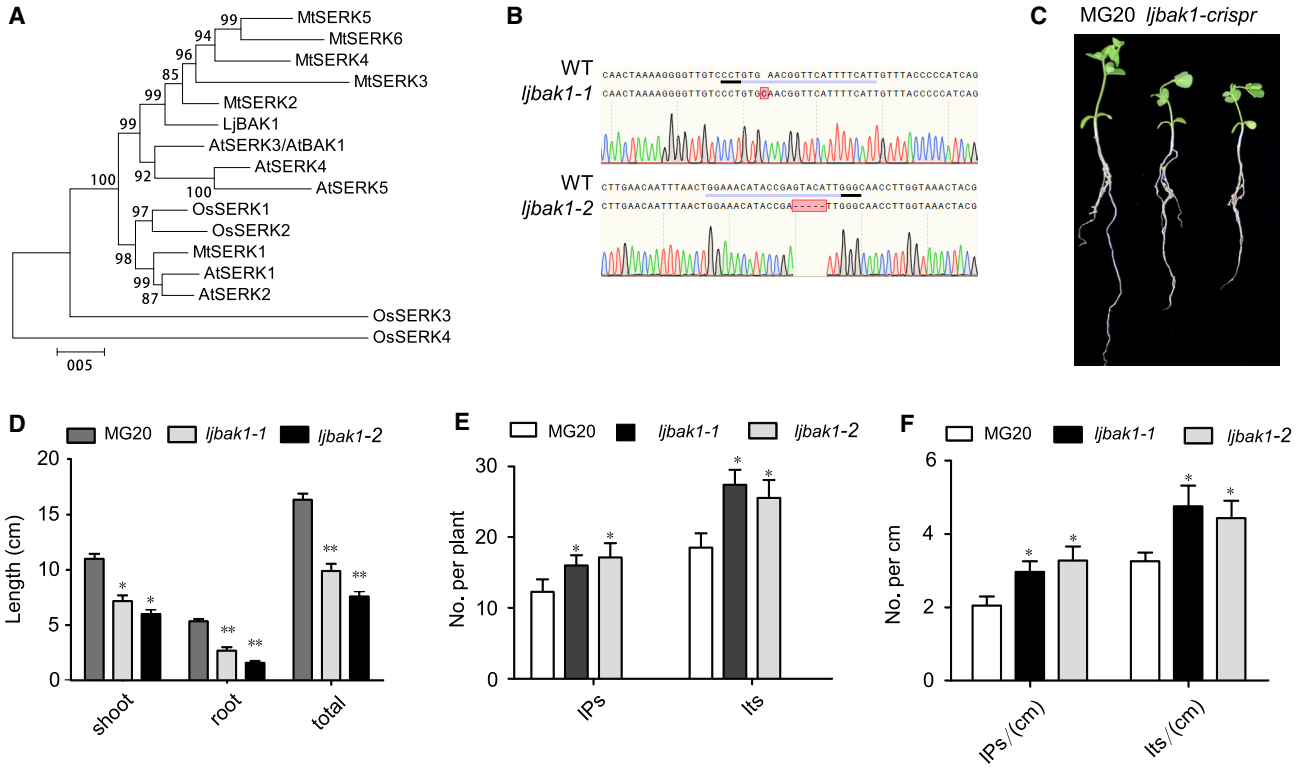

Figure 4. LjBAK1 suppresses rhizobial infection in *L. japonicus*.

(A) Phylogenetic tree of LjBAK1, AtSERKs, MtSERKs, and OsSERKs. Bootstrap values (%) obtained from 1,000 trials are indicated at the nodes.

(B) Two *ljbak1* null mutants were generated using CRISPR-Cas9 gene editing technology. Both the *ljbak1-1* and *ljbak1-2* mutant plants contain a frameshift mutation in the *LjBAK1 gene* that produces a prematurely terminated protein. *Ljbak1-1* has a cytosine insertion between 555 and 556 bp, and the *ljbak1-2* mutant has a 5-bp deletion between 410 and 414 bp.

(C–F) (C) and **(D)** Growth retardation phenotype of *ljbak1* mutant plants compared with wild-type plants. Two-week-old seedlings were used for shoot and root length measurements. **(E)** and **(F)** The numbers of ITs and IPs per plant **(E)** and per centimeter of root **(F)** were significantly greater in the *ljbak1* mutant lines than in the control plants at 5 dpi with *M. loti*. After germination, seedlings were grown in vermiculite and inoculated with rhizobia for 5 d before IT and IP measurements. Error bars represent ±SE. *$p < 0.05$ or **$p < 0.01$ (Student's t test, significant difference compared with the respective control). All experiments were performed with at least three biological replicates.

Consistent with these results, the expression of *NIN* and *ENOD40*, two genes involved in the early symbiotic signaling pathway, was higher in *ljbak1* mutant plants than in wild-type control plants (Supplemental Figures 4G and 4H). The nodule numbers produced on the *ljbak1* mutant plants were also compared with those of the wild-type plants. Because of the short root length of *ljbak1* mutant plants compared with wild-type plants, we compared nodule densities (nodule number per centimeter) between wild-type and *ljbak1* mutant plants 21 and 28 days post inoculation (dpi) with rhizobia (Supplemental Figure 4I). However, no significant differences in nodule density were observed between wild-type and *ljbak1* mutant plants (Supplemental Figure 4I), suggesting that increased rhizobial infection in the *ljbak1* mutant plants may compensate for reduced nodule numbers. These data indicate that LjBAK1 plays a negative role in mediating rhizobial symbiosis and functions at the early stage of rhizobial infection in plants.

LjBAK1 is involved in the intersection of innate immunity and symbiotic responses

The above results suggest that LjBAK1 negatively regulates symbiosis but plays a positive role in innate immunity. SymRK associates with and inhibits the kinase activity of LjBAK1. Hence, we tested whether LjBAK1 is involved in the intersection of innate immunity and symbiosis. MPK3/6 phosphorylation and immunity-related gene expression was compared between the *ljbak1-1* mutant and wild-type plants. Both flg22-and *M. loti*-triggered phosphorylation of MPK3/6 were slightly decreased in the *ljbak1-1* and *ljbak1-2* mutant plants compared with wild-type MG20 (Figure 5A and 5B). Rhizobial treatment inhibited MPK3/6 phosphorylation triggered by flg22, but no suppressive effect of rhizobial treatment on MPK3/6 phosphorylation was observed in the *ljbak1-1* mutant plants because of their weak MPK3/6 phosphorylation compared with wild-type plants (Figure 5C). Consistent with these findings, flg22-triggered expression of *LjCP450*, *Ljchitinase*, and *LjWRKY33* was inhibited by rhizobia pretreatment in wild-type but not *ljbak1-1* mutant plants (Figures 5D, 5E, and 5F).

LjBAK1 complemented *Arabidopsis bak1-4* mutant plants by rescuing both developmental defects and immune deficiency (Supplemental Figures 4D and 4E). Because BAK1 is an essential component in the regulation of brassinosteroid signaling, we compared brassinosteroid response in wild-type and *ljbak1* mutant plants. As shown in Supplemental Figure 4J, brassinosteroid treatment inhibited the root growth of wild-type

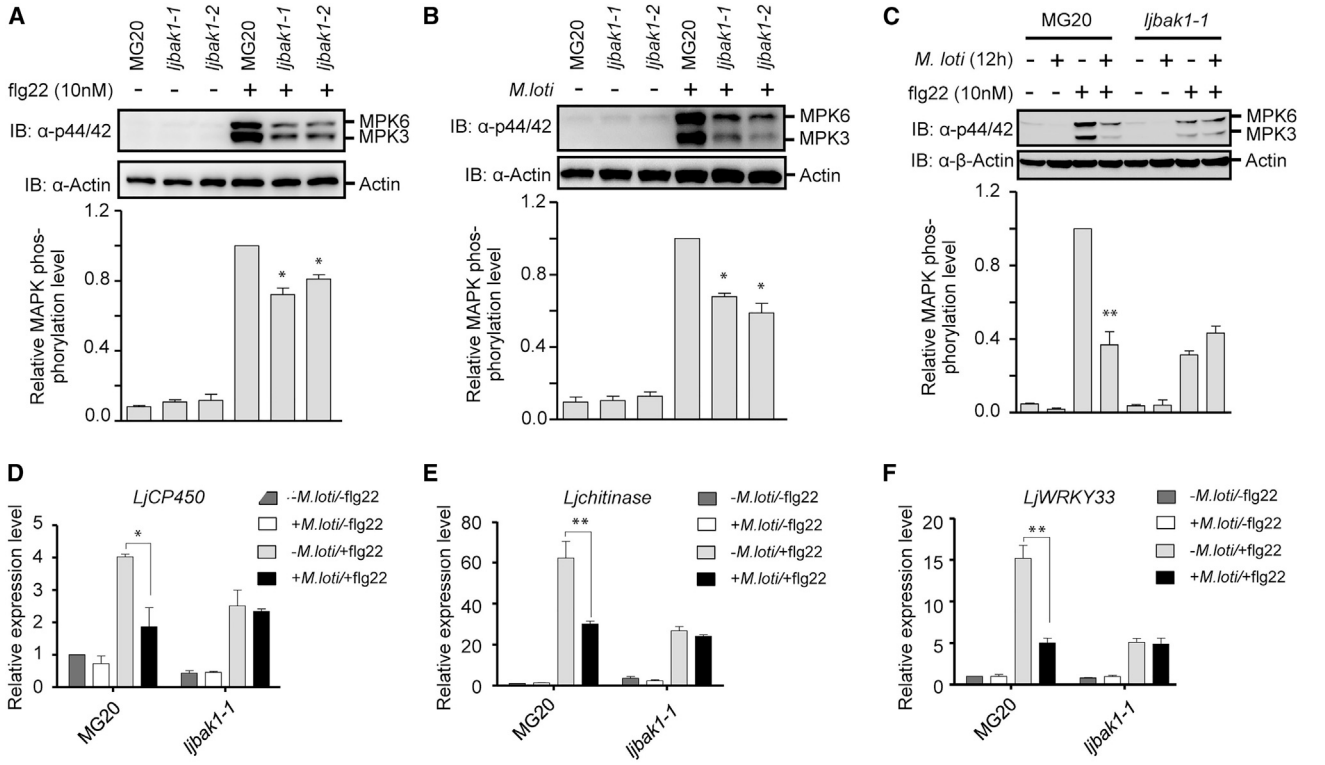

Figure 5. The *ljbak1* mutant plants have reduced immune responses after rhizobial treatment.

(A–C) MPK phosphorylation in roots of *L. japonicus* determined by immunoblotting with anti-P44/P42 antibody. The upper panel shows the phosphorylation of MPK3/6 under different treatments. The middle panel indicates similar loading for each lane represented by immunoblotting using anti-actin antibody. The lower panel shows the relative MAPK phosphorylation level for each lane quantified from three biological replicates using ImageJ software. The value of the control band in each figure was set to one for comparison. **(A** and **B)** Wild-type MG20 and two independent mutant lines of LjBAK1 treated with (+) or without (−) 10 nM flg22 **(A)** for 15 min or MAFF303099 **(B)** for 30 min. **(C)** Wild-type MG20 and *ljbak1-1* pretreated with (+) or without (−) MAFF303099 for 12 h, followed by treatment with 10 nM flg22.

(D–F) Roots of wild-type MG20 and *ljbak1-1* mutant *L. japonicus* pretreated with or without MAFF303099 for 12 h, followed by treatment with 10 nM flg22 for 60 min. Transcript levels of *LjWRKY33*, *Ljchitinase*, and *LjCP450* were determined by qPCR. Error bars represent ±SE (n = 3). *p < 0.05 or **p < 0.01 (Student's t test, significant difference between *M. loti* pretreatment and the mock control).

but not *ljbak1-1* mutant plants. To test whether LjBAK1 has a function similar to that of AtBAK1 in immunity, we investigated the function of LjBAK1 in *L. japonicus*. To this end, we measured flg22-triggered MPK3/6 phosphorylation and ROS production in *ljbak1-1* mutant plants that expressed LjBAK1. Flg22-triggered phosphorylation of MPK3 and MPK6 was detected at a low level in the *ljbak1-1* plants expressing the control vector compared with wild-type plants or *ljbak1-1* mutant plants expressing LjBAK1-FLAG (Supplemental Figure 4I). These data indicate that LjBAK1 plays a positive role in the regulation of plant immunity and development in *L. japonicus*.

Overexpression of SymRK suppresses plant innate immunity

The above data indicate that suppression of LjBAK1 by SymRK is required for the suppression of immune responses during rhizobial infection of *L. japonicus*; we therefore asked whether SymRK is required for the suppression of pathogenesis in other plants. To test this hypothesis, *SymRK*, *LjNFR1*, or *LjNFR5* was ectopically expressed in wild-type *Arabidopsis* (Col-0) (Supplemental Figure 5A–5C). Flg22-triggered MPK3/6 phosphorylation band intensity was decreased in *SymRK*- but not in *LjNFR1*- or *LjNFR5*-overexpressing transgenic *Arabidopsis* compared with the wild-type control (Figure 6A–6C). Pretreatment of *Arabidopsis* with flg22 induces plant immunity that reduces the growth of the pathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (Supplemental Figure 5D). However, reduction of *Pst* DC3000 growth by flg22 pretreatment was abolished in *SymRK*-overexpressing *Arabidopsis* plants (Supplemental Figure 5D). In wild-type *Arabidopsis*, flg22 treatment strongly induced the transcript levels of *AtFRK1*, *AtPER5*, and *AtGST*; however, the expression of these genes was significantly reduced in three independent *SymRK*-expressing lines (Figures 6D–6F). To test whether the suppressive effects of SymRK on plant immunity are dependent on LjBAK1, SymRK was overexpressed in the roots of *ljbak1-1* mutant plants, and MPK3/6 phosphorylation was detected after challenge with flg22 treatment (Supplemental Figure 5E). As shown in Supplemental Figure 5F, MPK3/6 phosphorylation levels were not detected at reduced levels in *ljbak1-1* mutant plants when *SymRK* was overexpressed compared with control transgenes. These results clearly indicate that negative regulation of innate immunity by SymRK probably arises from its ability to impact the function of BAK1.

In addition to suppressed plant immunity, transgenic *Arabidopsis* plants that overexpressed *SymRK* showed slightly reduced root

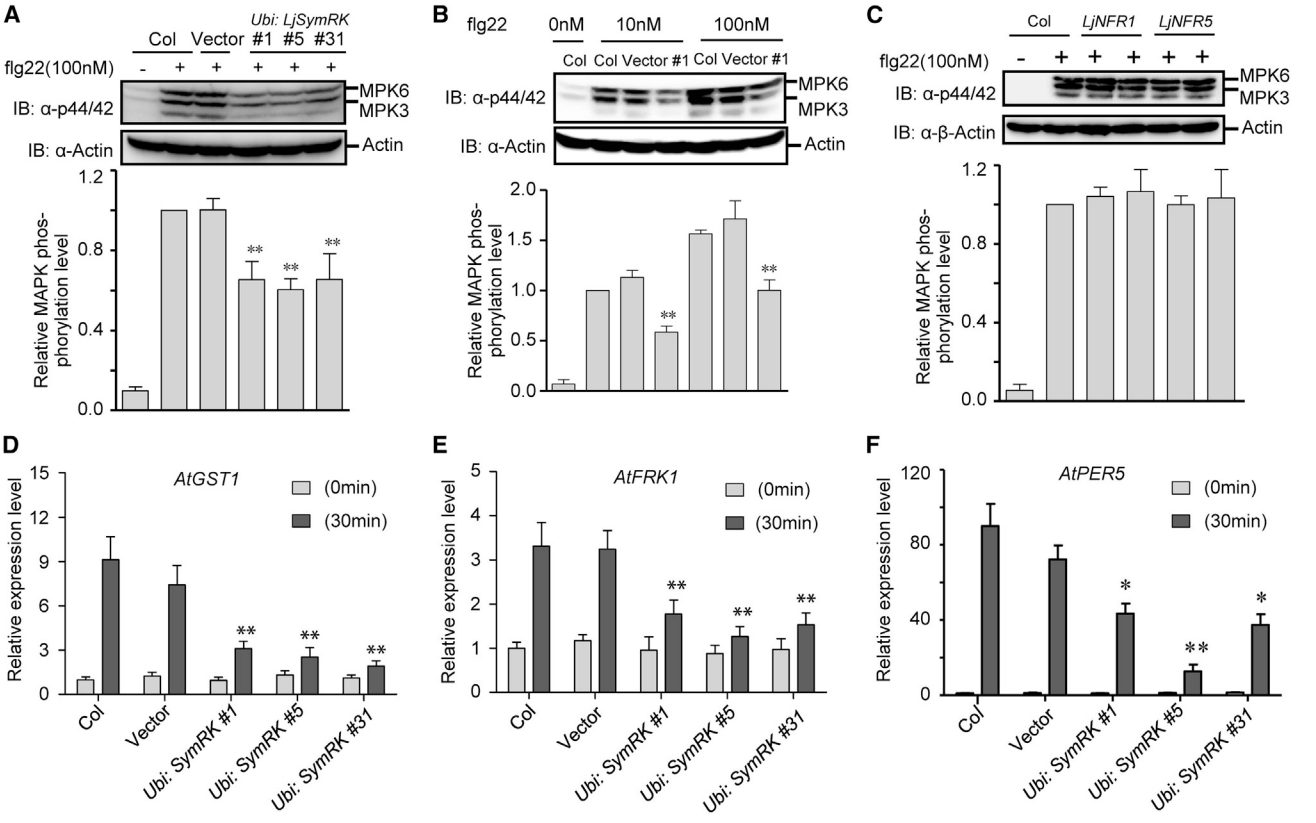

Figure 6. Ectopic expression of *SymRK* suppresses the flg22-induced immune response.

(A–C) MPK phosphorylation in *Arabidopsis* determined by immunoblotting with anti-P44/P42 antibody. Leaf discs (~0.2 cm^2) were punched from fully expanded leaves and floated in H_2O overnight before treatment. The upper panel shows the phosphorylation of MPK3/6 under different treatments. The middle panel indicates similar loading for each lane represented by immunoblotting with anti-actin antibody. The lower panel shows the relative MAPK phosphorylation level for each lane quantified from three biological replicates using ImageJ software. The value of the control band in each figure was set to one for comparison. **(A)** Three independent lines of *SymRK* under the control of the *L. japonicus ubiquitin (Ub)* promoter treated with 100 nM flg22 for 15 min. **(B)** Transgenic *Arabidopsis* expressing *SymRK* treated with different concentrations of flg22 for 15 min. **(C)** *Arabidopsis* Col-0 plants expressing *LjNFR1* or *LjNFR5* under the control of the *L. japonicus ubiquitin (pUb)* promoter were treated with 100 nM flg22 for 15 min. **(D–F)** Transcript levels of *AtGST1, AtFRK1*, and *AtPEPR5* in wild-type and *SymRK*-overexpressing *Arabidopsis* were determined using qPCR in the presence of flg22. Error bars represent ±SE (n = 3). *p < 0.05 or **p < 0.01 (Student's t test, significant difference between *SymRK* transgenic and Col-0 control plants). #1, #5, and #31 represent three independent transgenic lines expressing *SymRK*.

length (Supplemental Figure 5G and 5H), suggesting that SymRK also impacts plant development. To examine whether the kinase activity of SymRK is required for suppression of plant immunity, MPK3/6 phosphorylation and *AtFRK1* expression were measured in response to flg22 treatment in *Arabidopsis* that ectopically overexpressed a kinase-dead version of SymRK-km (Supplemental Figure 5A). As shown in Supplemental Figure 5I and 5J, MPK3/6 phosphorylation and *AtFRK1* expression induced by flg22 treatment were not detected at reduced levels in *Arabidopsis* overexpressing *SymRK-km* compared with control plants. These data indicate that the kinase activity of SymRK is important for suppression of the innate immunity induced by flg22 treatment, consistent with the requirement of SymRK-CD kinase activity for suppression of LjBAK1-CD kinase activity, as shown in the *in vitro* assay.

DISCUSSION

An optimized balance of innate immunity is important for successful rhizobial infection and colonization in leguminous plants. Increasing data suggest that innate immunity is activated in plants during the initial stages of infection but then subsides, probably owing to active suppression by both host and symbiont. How plants actively suppress innate immunity is largely unclear. In this study, we showed that SymRK contributes significantly to the suppression of plant innate immunity during symbiotic interaction with rhizobia. Overexpression of *SymRK* suppressed both rhizobial inoculation- and flg22-triggered immune responses in plants. SymRK directly targets and inhibits the kinase activity of LjBAK1, a positive regulator of plant immunity. In addition, LjBAK1 was shown to have a significant role in the suppression of rhizobial infection. The data indicate that SymRK and LjBAK1 act together as a physiological switch to regulate innate immunity and RNS in *L. japonicus* (Figure 7).

The most recent model for the evolution of legume-rhizobial symbiosis is that it arose within a single clade of angiosperms by co-option of existing pathways, with some species subsequently losing the ability to nodulate (Griesmann et al., 2018). A key aspect is that the rhizobial symbiosis evolved from the much older mycorrhizal

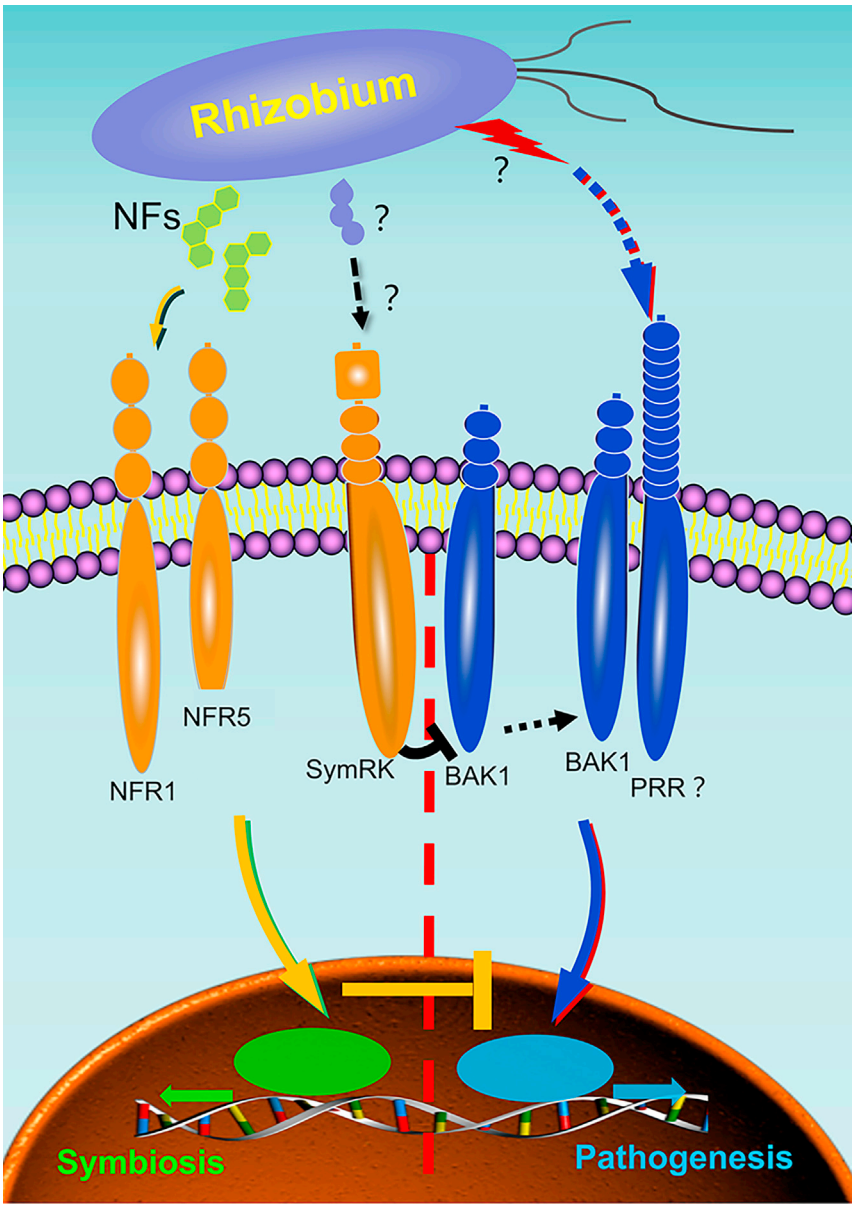

Figure 7. A proposed model for suppression of plant immunity by the SymRK-BAK1 complex during the legume-rhizobial symbiosis.
During the symbiotic interaction with host cells, rhizobial nod factors and other molecules may separately suppress the immunity triggered during the early interaction. SymRK, the central component involved in the symbiotic pathway, works together with BAK1 to form a physiological switch to regulate plant immunity during the legume-rhizobial symbiosis.

symbiosis, adapting key signaling components, such as those that comprise the CSP (Giraud et al., 2007; Cao et al., 2017; Zipfel and Oldroyd, 2017). Thus, it is not surprising that our data suggest that instead of developing novel components, rhizobia make use of conserved components of the innate immunity pathway to regulate the host response during infection. In this way, enhanced rhizobial infection is easily recognized, and this information is transferred directly to suppress detrimental defense responses.

Arabidopsis BAK1 was previously identified as a coreceptor for BRI1 to regulate plant development (Li et al., 2002; Nam and Li, 2002). However, BAK1 also appears to serve as a coreceptor for multiple plant receptors, including FLS2 and EFR, to mediate plant response to pathogens (Ma et al., 2016; Gong et al., 2019). BAK1 is a typical kinase, and transphosphorylation between PRRs and BAK1 is required for activation of MTI in plants. Because of its central role in regulating plant development and immune responses triggered by different microbial MAMPs, BAK1 may be a key component in the regulation of plant interactions with different microbes to establish symbiosis, pathogenesis, or commensalism. Therefore, BAK1 should be a direct target protein of different microbes to balance symbiosis and pathogenesis. Indeed, *Arabidopsis* BAK1 was found to be directly targeted and suppressed by bacterial effectors, such as AvrPtoB from *Pseudomonas syringae* (Shan et al., 2008), providing an effective means for pathogen infection of plant cells. With regard to its positive role in plant innate immunity, LjBAK1 was found to be inhibited by the host protein SymRK to favor rhizobial infection. Both kinase-dead and wild-type versions of SymRK could interact with LjBAK1; however, only wild-type SymRK was shown to suppress the kinase activity of LjBAK1, suggesting that the functions required for interaction and kinase suppression must be differentially regulated. The interaction between SymRK and LjBAK1 was enhanced after rhizobial inoculation. Although not studied in *L. japonicus*, DMI2 protein accumulates after rhizobial inoculation in an NF-independent manner in *M. truncatula* (Pan et al., 2018). Therefore, a possible scenario is that rhizobia have other uncharacterized molecules that target SymRK to advance the symbiotic pathway but also to enhance the suppression of LjBAK1, the common signaling component in immune signaling, thereby promoting rhizobial infection.

In addition to BAK1, another *Arabidopsis* SERK protein, AtSERK4 (also named BAK1-like1, BKK1) plays a redundant role with BAK1 to mediate plant innate immunity and suppress plant cell death. However, compared with BKK1, BAK1 plays the major role in mediating plant defense response. Therefore, as we observed in *L. japonicus*, *Ljbak1* mutant plants showed a significant but not complete decrease in immune response induced by both rhizobia and flg22 treatment, suggesting that another protein(s), perhaps an *L. japonicus* BKK1 homolog, may also play a role in rhizobial infection.

SymRK has been identified as an essential component involved in the RNS. SymRK was proposed to form a protein complex with

LjNFR5 (Antolín-Llovera et al., 2014b), one of the NF receptors, to transduce symbiotic signaling. However, rhizobia, as foreign microbes, were observed to activate plant immunity with the involvement of LjBAK1 during the early infection and colonization stages in plant cells. Therefore, suppression of immunity during rhizobial infection is important for the establishment of symbiosis. Indeed, different groups have found that NF has a role in the suppression of plant immune responses (Feng et al., 2019; Liang et al., 2013; Rey et al., 2019; Shaw and Long, 2003); however, the SymRK homolog DMI2 does not appear to be required for suppression of the immunity mediated by NF in *M. truncatula* (Feng et al., 2019; Rey et al., 2019; Shaw and Long, 2003). The finding that SymRK has another, apparently NF-independent, role in the suppression of plant innate immunity during rhizobial infection provides a new clue as to how plants can actively suppress the immune response activated during rhizobial infection. Because the *nodC*$^{-}$ rhizobial strain with impaired NF biosynthesis also suppresses immunity in *L. japonicus*, other rhizobial molecules besides NF may exist to suppress immunity, and SymRK may be involved in this pathway. How SymRK is activated to reduce BAK1 phosphorylation is of great interest for future research.

The intimate associations between plants and environmental microbes lead to the formation of different interactions, such as pathogenesis, commensalism, and mutualism. Numerous publications have made clear that pathogenesis includes multiple layers of alternate regulations that activate or suppress plant innate immunity. Variations in MAMPs, e.g., the flg22 epitope, and the suppression of plant immunity mediated by the type two secretion system seem to be prerequisites for the association of commensal symbionts with plants (Hacquard et al., 2017; Teixeira, et al., 2021). For example, as mutualistic symbionts, rhizobia have both uncharacterized MAMP(s) that trigger weak immunity in their host cells (Lopez-Gomez et al., 2011) and other molecules, such as NFs, that suppress plant innate immunity to allow successful infection and colonization of symbiotic cells. A rhizobial strain that is unable to produce NFs still has the ability to suppress weak immunity, suggesting that other molecules may be involved in this process. BAK1 was shown to be a universal coreceptor for multiple PRRs to transduce immune responses in plants, and it is therefore important for early interactions during rhizobial infection. The direct targeting of LjBAK1 and suppression of its kinase activity by SymRK may serve as a physiological switch by which rhizobia balance immunity and symbiosis to make successful infections. Because the interaction between SymRK and LjBAK1 is induced by rhizobial inoculation, the suppression of LjBAK1 seems to be actively mediated by rhizobia during their symbiotic interaction with host cells. The suppressive function of SymRK is not only limited to roots but also occurs in leaves, suggesting that the functions of SymRK in symbiosis and immune suppression may be differentially regulated. The fact that Medicago DMI2 (a SymRK homolog) protein abundance is enhanced after rhizobial inoculation in an NF-independent manner supports this hypothesis. Therefore, the mechanism by which SymRK is activated to suppress immunity and the exact role of LjBAK1 in the immune response to rhizobia will require further clarification. However, given that BAK1 serves as a coreceptor for multiple MAMP receptors, the most likely scenario is that LjBAK1 acts in conjunction with legume receptor(s) involved in the recognition of rhizobial MAMPs.

In addition to being an essential molecule for mediating most symbiotic interactions, rhizobial NF has a role in the suppression of plant immunity to favor rhizobial infection (Feng et al., 2019; Rey et al., 2019; Shaw and Long, 2003). The present study shows that SymRK-mediated suppression of plant immunity is not dependent on rhizobial NF, suggesting that rhizobia may possess other uncharacterized molecules with which to suppress immunity and that SymRK is also involved. If this were the case, SymRK would have a dual function, mediating the NF-signaling pathway and suppressing immunity during rhizobial infection.

In summary, the data presented here provide a mechanism by which rhizobia can suppress host immunity during the early infection process. Moreover, the data provide at least a partial mechanism for the activity of SymRK during symbiosis and, most importantly, reveal an interesting intersection between symbiotic and defense signaling.

MATERIALS AND METHODS

Plant material and growth conditions

Wild-type *A. thaliana* (Ws-2, Col-0), *bri1-5* and *bak1-4* mutant plants, and transgenic *bri1-5* and *bak1-4* plants expressing LjBAK1 were grown in a growth chamber at 23°C under a 16-h light/8-h dark photoperiod. Wild-type plants of *L. japonicus* (Handberg and Stougaard, 1992) '*Miyako jima* MG-20' and loss-of-function homozygous *ljbak1* mutants (*ljbak1-1* and *ljbak1-2*) were used for phenotype analysis. Seeds were scarified by immersion in H_2SO_4 for 8 min before surface sterilization in 1% NaClO supplemented with 0.1% Tween-20 for 20 min. Seeds were plated on 1/2 MS medium supplemented with 0.8% agar for germination at 28°C in the dark for 2 days, then transferred to a growth chamber with a 16-light/8-h dark cycle at 23°C. Rhizobial strain *M. loti* MAFF303099, *M. loti* MAFF303099 expressing GFP, and *M. loti* MAFF303099 *nodc*$^{-}$ were used in this study. Liquid culture of *M. loti* (OD_{600} = 1.0) grown in Tryptone Yeast medium was pelleted by centrifugation and resuspended in a half-strength Broughton and Dilworth (B&D) nitrogen-free medium supplemented with 0.5 mM KNO_3. Four plant seedlings were grown in one pot and inoculated with 50 mL (OD_{600} = 0.02) rhizobial strain *M. loti*. *Agrobacterium rhizogenes* strain LBA1334 was used for hairy root transformation. *L. japonicus* ecotype Gifu-129 mutant seeds (30010361) were provided by the Center for Carbohydrate Recognition and Signaling (https://lotus.au.dk/). Homozygous mutants were genotyped using a PCR-based screening approach (Małolepszy et al., 2016).

Vector construction

Primers LjBAK1-F and LjBAK1-R (Supplemental Table 1) were used to amplify the coding region of *LjBAK1* (KY131980.1) from cDNA of *L. japonicus*. The purified PCR product was cloned into the pEASY-Blunt cloning vector (TransGen Biotech, Beijing, China) and confirmed by sequencing. To generate the constructs for yeast two hybrid assays, the CDs of LjBAK1, MtBAK1, MtDMI2, OsBAK1, and OsSymRK were cloned into pGADT7 or pGBKT7 using the Gibson cloning method (Gibson et al., 2009). The full-length coding sequence of *SymRK* without the stop codon was cloned into the pUB1301-HA plasmid between the *Stu*I/*Xba*I sites to express SymRK-HA in plants. For expression of LjBAK1-FLAG protein, the *L. japonicus ubiquitin (Ub)* promoter and a 2X (G4S) linker were amplified and used to replace the 35S promoter in the p35S-GFP-FLAG plasmid at the *Kpn*I sites to generate pUb-GFP-FLAG. The full-length coding sequence of LjBAK1 without the stop codon was then cloned into pUb-GFP-FLAG between the *Stu*I/*Xba*I sites to express

LjBAK1-FLAG in plants. These constructs were introduced into *Agrobacterium tumefaciens* EHA105 and *Agrobacterium rhizogenes* strain LBA1334 for hand-infiltration in *N. benthamiana* and hairy root transformation in *L. japonicus*, respectively. For the complementation assay in the *symrk-409* null mutant, the full length of *SymRK* was cloned into the pUb-GFP vector between the *Stu*I/*Xba*I sites to express *SymRK* under the control of the *L. japonicus ubiquitin (Ub)* promoter. For *in vitro* kinase assays, the CD of SymRK was cloned into pMAL-c2X for expression of MBP-SymRK-CD in *Escherichia coli* based on a previously described protocol (Chen et al., 2012). The CD of LjBAK1 was amplified and inserted into pET28a at the *Eco*RI/*Xho*I sites for the expression of His-tagged LjBAK1-CD recombinant protein. The CRISPR–Cas9 system was used to knock out *LjBAK1* in *L. japonicus* as previously described (Wang et al., 2016). The web tool CRISPR-P 1.0 (http://cbi.hzau.edu.cn/cgi-bin/CRISPR) (Liu et al., 2017) was used to identify two specific single-gRNA sequences targeting *LjBAK1*. Two pairs of gRNA oligonucleotides were synthesized and cloned into the *Bbs*I-digested pBlueScript SK(+)-LjU6 vector. The resulting plasmid pBlueScript SK (+)-LjU6-sgRNA was digested with *Kpn*I and *Xba*I and ligated into pCAMBIA1300-sGFP-2X35s-Cas9 for hairy root transformation in *L. japonicus*. The gRNAs with high mutation efficiency detected in hairy roots were chosen for the generation of stable transgenic *L. japonicus* to create *ljbak1* mutant plants. All primers used in this study are listed in Supplemental Table 1.

Coimmunoprecipitation analysis

HA-tagged SymRK, LjNFR1, and LjNFR5 were expressed in the roots of *L. japonicus* plants expressing LjBAK1-FLAG. Crude proteins were extracted from each transgenic root in immunoprecipitation buffer (25 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 10 mM EDTA, 1 mM DTT) with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) at a proportion of 1:1 (weight: volume). Samples were incubated on ice for 40 min and centrifuged three times at 12,000 × *g* for 10 min at 4°C. For coimmunoprecipitation assays, the supernatant was incubated with 1:100 diluted anti-FLAG antibody (Sigma-Aldrich) for 2 h at 4°C with gentle rotation. After that, 50 μL of protein A/G magnetic beads (Genscript, Nanjing, China) were added to the supernatants and incubated overnight at 4°C with gentle rotation followed by magnetic separation. The beads were washed six times with washing buffer (25 mM HEPES [pH 7.5], 150 mM NaCl). The beads were eluted in 100 μL of 1X SDS loading buffer and boiled for 8 min at 100°C. The supernatant was separated by 8% SDS-PAGE followed by western blot analysis using horseradish peroxidase (HRP)-conjugated anti-HA (Sigma-Aldrich) and anti-FLAG antibodies. The coimmunoprecipitation analyses were performed with three biological replicates.

Yeast two-hybrid assay

To test the interactions between LjBAK1 and SymRK, the coding sequences of the intracellular domains of SymRK, LjNFR1, LjNFR5, and LjBAK1 were cloned into pGBKT7 (for expression of binding domain-fused protein) and pGADT7 (for expression of activation domain-fused protein) vectors. The BD and AD plasmids were transformed into the yeast strains Y187 and AH109, respectively. After mating, yeast cells were spread onto SD/-2 synthetic dropout medium (-Trp/-Leu) and SD/-4 selective medium (-Trp/-Leu/-His/-Ade) and incubated at 28°C for an additional 4 to 5 d. Each experiment was performed with at least three biological replicates.

MAPK3/6 phosphorylation assay

L. japonicus seeds were scarified in H_2SO_4 for 8 min, followed by surface sterilization with 2% NaClO supplemented with 0.1% Tween-20 for 20 min. Seeds were stratified at 4°C in the dark for 3 d and then plated onto ½ MS medium supplemented with 0.8% agar for 36 h of growth at 22°C. Seedlings were then transferred to ½ B&D medium (1.2% [w/v] agarose, pH 5.8) for root elongation in the dark in a greenhouse for an additional 10 d. The roots from 2-week-old *L. japonicus* plants were floated in H_2O overnight at room temperature. For MAPK3/6 phosphorylation assays, flg22 treatment was performed for 15 min, and *M. loti pretreatment was performed for* 12 h. For each treatment, about 100 mg of root tissue was pretreated with or without *M. loti* (OD_{600} ~0.5) before treatment with flg22. Crude protein was extracted from roots in a buffer that contained 50 mM 2-amino-2-(hydroxyl methyl)-1,3-propanediol (Tris)-HCl (pH 7.5), 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 4 M urea, and protease inhibitor cocktail Complete Mini tablets (Roche). MAPK activation was monitored by western blotting with anti-P44/P42 antibody that recognized the dual phosphorylation of the MAPK activation loop (Cell Signaling Technology, Danvers, MA). Membranes were stained with Ponceau dye or probed with anti-actin antibodies to verify equal loading. All experiments were performed with at least three independent biological replicates.

ROS measurement

Roots from 1-week-old *L. japonicus* seedlings were cut into 0.5-cm pieces and floated in H_2O with or without added *M. loti* (OD_{600} = ~0.2) suspension for 12 h in a 96-well polystyrene plate (Greiner, Kremsmunster, Austria). For the ROS assay, a reaction mixture containing 2.5 μM L-012 (Wako Chemicals), 5 μg/mL HRP (Sigma-Aldrich), and different concentrations of flg22 was added to each well for 30 min. Luminescence signals were monitored using a Mithras LB 940 Multimode Microplate Reader (Berthold, Germany). For each experiment, at least six technical replicates and three biological replicates were performed for each treatment. Data analyses and visualization were performed with GraphPad Prism software (5.01 version). ROS measurements were performed with at least three independent biological replicates.

Phylogenetic analysis

Phylogenetic analysis was performed using MEGA6 software (Tamura et al., 2013). Full-length protein sequences were aligned with Clustal X2 (Larkin et al., 2007). Maximum likelihood phylogeny estimation was performed using the Jones-Taylor-Thornton matrix-based model with discrete Gamma distribution (+G, five categories) and 1,000 bootstrap replicates. Sequence information can be found at GenBank under the following accession numbers: AtSERK1 (At1g71830), AtSERK2 (At1g34210), AtSERK3 (At4g33430), AtSERK4 (At2g13790), AtSERK5 (At2g13800), MtSERK1 (AY162176.1), MtSERK2 (HM640001.1), MtSERK3 (HM640008.1), MtSERK4 (HM640002.1), MtSERK5 (HM640003.1), and LjBAK1 (KY131980.1 in NCBI, LotjaGi6g1v0354800 in Gifu v1.2, Lj6g0001211.1 in *L. japonicus* MG20 genome (Li et al., 2020), and chr6.CM0314.410.r2.m in miyakogusa v2.5).

Real-time PCR

Total RNA was isolated from roots or leaves using TRIzol reagent (Invitrogen, Carlsbad, CA). The PrimeScript Real-Time Reagent kit (Takara, Kusatsu, Japan) was used to remove genomic DNA before first strand cDNA synthesis using oligo(dT) as a primer. Real-time quantitative reverse PCR (qPCR) was performed using the SYBR Select Master Mix reagent (ABI, Waltham, MA). All PCR reactions were performed using an ABI ViiA 7 Real-Time PCR System under the standard cycling mode. Transcript levels were analyzed and normalized using both *Actin* and *Ubiquitin* genes, which are constitutively expressed in all plant tissues. At least three biological replicates and three technical replicates were performed for each experiment. Primers used for qPCR are listed in Supplemental Table 1.

Recombinant protein purification

To purify recombinant proteins, *E. coli* BL21 (DE3) cells were transformed with plasmids containing different genes. *E. coli* cells were grown in Luria-Bertani (LB) medium to an OD600 value of ~0.6 before the addition of 0.3 mM isopropyl-D-1-thiogalactopyranoside (IPTG) for protein expression. Bacteria were collected and resuspended in buffer containing 20 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA, and 5% [v/v] glycerol (pH 7.4). His-tagged proteins were purified using nickel-agarose beads (GenScript, Nanjing, China) under native conditions and eluted with a buffer solution containing 200 mM imidazole. MBP fusion proteins were

purified using Amylose resin (New England Biolabs, Ipswich, MA), and GST fusion proteins were purified using Glutathione Resin (GenScript). Purified proteins were desalted using Millipore Amicon Ultra-15 and stored at −80°C for further analyses.

In vitro kinase assay

For protein kinase assays, about 0.25 μg of proteins and 1 μg of substrate protein were incubated in a buffer containing 20 mM HEPES (pH 7.4), 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 10 μM ATP, and 5 μCi of [γ-^{32}P] ATP at 26°C for 15 min. The reaction was terminated after the addition of 5× SDS loading buffer followed by boiling for 5 min. Protein samples were separated directly on an SDS-PAGE gel and detected by autoradiography using a phosphor screen and a FUJI BAS-2500 image analyzer (Fujifilm, Tokyo, Japan). The exact same amount of protein for each sample was separately loaded on another SDS-PAGE gel for Coomassie Blue staining and used as a loading control. All experiments were performed with at least three biological replicates.

In vivo phosphorylation assay

For detection of the *in vivo* phosphorylation status of LjBAK1, stable transgenic plants or transgenic roots expressing LjBAK1-3XFLAG tags under the control of the 35S promoter were generated in wild-type and *symrk-409* mutant *L. japonicus* (Gifu or MG20). After inoculation with or without *M. loti* for 12 h, root tissues from transgenic plants were treated with 10 nM flg22 for 15 min. Crude proteins from these samples were extracted using an immunoprecipitation buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 10 mM EDTA, 1 mM DTT, and protease inhibitor cocktail (Sigma-Aldrich) and immunoprecipitated with anti-FLAG agarose (Sigma-Aldrich) for 5 h at 4°C, followed by washing five times with washing buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM EDTA, and 0.5% (v/v) Triton X-100. The eluted samples were detected using anti-FLAG and anti-Phospho Ser/Thr/Tyr antibodies (Abcam, Cambridge, UK). The immunoblot analyses were performed with three biological replicates.

Arabidopsis transformation assay

All binary vectors were electroporated into *A. tumefaciens* EHA105 and transformed into wild-type *A. thaliana* (Col-0) or *bak1-4* mutant plants using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on 1/2 MS medium with 25 mg/L hygromycin after seed surface sterilization.

Primer sequences

All primer sequences used in this study are listed in Supplemental Table 1.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

Y.F., G.S., Z.Z., and Y.C. conceived the idea and designed the experiments. Y.F., C.L., P.W., L.P., T.W., C.W., Q.T., B.L., and Y.O. performed the experiments. H.Z., S.Y., and R.H. provided reagents and suggestions for research. Y.F., C.L., P.W., G.S., Z.Z., and Y.C. analyzed the data. Y.F., G.S., Z.Z., and Y.C. wrote the article.

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