Mitogen-activated protein kinase (MAPK) signaling cascades play critical roles in various cellular events in plants, including stress responses, innate immunity, hormone signaling, and cell specificity. MAPK-mediated stress signaling is also known to negatively regulate nitrogen-fixing symbiotic interactions, but the molecular mechanism of the MAPK signaling cascades underlying the symbiotic nodule development remains largely unknown. We show that the MtMKK5-MtMPK3/6 signaling module negatively regulates the early symbiotic nodule formation, probably upstream of ERN1 (ERF Required for Nodulation 1) and NSP1 (Nod factor Signaling Pathway 1) in Medicago truncatula. The overexpression of MtMKK5 stimulated stress and defense signaling pathways but also reduced nodule formation in M. truncatula roots. Conversely, a MAPK specific inhibitor, U0126, enhanced nodule formation and the expression of an early nodulation marker gene, MtNIN. We found that MtMKK5 directly activates MtMPK3/6 by phosphorylating the TEY motif within the activation loop and that the MtMPK3/6 proteins physically interact with the early nodulation-related transcription factors ERN1 and NSP1. These data suggest that the stress signaling-mediated MtMKK5/MtMPK3/6 module suppresses symbiotic nodule development via the action of early nodulation transcription factors.

Keywords: nitrogen fixation, legume, MAPK, signal transduction, symbiosis

INTRODUCTION

The nitrogen-fixing symbiotic interaction between leguminous plants and soil bacteria, collectively known as rhizobia, has essential roles in both natural and agricultural systems. The nitrogen fixing nodule symbiosis has been specifically adopted by a few evolutionarily related plant families, including the legumes (Geurts et al., 2016; Madsen et al., 2003; Oldroyd et al., 2011). This symbiosis is characterized by the formation of a new root lateral organ, the nodule, which provides an optimal environment for symbiotic nitrogen fixation by specific rhizobia (Madsen et al., 2010; Oldroyd, 2013; Oldroyd et al., 2011). Recent progress in understanding molecular mechanisms underlying symbiotic interactions between legume and rhizobia have revealed host plant signaling components involved in the perception of bacteria-driven signaling molecules, known as the Nod factors (Crespi and Frugier, 2008; Perret et al., 2000; Remigi et al., 2016). The characterization of nodulation-defective mutants in model legume plants such as M. truncatula and the cloning of genes encoding nodulation signaling components suggest that the signaling pathways for nitrogen fixing symbiosis are evolutionarily conserved in legumes and even in nitrogen fixing symbiotic non-legumes (Ane et al., 2004; Capoen and Oldroyd, 2008; Gherbi et al., 2008; Searle et al., 2003; Smit et al., 2005). The symbiotic nitrogen-fixing nodulation
is initiated by the recognition of rhizobia-secreted NF signals by host legumes, depending on a receptor complex containing MtLYK3/NFP (LysM-receptor like Kinase 3/Nod Factor Perception; Geurts et al., 2016; Madsen et al., 2003; Oldroyd et al., 2011). These events rapidly induce nuclear Ca\(^{2+}\) spiking, subsequently stimulating MTDNI3 (Does Not Make Infections 3, a Ca\(^{2+}\)-calmodulin-dependent kinase), and activate transcription factors including NSP1/NSP2 (GRAS family) and ERN1 (AP2/ERF family; Gleason et al., 2006; Smit et al., 2005; Soyano and Hayashi, 2014).

The number of nodules formed on the root system is controlled by a combination of positive and negative regulatory pathways. Among the positive pathways, cytokinin and auxin are critical for nodule organogenesis (Frugier et al., 2008; Suzuki et al., 2013). In contrast, several hormones linked to stress and defense responses, including salicylic acid (SA), jasmonate (JA), abscisic acid (ABA) and ethylene, negatively control NF signaling and infections by symbiotic rhizobia (Ryu et al., 2012). In addition, a systemic regulation of nodulation, which depends on peptides/receptor kinases is also involved (Oldroyd, 2013). Altogether, these regulatory mechanisms allow either local or systemic controls to determine the optimal nodule development in relation to the environmental conditions, i.e. nitrogen availability in soil and the plant ability to provide carbon molecules for assimilation of the fixed nitrogen.

In plants, MAP kinase signaling cascades are involved in diverse abiotic and biotic stress responses, and these stresses negatively affect the symbiotic nodule formation (Lopez-Gomez et al., 2012; Ryu et al., 2012). Conversely, symbiotic rhizobia infections induce the activation of MAPK signaling cascades and defense/stress related responses early during infection (Lopez-Gomez et al., 2012). However, molecular mechanisms associated to MAPK action in nitrogen fixing symbiotic interactions are still elusive. In this study, we show that the MKK5-MPK3/6 signaling cascade negatively regulates nitrogen fixing nodule formation in *M. truncatula* probably upstream of the NSP1/ERN1-dependent signaling pathway.

**MATERIALS AND METHODS**

**Plant materials and nodulation assays**

The *Medicago truncatula* cv Jemalong A17 was used as a wild-type control and as the genetic background for transient *Agrobacterium rhizogenes*-mediated root transformation. *M. truncatula* seeds were treated with concentrated sulfuric acid for 5 min with gentle agitation and washed with sterile water. The seeds were further sterilized with sodium hypochlorite for 2 min. The surface-sterilized seeds were then placed on inverted agar plates in the dark for 2 days at 4°C and germinated for 2 days at 23°C. The germinated seedlings were transferred onto Fahraeus agar plates (1 mM NH\(_4\)NO\(_3\)) for 2 weeks and were starved of nitrogen for 1 week, unless otherwise stated, by transferring them to Fahraeus agar plates lacking a nitrogen source, as described in the Medicago handbook (http://www.noble.org/MedicagoHandbook).

For nodulation experiments, nitrogen-starved *M. truncatula* seedlings were inoculated with 200 ml of the *Sinorhizobium mellotii* A857M strain expressing an aminolevulinic acid synthetase-lacZ fusion. An OD\(_{600nm}\) = 0.02 suspension was used for inoculation, which was distributed without or with 0, 1, 5 and 10 mM of U0126 and placed for further 2 weeks at 23°C (light-dark photoperiod: 16 hours/8 hours).

**Plasmid construction and BIFC assays**

The full-length cDNAs of MtMKKS5, MtMPK3 and MtMPK6 were cloned into plant expression vectors containing hemagglutinin (HA), myc, GUS or FLAG tags, as well as the 35S-C4PPDK promoter (Hwang and Sheen, 2001). MtMKKS5a (T229E and S235E) and MtMKKS5in (K113M) variants were generated using the manufacturer’s instructions for the QuickChange Site-Directed Mutagenesis kit (Stratagene, USA). For transient root transformation, the MtMKKS5 cDNA was sub-cloned into the pBI121 vector (Clontech) and transformed into the ARqua1 *Agrobacterium rhizogenes* strain (Boisson-Dernier et al., 2001). For the GST-fused recombinant proteins, the MtMKKS5a, MtMPK3, MtMPK6 and MtMPK13 cDNAs were cloned into the pGEX 5X-1 vector. For the BIFC assay, 2 × 10\(^4\) *Arabidopsis thaliana* mesophyll protoplasts were typically transfected with 20 μg of total plasmid DNA (Hwang and Sheen, 2001). The transfected protoplasts were then incubated at 1 × 10\(^4\) cells per ml for 6 hours. All of the assays were carried out at least three times and a representative experiment is shown in figures.

**Real-time RT-PCR analysis**

To determine the expression levels of transcripts, total RNAs were isolated using the Trizol reagent (Invitrogen). Double strand cDNAs were synthesized from 1 μg of RNA with oligo dT primers and the ImProm-II reverse transcriptase (Promega). Gene-specific primers used in real-time RT-PCR are described in Supplementary Table 1.

**In vitro kinase and yeast two-hybrid assays**

For the *in vitro* kinase assay, 5 μg of GST-MtMPK was typically incubated with or without 5 μg of GST-MtMKKS5a in a kinase buffer [20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 12 mM MgCl\(_2\), 100 μM ATP, and 10 μCi of (γ\(^{32}\)P)ATP] for 1.5 hours at 23°C. To test the phosphorylation of MtERN1, the MtMKKS5a-activated GST-MtMPK6 was used to phosphorylate recombinant GST-MtERN1 and MtERN1\(^{593A}\) (1:20 enzyme/substrate ratio) in the same reaction buffer. The reactions were stopped by the addition of a SDS-sample buffer. After 30 min, proteins were subjected to a 10% SDS-PAGE and the phosphorylated proteins were visualized by autoradiography. For kinase assays in *M. truncatula* roots, protein extracts of salt stress-treated roots were run on 10% SDS-polyacrylamide electrophoresis gels embedded with 0.25 mg/ml of Myelin Basic Protein (MBP) in the separating gel as a substrate for the kinase. After electrophoresis, SDS was removed by washing three times the gel with a washing buffer [25 mM Tris-Cl (pH 7.5), 0.5 mM DTT, 5 mM NaF, 0.5 mg/ml BSA, 0.1% Triton X-100 (v/v)], each time for 30 min at room temperature. The kinases were allowed to re-associate in 25 mM Tris-Cl (pH 7.5), 1 mM DTT, 5 mM NaF at 4°C overnight with three changes of buffer. The gel was
then incubated in the reaction buffer [25 mM Tris-HCl (pH 7.5), 2 mM EGTA, 12 mM MgCl₂, 1 mM DTT, 0.1 mM Na₂VO₃] with 200 nM ATP and 10 μCi of [γ-³²P]ATP for 1 hour at 23°C. The reaction was stopped by transferring the gel into 5% trichloroacetic acid (w/v) and 1% NaPPi (w/v). The phosphorylated proteins were visualized by autoradiography. Protein size markers (Invitrogen) were used to determine the size of protein kinases.

For yeast two-hybrid assays, the yeast strain AH109 was transformed with pGBK7-MtMPKs and pGADT7-MtERN1, MtNSP1 or MtMKK5 using the Lithium Acetate method. The transformed yeasts were grown on a synthetic selective medium lacking Leu, Trp, and His but containing 3-aminotriazole, or a non-selective medium lacking Leu and Trp.

RESULTS

A MAPK signaling cascade negatively regulates symbiotic nodule development

To investigate the potential link between stress- and defense-induced MAPK signaling activation and nitrogen-fixing symbiotic nodule formation, we first determined the effects of a MAPKK specific inhibitor, U0126 (Yoo et al., 2008), on the formation of nitrogen-fixing nodules in M. truncatula. Nodule formation was gradually increased in a dosagel-dependent manner in response to U0126 1-5 μM, and root development was slightly impaired by the U0126 treatment (Figs. 1A, 2B, and Supplementary Fig. S1A). However, the positive effect of U0126 on symbiotic nodule formation was reduced at 10 μM, likely due to severe root growth defects (Fig. 1B and Supplementary Fig. S1A). U0126 did not change the rate of cell division of S. meliloti (Supplementary Fig. S1B). Because several biotic/abiotic stresses negatively affect symbiotic nodulation, and because these environmental conditions also rapidly activate MAPK signaling cascades in non-legume plants (Harnel et al., 2006; 2012; Tena et al., 2011), we tested whether the inhibition of nodulation by a salt stress is associated to a MtMPK3/MtMPK6 activation in M. truncatula. High salinity indeed reduced nodule formation, as expected, and also rapidly induced MtMPK3/MtMPK6 kinase activity (Fig. 1C and Supplementary Fig. S2A). Conversely, MAPK inhibition by U0126 compromised the inhibition of nodulation by salt stress (Fig. 1C). This indicates that stress-related MAP kinases, such as MtMPK3/MtMPK6, may be involved in the inhibition of nodulation in response to stress conditions. Accordingly, the rapid induction of a stress-related gene, MtWRKY46 (Lohar et al., 2006), after S. meliloti inoculation was significantly decreased in the presence of U0126 (Fig. 1D), suggesting a putative link between MAPK-mediated stress and nodulation responses. We next monitored the expression pattern of the early nodulation marker gene MtNIN, which encodes a transcription factor that is rapidly induced by rhizobial NFs (Schauer et al., 1999). M. truncatula roots were inoculated with S. meliloti in the presence or absence of U0126 and NaCl for 1, 6 and 12 hours. A gradual induction of MtNIN expression was observed after rhizobium application, and the U0126 treatment further induced MtNIN expression (Fig. 1E). A 12 hours salt stress reduced the activation of MtNIN expression in nodulated roots, and this inhibition was not observed after the MAPK inhibitory treatment (Supplementary Fig. S2B). This suggests that MAPK signaling cascades may negatively regulate the NF signaling pathway.

The MtMKK5 stress-related pathway plays a negative role in symbiotic nodulation

In the Arabidopsis model, AtMKK4/5 proteins rapidly stimulate specific downstream MAPKs in response to diverse biotic and abiotic stress conditions (Tena et al., 2001). In addition, the Medicago sativa ortholog of AtMKK4/5, SIMKK
A Negative Role for MAPK Signaling in Symbiotic Nodule Formation
Hojin Ryu et al.

Fig. 2. MtMKK5 negatively regulates the M. truncatula nodulation. (A) MtMKK5 enhances the expression of the FRK1 marker. Expression was determined using real time RT-PCR in Arabidopsis protoplasts transfected with the indicated genes (KKa, KK activated; KKin, KK inactivated). Error bars indicate Standard Deviation (SD; n = 2 biological replicates). (B, C) Ectopic expression of MtMKK5a reduces nodule formation. Three-week-old M. truncatula roots expressing p35S-GUS or p35S-MtMKK5a-HA were inoculated with S. meliloti for two weeks. Representative nodule phenotypes of the transgenic roots are shown in (B), and the number of nodules is shown in (C). Error bars indicate SE (n = 14), and a Student’s t-test was performed (**P < 0.01) to assess significant differences. (D) MtMKK5a activates the expression of a subset of stress-related MtWRKY genes. Expression levels were determined by real-time RT-PCR in roots expressing p35S-GUS or p35S-MtMKK5a-GUS constructs. Error bars indicate SE (n = 3).
A Negative Role for MAPK Signaling in Symbiotic Nodule Formation
Hojin Ryu et al.

Mol. Cells 2017; 40(1): 17-23  21

A                   B

C

Fig. 3. The M. truncatula MtMKK5 protein activates MtMPK6.
(A) Physical interaction between MtMKK5 and MtMPK3/6 in a yeast two-hybrid assay. The transformed yeasts were selected on a synthetic medium lacking Leu and Trp (-LT, upper panels) or lacking Leu, Trp and His (-LTH) and with 3-AT 5 mM (lower panels). (B) A Bimolecular Functional Complementation (BiFC) assay showed that the interaction between MtMKK5 and MtMPK3/6 occurs in the cytoplasm and nucleus. cVENUS (cV) tagged MtMKP3/6 were co-transfected with nVENUS (nV) tagged MtMKK5 into Arabidopsis mesophyll protoplasts. The complemented fluorescence of the VENUS reporter is observed in green, and the red fluorescence corresponds to the auto-fluorescence of chloroplasts. (C) MtMKK5 activates MtMPK6 in an in vitro kinase assay using the Myelin Basic Protein (MBP) as a substrate.

through phosphorylation of transcription factors (Hamel and Beaudoin, 2010; Rodriguez et al., 2010). Because MtMKK5 negatively regulates nodulation, and the stress-related MtMKK5-MtMPK3/6 cascade is conserved in M. truncatula roots, we tested if the MtMPK3/6 kinases could interact and phosphorylate transcription factors associated with NF signaling. Both MtMPK3 and MtMPK6 can physically interact with two early nodulation-related transcription factors, MtERN1 and MtNSP1 (Fig. 4A). These protein interactions were independently confirmed by a BiFC assay, which also showed that MtERN1 and MtNSP1 mainly associate with the MtMPK3/6 proteins in the nucleus (Fig. 4B). In addition, the MtMKK5-activated MtMPK6 can directly phosphorylate MtERN1, and this phosphorylation was abolished by a Serine to Alanine mutation of MtERN1 at position 93 (from the predicted ATG; Fig. 4C). These results suggest that the MtMKK5-MtMPK3/6 modules directly regulate at least MtERN1 during symbiotic nodule development.

DISCUSSION

It has been well established that abiotic and biotic stresses are major negative regulators of plant-microbe symbiotic interactions (Ding et al., 2008; Duzan et al., 2004; Lopez-Gomez et al., 2012). In this study, we have identified an inhibitory role of the defense/stress-activated MAPK signaling cascades in symbiotic nodule formation. Our results show that the stress-activated MKK5-MPK3/6 modules negatively regulate symbiotic interactions between M. truncatula and S. meliloti. Interestingly, MtMPK3 and MtMPK6 interacted with key early nodulation transcriptional regulators related to NF signaling, ERN1 and NSP1. This integrates MAPK cascades into the regulation of the early transcriptional events required for symbiotic recognition, rhizobial infection and/or nodule organogenesis. Our data additionally provide some clues, at the molecular level, about how environmental stresses and pathogens may negatively affect beneficial plant-microbe interactions.

The negative impacts of both abiotic and biotic stresses in symbiotic plant-microbe interactions have been well characterized at the physiological level (Lopez-Gomez et al., 2012; Ryu et al., 2012). However, the underlying molecular mechanisms are still unclear. Our data revealed that the abiotic stress-activated MAPK signaling cascades can affect the transcriptional regulation of early nodulation-related transcription factors (Supplementary Fig. S6). Although our data do not clearly define the biological roles of MAPK-mediated phosphorylation on the function of these nodule related transcription factors, data obtained in M. truncatula roots
clearly show a negative function for stress-activated MAPK signaling cascades in nodule formation. A previous study reported, in the *L. japonicus* model, a positive role of a MAPKK closely related to MtMKK5 in symbiotic nodulation, in contrast to our study (Chen et al., 2012). An interaction between the LjSymRK (Symbiosis Receptor Kinase, corresponding to DMI2 in *M. truncatula*) protein and LjSIP2, a MAPKK closely related to MtMKK5 (Supplementary Fig. S4), was additionally identified (Chen et al., 2012). We therefore tested the interaction between MtDM2 and MtMKK5 in a yeast two hybrid system, using similar conditions as in Chen et al., 2012 (Supplementary Fig. S7). No interaction could be detected in Chen et al. (2012) between the alfalfa proteins MsSIP and MsNORK (Nodulation Receptor Kinase, orthologous of MtDM2 and LjSymRK) using the same yeast two hybrid assay as was used for LjSymRK and LjSIP2. Therefore, these results suggest that different regulatory mechanisms may be controlled by MAPK signaling cascades depending on legume plants (*L. japonicus* vs *Medicago* sp.), leading to positive or negative effects on symbiotic nodulation. *Medicago* sp. plants have an indeterminate type nodulation, unlike *L. japonicus*, and the differential requirement of the MAPKK5/DM2-SymRK interaction may be associated to this developmental feature. More systematic studies on how the different molecular components of the NF signaling pathway integrate with MAPK signaling pathways are required to determine differences in the regulation of this signaling pathway by MAPKs across legumes and their nodulation-type diversity.

Several stress-related plant hormones, including ethylene, JA, SA, and ABA, negatively affect early symbiotic nodulation, and notably the calcium spiking essential for the NF signaling pathway (Ryu et al., 2012). Interestingly, these hormonal signals are also known activators of MAPK cascades in various plants (Rodriguez et al., 2010). These results suggest a model where stress-related hormones may negatively regulate early nodulation stages by activating MAPK signaling cascades. It is also possible that the well-known MAPK function in immune responses may be related to the inhibitory effect observed on symbiotic nodulation (Tena et al., 2001). Several studies have reported that symbiotic bacteria can activate defense-related MAPK signaling cascades to activate defense- and stress-response genes (Hamel and Beaudoin, 2010; Lopez-Gomez et al., 2012; Ryu et al., 2012). Interestingly, these responses were rapidly attenuated during early symbiotic nodule development (Lohar et al., 2006). Taken together, these results and our findings support a model where the MAPK signaling cascades, which are activated in host legume plants by stress or defense responses, can negatively affect symbiotic nodulation by modulating the NF signaling pathway (Supplementary Fig. S6).

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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