Molecules and Cells

MAP Kinase-Mediated Negative Regulation of Symbiotic Nodule Formation in *Medicago truncatula*

Hojin Ryu[1,2], Carole Laffont[3], Florian Frugier[3], and Ildoo Hwang[1,*]

[1]Department of Life Sciences, POSTECH Biotech Center, Pohang University of Science and Technology, Pohang 37673, Korea, [2]Department of Biology, Chungbuk National University, Cheongju 28644, Korea, [3]Institute of Plant Sciences-Paris-Saclay (IPS2), CNRS, INRA, Univ Paris-Sud, Univ Paris-Diderot, Univ d'Evry, Université Paris-Saclay, Bâtiment 630, 91190 Gif-sur-Yvette, France
*Correspondence: ihwang@postech.ac.kr
http://dx.doi.org/10.14348/molcells.2017.2211
www.molcells.org

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

Received 31 August, 2016; revised 22 December, 2016; accepted 27 December, 2016; published online 31 January, 2017

eISSN: 0219-1032

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 MtDMI3 (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; Suzaki 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℃ and germinated for 2 days at 23℃. The germinated seedlings were transferred onto Fahraeus agar plates (1 mM NH_4NO_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 meliloti* ABS7M 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 μM of U0126 and placed for further 2 weeks at 23℃ (light-dark photoperiod: 16 hours/8 hours).

Plasmid construction and BiFC assays

The full-length cDNAs of *MtMKK5, 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). *MtMKK5a* (T229E and S235E) and *MtMKK5in* (K113M) variants were generated using the manufacturer's instructions for the QuickChange Site-Directed Mutagenesis kit (Stratagene, USA). For transient root transformation, the *MtMKK5* 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 *MtMKK5a, 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-MtMKK5a in a kinase buffer [20 mM Tris-HCl (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℃. To test the phosphorylation of MtERN1, the MtMKK5a-activated GST-MtMPK6 was used to phosphorylate recombinant GST-MtERN1 and MtERN1^{S93A} (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-HCl (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 renature in 25 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM NaF at 4℃ 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 $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4) with 200 nM ATP and 10 μCi of (γ-^{32}P)ATP] for 1 hour at 23℃. 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 *pGBKT7-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 dosage-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 (Hamel 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 (Schauser 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

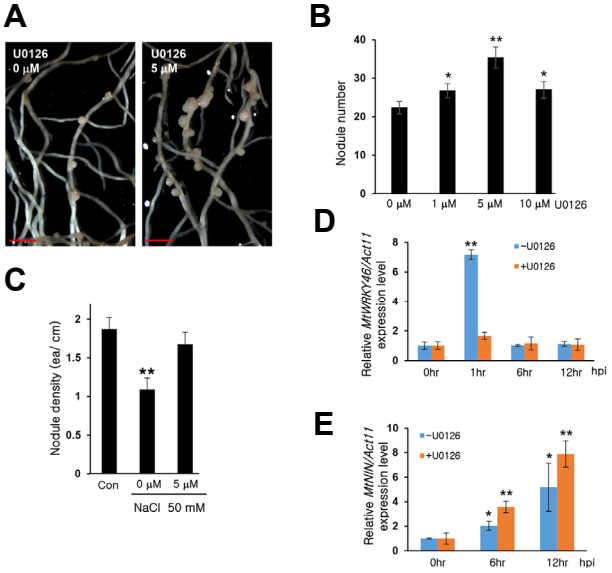

Fig. 1. MAPK signaling cascades negatively regulate symbiotic nodulation in *M. truncatula*. (A, B) A treatment with the MAPKK inhibitor U0126 increases symbiotic nodulation in *M. truncatula*. Nodule formation was analyzed on roots infected with *S. meliloti* for 3 weeks without or with U0126 5 μM, and representative pictures are shown (A). Nodule number of 3-week-old *M. truncatula* roots infected with *S. meliloti* was determined after 2 weeks without or with U0126 at 1, 5 or 10 μM (B). (C) A U0126 treatment suppresses the salt stress-mediated inhibition of nodulation. The nodulation assay was performed with *S. meliloti*, without or with U0126 5 μM, and in the presence of 50 mM NaCl. The nodule number of 10-day-old *M. truncatula* roots infected with *S. meliloti* was determined after 2 weeks. (D, E) A Rhizobium inoculation rapidly activates the expression of the defense-related *MtWRKY46* gene depending on a MAPK signaling cascade (D) and the inhibition of MAPK signaling by U0126 enhances the expression of the nodule specific marker gene *MtNIN* (E). After an incubation for 1 hour without or with U0126 5 μM, *M. truncatula* roots were inoculated with *S. meliloti* and collected 6 to 12 hours post inoculation (hpi). The expression levels of *MtWRKY46* (D) and *MtNIN* (E) were determined using real time RT-PCR (n = 3 biological replicates). Error bars indicate Standard Errors (SE; n > 10 plants per biological replicate), and Student's *t*-test were performed (*$P < 0.05$; **$P < 0.01$) to assess significant differences.

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

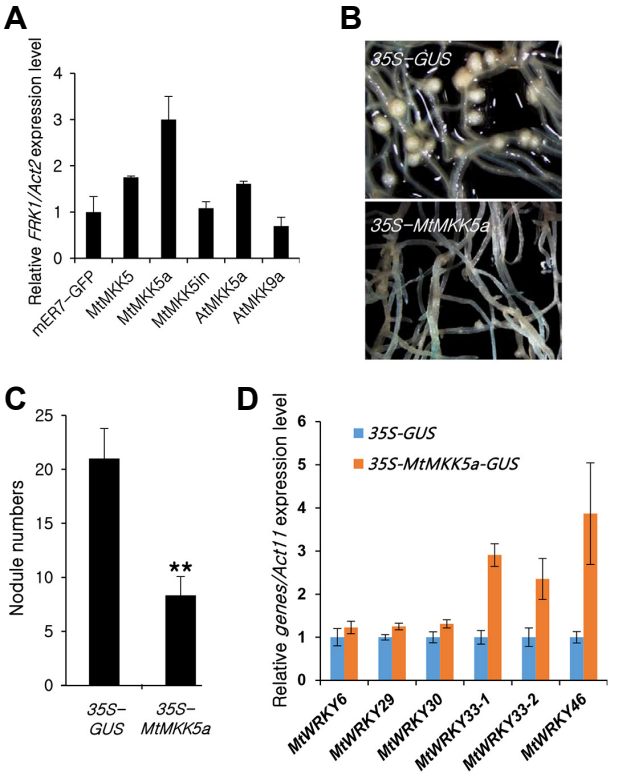

Fig. 2. MtMKK5 negatively regulates the *M. truncatula* nodulation. (A) MtMKK5 enhances the expression of the *FRK1* marker. Expression was determined using real time RT-PCR in *Arabidopsis* protoplasts transfected with the indicated genes (KKa, KK activated; KKin, KK inactivated). Error bars indicate Standard Deviations (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).

(Stress-Induced MAPKK), acts as a positive regulator of salt stress responses via its activation in MAPK signaling cascades (Rodriguez et al., 2010). This suggests that the previously observed negative effects of MAPK signaling cascades in *M. truncatula* nitrogen-fixing symbiotic nodulation may be linked to orthologues of SIMKK. To test this, we first identified the MAPKK protein which is most closely related to SIMKK and AtMKK5 in the *M. truncatula* genome, which was therefore named MtMKK5. The MtMKK5 protein shares 69.2, 72 and 95% identity with AtMKK4, AtMKK5 and SIMKK, respectively (Supplementary Figs. S3 and S4), suggesting that MtMKK5 might have similar functions to AtMKK4/5 and SIMKK. To test this, we first determined whether MtMKK5 could enhance the expression of an *Arabidopsis* AtMKK4/5-induced gene, *FRK1* (Tena et al., 2001, Fig. 2A) in *Arabidopsis* mesophyll protoplasts. MtMKK5 indeed induced the expression of the *FRK1* MKK4/5 pathway marker to a higher level than the induction provoked by a constitutively active AtMKK4 protein. Furthermore, a constitutively activated variant of MtMKK5, called MtMKK5a (for "activated"), induced even more strongly *FRK1* expression; conversely, a "kinase dead" MtMKK5 version, called MtMKK5in (for "inactivated"), did not affect *FRK1* expression. These results suggest that the MtMKK5 function in MAPK stress signaling cascades is at least partially conserved between *Arabidopsis* and *M. truncatula*.

To test *in vivo* the role of *MtMKK5*-activated downstream signaling pathways in symbiotic nodulation, we ectopically expressed the *MtMKK5a* variant in *M. truncatula* roots. Nodule formation was significantly reduced by *MtMKK5a* overexpression (Figs. 2B, 2C and Supplementary Fig. S5A). In addition, the expression of stress-related *MtWRKY* genes, such as *MtWRKY33-1, MtWRKY33-2* and *MtWRKY46*, was increased in *MtMKK5a* overexpressing *M. truncatula* roots compared to control roots, even in the absence of stress (Fig. 2D). Taken together, these results suggest that MtMKK5-induced MAPK signaling cascades negatively regulate symbiotic nodule formation.

The *M. truncatula* MtMKK5 kinase activates MtMPK3/6

Under stress conditions, the *M. sativa* SIMKK protein and as well as homologous MAPKK proteins in other plants activate MPK3 and MPK6 by a direct phosphorylation of the TEY motif, thereby stimulating downstream stress responses (Tena et al., 2001). To test whether the activation of MtMPK3 and/or MtMPK6 is involved in the MtMKK5-dependent inhibition of symbiotic nodulation, we next determined whether MtMKK5 interacts with MtMPK3/6 using yeast two-hybrid (Y2H) and Bimolecular Fluorescence Complementation (BiFC) assays (Figs. 3A and 3B). Both MtMPK3 and MtMPK6 interacted with MtMKK5 in yeast cells (Fig. 3A). This interaction was independently confirmed using a BiFC assay, which also revealed that the interaction mainly occurred in the cytoplasm and nucleus (Fig. 3B). An in-gel kinase assay was then carried out to determine if MtMKK5a induces MtMPK3 and/or MtMPK6 kinase activity, using Myelin Basic Protein (MBP) as a typical substrate to test for MPK activity (Tena et al., 2001; Fig. 3C and Supplementary Fig. S5B). MBP was strongly phosphorylated by the MtMKK5a-activated MtMPK6, in contrast to MtMPK13 which was used as a negative control (Fig. 3C). Surprisingly, the MBP-kinase activity of MtMPK3 was very weak (Supplementary Fig. S5, red arrow), suggesting that MtMPK6 might play a more important role than MtMPK3 in MtMKK5-activated stress signaling pathways. This idea was further supported by the observation that a stronger activation of MtMPK6 (46 KDa molecular mass), compared to MtMPK3 (44 KDa), was also identified in *M. truncatula* roots in response to a salt stress (Supplementary Fig. S2B).

The MtMKK5-MtMPK3/6 module can phosphorylate transcription factors essential for early symbiotic nodulation

Activated MPKs usually directly modulate signaling pathways

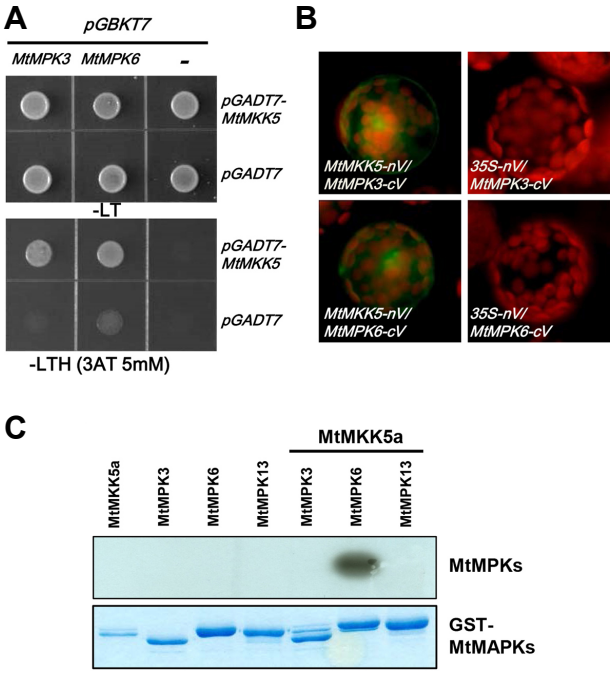

Fig. 3. The M. truncatula MtMKK5 protein activates MtMPK6. (A) Physical interaction between MtMKK5 and MtMPK3/6 in a yeast two-hybrid assay. The transformed yeasts were selected on a synthetic medium lacking Leu and Trp (-LT, upper panels) or lacking Leu, Trp and His (-LTH) and with 3-AT 5 mM (lower 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 *MtMPK3/6* were co-transfected with nVENUS (nV) tagged *MtMKK5* into *Arabidopsis* mesophyll protoplasts. The complemented florescence 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.

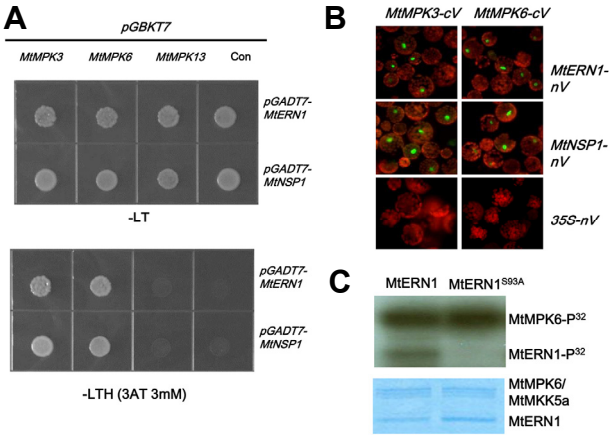

Fig. 4. The MtMKK5-MtMPK3/6 module interacts with early nodulation signaling factors and can phosphorylate MtERN1. (A) MtERN1 and MtNSP1 interact with MtMPK3 and MtMPK6, but not with MtMPK13, in a yeast two-hybrid assay. The transformed yeasts were selected on a synthetic medium lacking Leu and Trp (-LT, upper panels) or lacking Leu, Trp and His (-LTH) and with 3-AT 3 mM (lower panels). (B) MtERN1 and MtNSP1 interact in the nucleus with MtMPK3 and MtMPK6 in a Bimolecular Functional Complementation (BiFC) assay. (C) Phosphorylation of MtERN1 in an *in vitro* kinase assay at the residue Serine^{93} by the MtMPKK5-activated MtMPK6 protein.

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 MtKKK5-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 symbiont 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 MtDMI2 and MtMKK5 in a yeast two hybrid system, using similar conditions as in Chen et al., 2012 (Supplementary Fig. S7). No interaction could be identified, in agreement with the very weak interaction detected in Chen et al. (2012) between the alfalfa proteins MsSIP and MsNORK (Nodulation Receptor Kinase, orthologous of MtDMI2 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/DMI2-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).

ACKNOWLEDGMENTS

This work was supported by a grant to I. H. from the Cooperative Research Program for Agriculture Science & Technolgy Development (PJ010953022016), and a grant to H. R. from Basic Science Research Program through the National Research Foundation of Korea (2015R1A4A1041869). The F.F. laboratory was supported by the CNRS.

REFERENCES

Ane, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Oldroyd, G.E., Ayax, C., Levy, J., Debelle, F., Baek, J.M., Kalo, P., et al. (2004). *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. Science *303*, 1364-1367.

Capoen, W., and Oldroyd, G. (2008). How CYCLOPS keeps an eye on plant symbiosis. Proc. Natl. Acad. Sci. USA *105*, 20053-20054.

Chen, T., Zhu, H., Ke, D., Cai, K., Wang, C., Gou, H., Hong, Z., and Zhang, Z. (2012). A MAP kinase kinase interacts with SymRK and regulates nodule organogenesis in *Lotus japonicus*. Plant Cell *24*, 823-838.

Crespi, M., and Frugier, F. (2008). De novo organ formation from differentiated cells: root nodule organogenesis. Sci. Signal. *1*, re11.

Ding, Y., Kalo, P., Yendrek, C., Sun, J., Liang, Y., Marsh, J.F., Harris, J.M., and Oldroyd, G.E. (2008). Abscisic acid coordinates Nod factor and cytokinin signaling during the regulation of nodulation in *Medicago truncatula*. Plant Cell *20*, 2681-2695.

Duzan, H.M., Zhou, X., Souleimanov, A., and Smith, D.L. (2004). Perception of Bradyrhizobium japonicum Nod factor by soybean [*Glycine max (L.)* Merr.] root hairs under abiotic stress conditions. J. Exp. Bot. *55*, 2641-2646.

Geurts, R., Xiao, T.T., and Reinhold-Hurek, B. (2016). What does it take to evolve a nitrogen-fixing endosymbiosis? Trends Plant Sci. *21*, 199-208.

Gherbi, H., Markmann, K., Svistoonoff, S., Estevan, J., Autran, D., Giczey, G., Auguy, F., Peret, B., Laplaze, L., Franche, C., et al. (2008). SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and *Frankiabacteria*. Proc. Natl. Acad. Sci. USA *105*, 4928-4932.

Gleason, C., Chaudhuri, S., Yang, T., Munoz, A., Poovaiah, B.W., and Oldroyd, G.E. (2006). Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. Nature *441*, 1149-1152.

Hamel, L.P., and Beaudoin, N. (2010). Chitooligosaccharide sensing and downstream signaling: contrasted outcomes in pathogenic and beneficial plant-microbe interactions. Planta *232*, 787-806.

Hamel, L.P., Nicole, M.C., Sritubtim, S., Morency, M.J., Ellis, M., Ehlting, J., Beaudoin, N., Barbazuk, B., Klessig, D., Lee, J., et al. (2006). Ancient signals: comparative genomics of plant MAPK and MAPKK gene families. Trends Plant Sci. *11*, 192-198.

Hamel, L.P., Nicole, M.C., Duplessis, S., and Ellis, B.E. (2012). Mitogen-activated protein kinase signaling in plant-interacting fungi: distinct messages from conserved messengers. Plant Cell *24*, 1327-1351.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature *413*, 383-389.

Lohar, D.P., Sharopova, N., Endre, G., Penuela, S., Samac, D., Town, C., Silverstein, K.A., and VandenBosch, K.A. (2006). Transcript analysis of early nodulation events in *Medicago truncatula*. Plant Physiol. *140*, 221-234.

Lopez-Gomez, M., Sandal, N., Stougaard, J., and Boller, T. (2012). Interplay of flg22-induced defence responses and nodulation in *Lotus japonicus*. J. Exp. Bot. *63*, 393-401.

Madsen, E.B., Madsen, L.H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczyglowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., et al.

(2003). A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425, 637-640.

Madsen, L.H., Tirichine, L., Jurkiewicz, A., Sullivan, J.T., Heckmann, A.B., Bek, A.S., Ronson, C.W., James, E.K., and Stougaard, J. (2010). The molecular network governing nodule organogenesis and infection in the model legume *Lotus japonicus*. Nat. Commun. *1*, 10.

Oldroyd, G.E. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat. Rev. Microbiol. *11*, 252-263.

Oldroyd, G.E., Murray, J.D., Poole, P.S., and Downie, J.A. (2011). The rules of engagement in the legume-rhizobial symbiosis. Annu. Rev. Gene. *45*, 119-144.

Perret, X., Staehelin, C., and Broughton, W.J. (2000). Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. *64,* 180-201.

Remigi, P., Zhu, J., Young, J.P., and Masson-Boivin, C. (2016). Symbiosis within symbiosis: evolving nitrogen-Fixing legume symbionts. Trends Microbiol. *24,* 63-75.

Rodriguez, M.C., Petersen, M., and Mundy, J. (2010). Mitogen-activated protein kinase signaling in plants. Annu. Rev. Plant Biol. *61*, 621-649.

Ryu, H., Cho, H., Choi, D., and Hwang, I. (2012). Plant hormonal regulation of nitrogen-fixing nodule organogenesis. Mol. Cells *34*, 117-126.

Schauser, L., Roussis, A., Stiller, J., and Stougaard, J. (1999). A plant regulator controlling development of symbiotic root nodules. Nature *402*, 191-195.

Searle, I.R., Men, A.E., Laniya, T.S., Buzas, D.M., Iturbe-Ormaetxe, I., Carroll, B.J., and Gresshoff, P.M. (2003). Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. Science *299*, 109-112.

Smit, P., Raedts, J., Portyanko, V., Debelle, F., Gough, C., Bisseling, T., and Geurts, R. (2005). NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. Science *308*, 1789-1791.

Soyano, T., and Hayashi, M. (2014). Transcriptional networks leading to symbiotic nodule organogenesis. Curr. Opin. Plant Biol. *20,* 146-154.

Tena, G., Asai, T., Chiu, W.L., and Sheen, J. (2001). Plant mitogen-activated protein kinase signaling cascades. Curr. Opin. Plant. Biol. *4*, 392-400.

Tena, G., Boudsocq, M., and Sheen, J. (2011). Protein kinase signaling networks in plant innate immunity. Curr. Opin. Plant. Biol. *14*, 519-529.

Yoo, S.D., Cho, Y.H., Tena, G., Xiong, Y., and Sheen, J. (2008). Dual control of nuclear EIN3 by bifurcate MAPK cascades in C2H4 signalling. Nature *451*, 789-795.