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Mitogen-Activated Protein Kinase Is Involved in the Symbiotic Interaction between *Bradyrhizobium japonicum* USDA110 and Soybean

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Mitogen-activated protein kinase (MAPK) plays an important role in mediating the intracellular transmission and amplification of extracellular stimuli. We examined whether MAPK is involved in the signaling process during the early step of nodule formation. A genistein induced culture filtrate (GCF) of *Bradyrhizobium japonicum* was prepared for inducing an early response by soybean root hairs via Nod factor. Upon treatment, several types of deformations were seen, demonstrating that GCF contains active Nod factor molecules. In-gel kinase assays showed that treating soybean roots with GCF induced the rapid activation of two protein kinases (molecular masses of 47 kD and 44 kD), which phosphorylate myelin basic protein (MBP). To identify the activated kinase, we prepared an antibody against *GMK1* (*Glycine max* MAP kinase 1), based on information from SIMK (an alfalfa MAP kinase) and a soybean EST database. An immunocomplex kinase assay with the GMK1-specific antibody revealed that the 47-kD kinase in GCF-treated seedlings is indeed GMK1. Consistent with many other MAP kinases, GMK1 is likely to be under post-translational regulation. Considering these results and previous reports from soybean, GMK1 seems to be a signaling mediator with a broad range of stimuli, including a fungal elicitor, wounding, and the symbiotic interaction between soybean and *B. japonicum*.

Keywords: *Bradyrhizobium japonicum*, in-gel kinase assay, MAPK, root nodule, soybean, symbiosis

Mitogen-activated protein kinases (MAPKs), also called extracellular signal-regulated kinases (ERKs), are a family of eukaryotic, serine-threonine, protein kinases that participate in the cascade of protein phosphorylations, by which numerous and diverse signals are transduced within the cell (Ferrell, 1996). Several MAPKs have been identified in a variety of plant species, including *Arabidopsis* (MAPK Group, 2002), tobacco (Seo et al., 1995; Zhang and Klessig, 1998), alfalfa (Jonak et al., 1996; Cardinale et al., 2002), rice (Agrawal et al., 2003), tomato (Stratmann and Ryan, 1997; Holley et al., 2003), and soybean (Taylor et al., 2001; Daxberger et al., 2007). They are related to a wide range of actions during plant growth and development, including cellular processes, responses to pathogens, cell cycle regulation, wounding, cytoskeleton associations, hormone signal transduction, and osmotic and oxidative stresses (Tena et al., 2001; Zhang and Klessig, 2001; Jonak et al., 2002; Cessna et al., 2003; Cheong and Yun, 2007).

Nitrogen-fixing nodules form on the roots of legumes as a result of infection by the genus *Rhizobium*. The establishment of a root nodule and its subsequent development encompass complex interactions between the two organisms, resulting in structural and biochemical changes in both partners (Beringer et al., 1979). Several events occur during this plant-bacterium interaction, including secretion of Nod factor and perception by the host, invasion of the root hair followed by development of an infection thread, release of bacteria into cortical cells, formation of nodule primordia, elongation, and differentiation into a mature nodule (Stougaard, 2000). Within the signaling pathway after Nod factor perception, activated trimeric G-protein is important in *Medicago truncatula* (Pingret et al., 1998), and an increase in the phosphatidic acid (PA) concentration is caused by the activation of phospholipase C (PLC) as well as phospholipase D (PLD), action that is essential for the induction of root hair deformations (den Hartog et al., 2001). These events are closely related to MAPK activation in both *Arabidopsis* and animal cells (Miles et al., 2004), suggesting a possible role for this process during root nodule development in legumes.

Fernandez-Pascual et al. (2006) have shown that two different MAPKs are involved in the symbiosis between *Bradyrhizobium* sp. and *Lupinus albus*. Activities of SIMK and SAMK homologues from *L. albus* after plants are treated with living *B. sp.* are much higher than those treated with dead *B. sp.* However, MAPK activity has neither been reported in the nodulation of a model legume species, nor has its gene been identified. Here, we examined the relationship between soybean and *B. japonicum*. This is widely accepted as the model system for symbiosis in a crop species.

MATERIALS AND METHODS

Bacterial Culture and Preparation of Culture Filtrate

Bradyrhizobium japonicum strain USDA110 was grown at

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28°C in 100 mL of yeast mannitol broth (YEM; Vincent, 1970). Culturing conditions included shaking at 150 rpm for 3 d on an orbital shaker, followed by sub-culturing into 200 mL of a YEM medium. After 5 d (OD_{600} of 0.4 to 0.6), the *B. japonicum* culture filtrate was prepared as described previously (Ghelue et al., 1997). Briefly, 5 μM genistein was added to bacterial cultures to induce the synthesis and production of Nod factor, and the same volume of DMSO was added to the control reaction. After another 3 d of incubation, the cultures were centrifuged and the supernatants were filter-sterilized with a 0.22 μm syringe filter (Millipore, Billerica, MA, USA). The resulting cell-free culture filtrates were used for our root hair deformation assays and plant treatments.

Plant Material and Root Hair Deformation Assay

Seeds of soybean [*Glycine max* (L.)] cultivar Backtae were surface-sterilized in 2% sodium hypochlorite for 5 min and washed several times with distilled sterilized water. They were then immersed in sterilized water and incubated at 4°C for 24 h before being transferred to a humidified dark box at 27°C in a growth chamber. After 7 d of incubation, a variation on the methods of Prithiviraj et al. (2000) was used for the root hair deformation assay. Lateral roots of similar length and showing abundant root hairs were selected for uniformity, then aseptically excised and placed on slides with a droplet of either YEM or *B. japonicum* filtrate. The slides were kept in a closed, moist chamber for a defined period. Roots were fixed with a staining solution [0.02% (w/v) methylene blue, 20% (v/v) glycerol, and 10% (w/v) phenol], and root hair deformations were observed via light microscopy (Nikon E600). Each experimental unit comprised at least four lateral roots on the same slide.

Protein Sample Preparation and in-Gel Kinase Assay

At the desired time points, plants were frozen in liquid nitrogen and finely ground with a mortar and pestle. The powder was solubilized in 0.5 mL of modified RIPA buffer [50 mM Tris (pH 7.4), 1% (v/v) nonidet P40, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM PMSF, 1 μg mL^{-1} leupeptin, and 1 μg mL^{-1} pepstatin]. Samples were centrifuged at 4°C for 15 min at 15,000*g*, then centrifuged twice more. Extracts containing 30 μg of protein were electrophoresed on 10% (w/v) SDS-PAGE embedded with 0.33 mg mL^{-1} myelin basic protein (MBP) in the separating gel as a substrate for the kinases. Afterward, we performed in-gel kinase assays as described previously (Taylor et al., 2001). The gels were then dried before being exposed to X-ray film (Fuji, Tokyo, Japan).

Cloning and Antibody Production for GMK1

Specific PCR primers corresponding to the putative 5′ and 3′ untranslated regions of an EST sequence (Soybean Gene Index, www.tigr.org, TC206197) were designed and synthesized by IDT (Coralville, IA, USA). These were used to amplify cDNA that was reverse-transcribed with total RNA prepared from soybean seedlings. The PCR product was cloned into the pCRII-topo vector (Invitrogen, Carlsbad, CA, USA). To prepare an antibody specific to GMK1, peptide FNPEYQQ, corresponding to the C-terminus of GMK1, was synthesized and conjugated to the carrier, keyhole limpet hemacyanin. Polyclonal antibodies were raised in rabbits and purified by protein A column chromatography (Peptron, Daejeon, Korea).

Immunoblotting

Extracted proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane at 100 V for 1 h. Blocking was performed in phosphate-buffered saline [PBS: 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 (pH 7.4), 137 mM NaCl, 2.7 mM KCl, and 0.05% (v/v) Tween 20] with 5% (w/v) nonfat dried milk. The blot was incubated with a 1:1000 dilution of GMK1 antibody in blocking buffer for 2 h at room temperature, followed by three washes (10 min each) with PBS. A secondary probe with a 1:4000 dilution of horseradish peroxidase-linked goat anti-rabbit antibody in PBS was incubated for 1 h. After three washes, GMK1 was analyzed with the ECL Plus detection system as specified by the manufacturer (Amersham, Buckinghamshire, UK).

Total RNA Extraction and RNA Gel Blot Analysis

Total RNA extraction and RNA gel blot analysis from soybean seedlings were carried out as previously described (Lee et al., 2004). Briefly, 20 μg of total RNA was separated on a 1.2% (w/v) formaldehyde agarose gel and transferred to a nylon membrane. Following hybridization and membrane-washing, the gel was exposed to X-ray film.

Immunocomplex Kinase Assay

For our immunocomplex kinase assay, 1 mg of protein extract was incubated overnight at 4°C with anti-GMK1 antibody in immunoprecipitation buffer [25 mM HEPES (pH 7.5), 5 mM EGTA, 5 mM EDTA, and 5 mM DTT]. Anti-GMK1 was not added for our (–)α-GMK1 control. Approximately 10 μL of protein A-agarose washed in immunoprecipitation buffer was added, and incubation was continued for another 6 h. Agarose bead-protein complexes were pelleted by brief centrifugation and washed three times with 1 mL of immunoprecipitation buffer. The resulting pellet was suspended in 30 μL of 2X SDS sample buffer and boiled for 5 min. After brief centrifugation, the supernatant was used for in-gel kinase assays.

RESULTS AND DISCUSSION

Preparation of Culture Filtrates and Root Hair Deformation Assay

We undertook a series of control experiments to establish whether our model system – soybean seedlings and culture filtrates from *Bradyrhizobium japonicum* USDA110 – would perform as well as that of *Alnus glutinosa* and *Frankia* strain ArI3 (Ghelue et al., 1997). Here, we prepared GCF (Genistein induced *B. japonicum* Culture Filtrate) and UCF (Uninduced *B. japonicum* Culture Filtrate). When GCF was applied to soybean root hairs, four types of deformation were observed: wiggling, bulging, curling, and branching

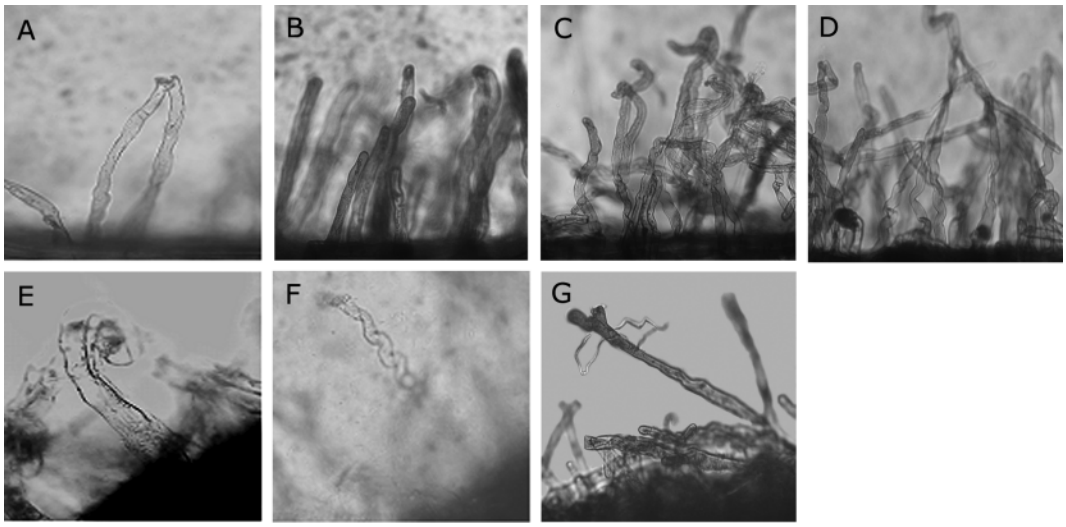

Figure 1. Types of root hair deformations manifested by *Bradyrhizobium japonicum* culture filtrate treatments. **A,** root hairs 12 h after control treatment in YEM medium. **B,** root hairs 12 h after UCF treatment. **C, D,** root hairs at 3 and 6 h, respectively, after treatment with GCF. **E, F, G,** typical root hairs 12 h after GCF treatment.

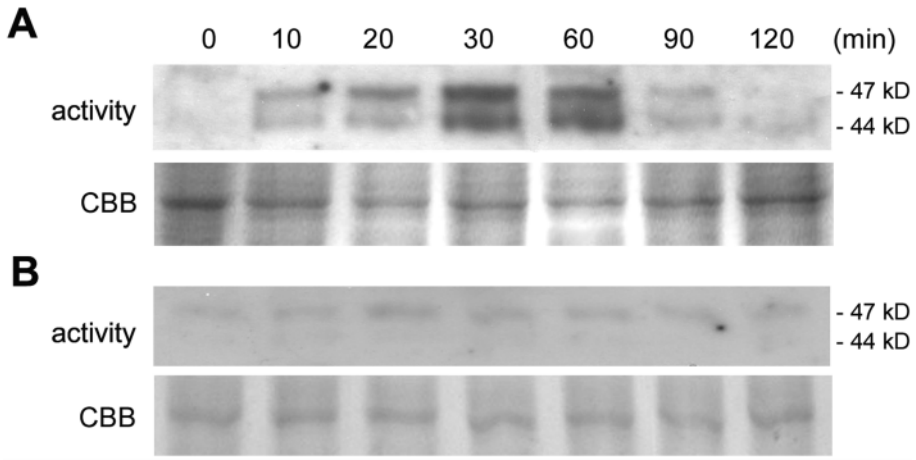

Figure 2. Two different kinases are activated by GCF. Seedlings (10-day-old) were treated with GCF **(A)** or UCF **(B)**. For in-gel kinase assays, extracts containing 30 μg of total protein were separated by SDS-PAGE with 0.33 mg mL^{-1} myelin basic protein. After protein re-naturation, kinase reactions were performed in-gel and analyzed by autoradiography. Coomassie brilliant blue (CBB)-stained band for unknown protein represents loading control.

(Fig. 1). This has also been reported from previous studies using purified Nod factor (Prithiviraj et al., 2000; Duzan et al., 2004). Root hairs started to wiggle and show some deformation 3 h after the treatment, and many were deformed after 12 h. However, none of the typical signs of Nod factor-induced hair deformation (HAD) were seen on slides treated with UCF. In all experiments, GCF treatment increased root hair deformation compared with that of UCF. Concluding that GCF contains active Nod factor molecules, we used it for further experiments.

MAPK Activation by Culture Filtrates

One feature of MAPKs is their apparent ability to phosphorylate myelin basic protein as an artificial substrate. To demonstrate whether culture filtrates can activate MAPK from soybean seedlings, we subjected protein extracts to in-gel protein kinase assay, with MBP as substrate. Here, 44- and 47-kD kinase bands were observed in extracts from seedlings treated with GCF (Fig. 2A), but not in those from seedlings exposed to UCF (Fig. 2B). Activity of these kinases was evident within 10 min, reaching a maximum by 30 min before being dramatically reduced by 90 min, and finally returning to a basal level by 2 h after GCF treatment. These data suggest that the Nod factor-containing culture filtrate can activate at least two different kinases in soybean. This implies that they are MAP kinases with a role in the signal transduction pathways induced by Nod factor.

Immunocomplex Kinase Assay

SIMK-specific antibody can immuno-precipitate MAPK that is involved in the establishment of root nodules for the symbiosis between *Lupinus* and *Bradyrhizobium* (Fernandez-Pascual et al., 2006). MAPK also is activated by wounding in soybean (Lee et al., 2001). Therefore, it is reasonable to

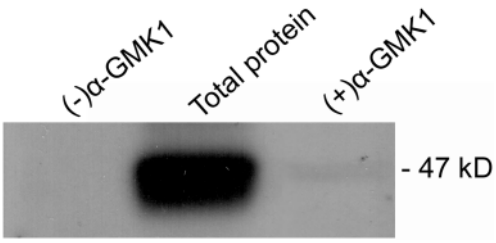

Figure 3. GCF-activated (47-kD) protein kinase is encoded by *GMK1*. Protein extracts were prepared from soybean seedlings treated with GCF for 30 min. Total protein (30 μg) and immunoprecipitated products from 1 mg of total protein, with or without 10 μg of protein A-purified GMK1 antibody, were separated by SDS-PAGE containing 0.33 mg mL^{-1} myelin basic protein. After protein re-naturation, kinase reactions were performed in-gel and analyzed by autoradiography.

hypothesize that soybean has a gene homologous to SIMK, which might be one of the two kinases activated by GCF. To test this theory, we cloned GMK1 (*Glycine max* MAP Kinase 1) from a cDNA sequence corresponding to a soybean EST with high similarity to SIMK. We then raised an antibody specific to a unique C-terminal sequence of GMK1 (FNPEYQQ).

Based on our immunocomplex kinase assay with GCF-treated seedlings (Fig. 3), when antibody against GMK1 was used, the immunoprecipitates of protein extracts at 30 min post-treatment showed faint kinase activity, with an apparent molecular mass of 47 kD. No activity signal was seen for protein precipitates without antibody. Because of the long period of exposure, two kinase bands for total protein had merged into one strong band whereas two separate bands were identified on a film with shorter exposure (data not shown). This indicates that the activated GMK1 is responsible for activity in the 47-kD band from the in-gel kinase assay. GMK1 is homologous to alfalfa SIMK and the same with previously reported as GmMPK6 (Daxberger et al., 2007), which is activated by wounding (Lee et al., 2001) and β-glucan elicitor (Daxberger et al., 2007). Because various stimuli, such as salicylic acid, avirulence factors, and hypo- or hyperosmotic stress, can activate alfalfa SIMK (Munnik et al., 1999) as well as its homologues, e.g., SIPK (Zhang and Klessig, 2001; Droillard et al., 2004) and *Arabidopsis* AtMPK6 (Nuhse et al., 2000), it is reasonable to hypothesize that GMK1 is one of the kinases activated by various environmental stimuli.

Activity of GMK1 Is Under Post-Translational Control

To determine whether the protein amounts for GMK1 changed in response to GCF, total protein from GCF-treated soybean seedlings were separated by SDS-PAGE and immuno-blotted with GMK1-specific antibody (Fig. 4A). In contrast to the alterations seen in kinase activities (Fig. 2A), the amounts of the different MAPK proteins did not vary over the 2h experimental period. We also examined mRNA transcript levels of GMK1, and found little variation among samples, the difference for which could not account for any dramatic change in activity (Fig. 4B). This indicates that the GMK1 protein is maintained at steady-state levels in the cell, thereby permitting the plant to respond rapidly to unpredictable external stimuli. These observations suggest that GMK1 activation is primarily achieved by post-translational modification, which is consistent with other plant MAPKs, such as SIPK from tobacco (Zhang and Klessig, 1998) and SIMK from alfalfa (Cardinale et al., 2002).

CONCLUSION

In this study, we found that GCF can induce root hair deformations and activate two different kinases (44 kD and 47 kD) from soybean seedlings. Direct biochemical evidence, based on our use of a C-terminus-specific antibody, showed that the 47-kD kinase, which is involved in the pathway for Nod factor signaling, is GMK1. Thus, by employing immunoprecipitation followed by an in-gel kinase assay, we have demonstrated that activated GMK1 is responsible for the

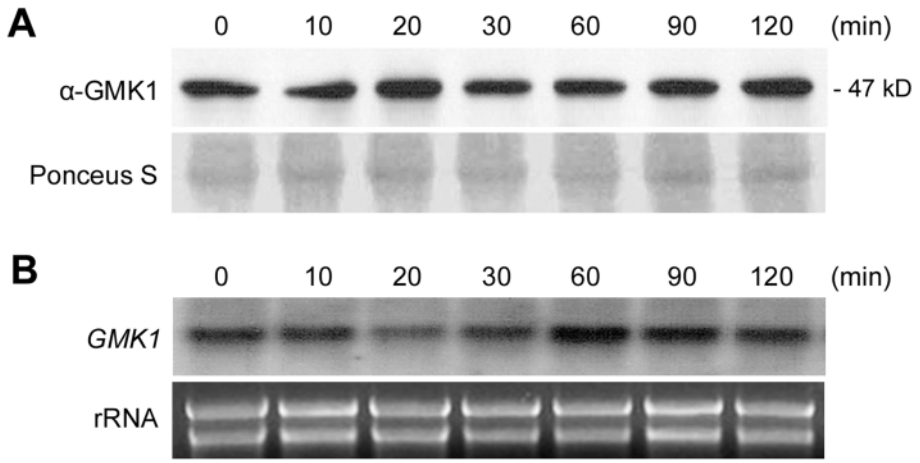

Figure 4. GMK1 protein **(A)** and its transcript level **(B)** by GCF. After 10-day-old seedlings were treated with GCF, all samples were frozen in liquid nitrogen at indicated time points following treatment. Total proteins (30 μg) were electro-blotted to nitrocellulose membrane, which was then stained with 0.1% (w/v) Ponceus S to confirm equal loading and appropriate transfer. Blot was used for immunoblot analysis to determine GMK1 protein level, with GMK1-specific antibody. Total RNA (20 μg) was used for RNA blot analysis to determine *GMK1* mRNA level. rRNA bands are presented as loading control.

activity in that 47-kD band. Because GMK1 is activated by post-translational mechanisms, ectopic expression *in planta* might be problematic. However, a gene knock-down system that incorporates a soybean hairy root transformation method is still challengeable to confirm its biological functioning during the formation of soybean root nodules. Overall, our study indicates a role for MAPK in the establishment of symbiosis between soybean and *Bradyrhizobium japonicum*. Experiments are in progress for identifying upstream MAPK kinase as a positive regulator and MAPK phosphatase as a negative regulator specific to GMK1. Successful completion of this research will facilitate better understanding of the signaling process that mediates such a symbiotic relationship.

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ABBREVIATIONS

BNM, buffered nodulation medium; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; NCBI, National Center for Biological Information; YEM, yeast mannitol medium.

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