Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2015) **16**(1), 61–70 DOI: 10.1111/mpp.12163

PsMPK7, a stress-associated mitogen-activated protein kinase (MAPK) in *Phytophthora sojae*, is required for stress tolerance, reactive oxygenated species detoxification, cyst germination, sexual reproduction and infection of soybean

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SUMMARY

The sensing of stress signals and their transduction into appropriate responses are crucial for the adaptation, survival and infection of phytopathogenic fungi and oomycetes. Amongst evolutionarily conserved pathways, mitogen-activated protein kinase (MAPK) cascades function as key signal transducers that use phosphorylation to convey information. In this study, we identified a gene, designated *PsMPK7*, one of 14 predicted genes encoding MAPKs in *Phytophthora sojae*. *PsMPK7* was highly transcribed in each tested stage, but was up-regulated in the zoospore, cyst and cyst germination stages. Silencing of *PsMPK7* affected the growth of germinated cysts, oospore production and the pathogenicity of soybean. *PsMPK7* transcription was induced by stresses from sorbitol, NaCl and hydrogen peroxide. Transformants in which *PsMPK7* expression was silenced (*PsMPK7*-silenced) were significantly more sensitive to osmotic and oxidative stress. Aniline blue and diaminobenzidine staining revealed that the silenced lines did not suppress the host reactive oxygen species (ROS) burst, indicating that either the inoculated plants activated stronger defence responses to the transformants and/or the *PsMPK7*-silenced transformants failed to overcome plant defences. In addition, extracellular secretion of laccase decreased in the silenced lines. Overall, our results indicate that the *PsMPK7* gene encodes a stress-associated MAPK in *P. sojae* that is important not only for responses to various stresses, but also for ROS detoxification, cyst germination, sexual oospore production and infection of soybean.

Keywords: cyst germination, MAPK, oospore production, pathogenicity, *Phytophthora sojae*, stress-activated protein kinase.

INTRODUCTION

Phytophthora species are destructive plant pathogens that attack a wide range of ornamentally and agriculturally important plants. At least 116 pathogenic *Phytophthora* species are currently known, and new species, or new variants of known species, are still being discovered (Kroon *et al*., 2012). Although *Phytophthora* species exhibit a filamentous growth morphology that is very similar to that of many fungi, they are not fungi or even related to fungi. The genus *Phytophthora* belongs to the oomycete group in the Stramenopila kingdom, which constitutes a distinct, major branch of the eukaryotic evolutionary tree and comprises saprophytes and pathogens of plants, animals and insects (Baldauf, 2003; Latijnhouwers and Govers, 2003; Tyler, 2007). One representative plant pathogen is *Phytophthora sojae*, which is the causal agent of root and stem rot in soybean. *Phytophthora sojae* has been reported throughout most soybean-growing regions globally, and causes plant stand reductions and even complete yield losses with damage valued at approximately $1–2 billion each year (Tyler, 2007; Wrather and Koenning, 2006).

In the field, the survival and dispersal of spores are essential for the success of many plant pathogens. *Phytophthora sojae* produces asexual spores called zoospores and sexual spores called oospores. Zoospores are the most important means of soybean infection, especially in flooded fields. The zoospores swim chemotactically towards compounds released by roots, including the isoflavones daidzen and genistein, and are then triggered to form adhesive cysts, which, in turn, germinate to produce hyphae or secondary zoospores. The hyphae penetrate the plant epidermis directly, but sometimes form appressoria for penetration (Enkerli *et al*., 1997; Tyler, 2007). The normal behaviour of zoospores has been demonstrated previously to be controlled by a G-protein α subunit in *P. sojae* and *Phytophthora infestans* (Hua *et al*., 2008; Latijnhouwers *et al*., 2004). However, the molecular mechanisms of development that occur after zoospore cyst formation, especially germination and the formation of infectious hyphae that penetrate soybean cells, are still largely unknown. As a result of fragility, asexual zoospores are ineffective resting structures, but the thick-walled and durable sexual oospores, which can survive in inhospitable environments, such as freezing or desiccating conditions, act as resting structures. In the subsequent year, oospores germinate to produce either a

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hyphal tube to infect the soybean plant or a germ sporangium to release zoospores (Blanco and Judelson, 2005). In *Phytophthora*, although some developmental stage-dependent genes have been identified that are active during sexual reproduction (Fabritius *et al*., 2002; Prakob and Judelson, 2007; Zhao *et al*., 2011), the signalling pathways governing sexual development are still unknown.

In plants, animals and other eukaryotic organisms, a family of serine/threonine protein kinases, known as mitogen-activated protein kinases (MAPKs), is involved in the transduction of a variety of extracellular signals and in the regulation of growth and developmental processes. Accumulating evidence has implicated the important roles of MAPK signalling pathways in the control of spore development, infection and sexual reproduction in fungi. For example, the budding yeast *Saccharomyces cerevisiae* has five MAPK pathways that are involved in sporulation, osmoregulation, cell wall integrity, pheromone response and filamentation (Chen and Thorner, 2007). MAPKs that specifically transmit environmental stress signals are known as stress-activated protein kinases (SAPKs). The SAPKs have been well studied in fungi and mammalian systems, and are responsible for responses to a wide range of stresses, such as heat shock, hyperosmolarity, ultraviolet (UV) light irradiation and oxidative stress (Degols *et al*., 1996; Hohmann, 2002; Kato *et al*., 1996; Kyriakis and Avruch, 1996; Millar *et al*., 1995; Shiozaki and Russell, 1995). SAPK gene-silenced transformants of phytopathogenic fungi exhibit impaired osmoregulation and stress responses. For example, Osm1 of *Magnaporthe oryzae* controls the accumulation of arabitol in response to hyperosmolarity in hyphae (Dixon *et al*., 1999); MgHog1 of *Mycosphaerella graminicola* controls pathogenicity, tolerance to osmosensitivity and the transition from yeast-like growth to filamentous growth (Mehrabi *et al*., 2006). SRM1 (a Hog1-type MAPK homologue) of *Bipolaris oryzae* controls tolerance to hyperosmolarity, hydrogen peroxide (H_2O_2) and UV exposure (Moriwaki *et al*., 2006).

Similar to the phytopathogenic fungi, successful infection by oomycetes depends on their ability to overcome defence responses in their hosts, and therefore MAPK signalling pathways are likely to play similar roles in the eukaryotic oomycete plant pathogens. However, because the oomycetes are distantly related and evolutionarily distinct from fungi, major homologues of fungal *MAPK* genes are difficult to identify in *Phytophthora* using sequence-based bioinformatics search methods. In our previous work, we identified 14 *MAPK* gene candidates in *P. sojae* (Ye *et al*., unpublished data) and characterized two of them. *PsMPK1* is involved in zoospore reproduction, cell wall integrity and pathogenicity (Li *et al*., 2014), and *PsMPK3* (or *PsSAK1*) is involved in premature encystment, germination and penetration (Li *et al*., 2010). In this study, we characterized another *P. sojae MAPK* gene, designated *PsMPK7*, which was transcriptionally up-regulated in the zoospore, cyst and cyst germination stages, and induced by osmotic and oxidative stresses mediated by sorbitol, NaCl and H_2O_2. In addition, *P. sojae* transformants in which *PsMPK7* expression was silenced were more sensitive to environmental stresses and exhibited abnormal growth during cyst germination and reduced virulence to soybean.

RESULTS

PsMPK7 gene encodes a MAPK

We identified a MAPK gene from *P. sojae*, designated *PsMPK7* (http://www.jgi.doe.gov; JGI v1.1, protein ID: 129213). The corrected *PsMPK7* gene model is 4185 bp in length, intronless and encodes a protein of 1394 amino acids [scaffold_7:138979–143163(-); Fig. S1, see Supporting Information]. PsMPK7 contains a typical serine/threonine protein kinase catalytic (STKc) MAPK domain (E = 5.74e-172; amino acids 1008–1334) and a WW domain (E = 4.58e-03; amino acids 1360–1390) which were predicted using the CD-Search program at the National Center for Biotechnology Information (NCBI) website (Marchler-Bauer *et al*., 2011). The PsMPK7 full-length protein is highly conserved with its orthologues in *Phytophthora ramorum* (87%), *Phytophthora parasitica* (88%), *Phytophthora capsici* (88%) and *Hyaloperonospora arabidopsidis* (81%). PsMPK7 and its orthologues have a predicted dual phosphorylation lip sequence TEY (amino acids 1168–1170; Fig. S2, see Supporting Information).

PsMPK7 transcripts were abundant in various developmental and infection stages, and were up-regulated twofold in the zoospore and cyst stages and more than fivefold during cyst germination, when compared with mycelia (Fig. 1A). A consistent transcription pattern was found from the published Digital Gene Expression profiling data (Ye *et al*., 2011), although the strong up-regulation during the germinating cyst stage was not detected (Fig. S3, see Supporting Information). This difference may be a result of differences in technologies or samples. However, the stage-specific up-regulation suggested that PsMPK7 may play important roles in the physiological processes of zoospores and cysts, and during cyst germination.

Association of *PsMPK7* with *P. sojae* pathogenicity

To determine whether *PsMPK7* was required for *P. sojae* pathogenicity, stable *PsMPK7*-silenced strains of *P. sojae* (P6497) were generated using a polyethylene glycol (PEG) transformation-mediated gene silencing method (see Experimental procedures). We obtained three *PsMPK7*-silenced lines, T16, T6 and T41, in which *PsMPK7* expression was reduced by 65%, 75% and 91%, respectively, compared with the wild-type (WT) strain, whereas *PsMPK7* expression was not affected in the control strain [CK; green fluorescent protein (GFP)-expressing transformant; see Experimental procedures] (Fig. 1B).

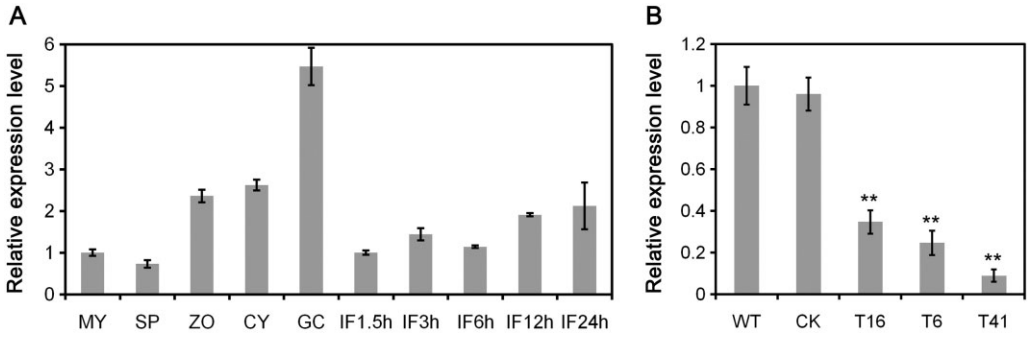

Fig. 1 *PsMPK7* gene expression. (A) *PsMPK7* expression was profiled using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) throughout the *Phytophthora sojae* life cycle, including vegetative mycelia (MY), sporulating hyphae (SP), zoospores (ZO), cysts (CY), cyst germination (GC) and during the early stages of infection, including 1.5, 3, 6, 12 and 24 h post-inoculation (hpi) of the susceptible soybean cv. Williams. (B) Relative gene expression levels of the control strain (CK) and *PsMPK7*-silenced transformants (T16, T6 and T41) using the wild-type (WT) as a reference (value, 1.0). The qRT-PCR assays were performed using cDNAs synthesized from mycelial RNAs. Expression of the *P. sojae actinA* gene was used as a constitutively expressed endogenous gene control, and the level of *PsMPK7* expression was determined relative to that of *actinA*. Three biological replicates, each containing three technical replicates for each sample, were performed. Statistical significance was analysed using Student's *t*-test between the WT/CK and WT/transformant comparisons (**$P < 0.01$).

To examine the roles of *PsMPK7* in *P. sojae* pathogenicity in its native host (soybean), inoculation assays were performed by applying zoospore suspensions of WT, CK and the three silenced transformants (T16, T6 and T41) onto etiolated seedlings of the soybean cultivar Hefeng 47. At 2 days post-inoculation (dpi), the hypocotyls of the etiolated seedlings inoculated with WT and CK zoospores showed typical disease symptoms and water-soaked lesions (Fig. 2). In contrast, the three silenced transformants produced almost no lesions or very small lesions that did not expand beyond the inoculation site (Fig. 2). To determine whether the pathogenicity defect was associated with penetration, the hypocotyls of etiolated seedlings were wounded with a pipette tip prior to inoculation with the zoospore suspensions. At 2 dpi under the same conditions, disease symptoms did not expand on the hypocotyls inoculated with zoospores of the silenced transformants (Fig. 2). However, the hypocotyls of the etiolated seedlings inoculated with WT and CK zoospores showed typical disease symptoms. These results indicated that *PsMPK7* was associated with *P. sojae* pathogenicity in soybean and that the loss of pathogenicity was not caused directly by an impaired ability to penetrate.

PsMPK7 is required for reactive oxygen species (ROS) detoxification

Plant defence responses play important roles during plant—microbe interactions. The recognition of microbe-associated molecules by plant immune systems causes reinforcement of the cell wall (e.g. callose deposition), the accumulation of pathogenesis-related (PR) proteins and rapid generation of ROS in the infected cell (Abramovitch *et al*., 2003; Bradley *et al*., 1992; Levine *et al*., 1994; Tanaka *et al*., 2006; Torres and Dangl, 2005). Callose deposition was observed at the infection sites of soybean epidermal cells at 16 h post-inoculation (hpi) in the transformants. It was normal and similar to that of WT (Fig. 3A). ROS accumulation at the infection site, also known as the oxidative burst, is regarded as one of the earliest events during incompatible interactions between plants and pathogens (Apostolet *et al*., 1989; Yoshioka *et al*., 2008). Thus, we examined host-derived ROS levels by staining with 3,3'-diaminobenzidine (DAB) at 10 hpi. After DAB staining, a limited reddish-brown precipitate was observed near the infection site of WT *P. sojae*. However, increased amounts of reddish-brown precipitate were observed in soybean cells around the infection site of *PsMPK7*-silenced transformants (Fig. 3B), indicating that the *PsMPK7*-silenced transformants failed to scavenge ROS that accumulated at the infection site.

PsMPK7 plays a role in cyst germination

Because increased *PsMPK7* transcript levels were detected during the zoospore, cyst and cyst germination stages, and these stages are the primary steps for host infection by *Phytophthora* pathogens, we examined the role of *PsMPK7* in cyst germination. Zoospore suspensions of the three transformants, WT and CK were centrifuged to induce encystment, and then incubated in 1% V8 liquid at 25 °C for 48 h. Although we found no differences in the formation or morphology of sporangia, or in the development, release or encystment of zoospores, abnormal germinated cysts were found in the three transformants. As shown in Fig. 4A, the abnormal germinated cysts revealed apical swelling, indicating that polarized growth might be affected. The rates of abnormal cysts ranged from approximately 15% to 23%, which were significantly ($P < 0.01$) higher than the values of 3% and 4% for WT and CK, respectively (Fig. 4B). Although the germination of cysts was impaired by *PsMPK7* silencing, the colony growth rates of

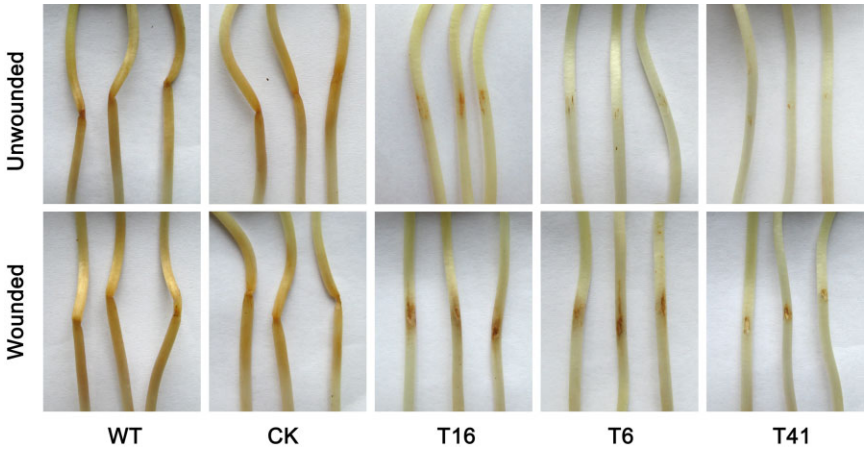

Fig. 2 Pathogenicity assay performed on etiolated soybean seedlings. Unwounded and wounded etiolated soybean seedlings (Hefeng 47) were inoculated with zoospores [approximately 100 in 10 μL for wild-type (WT) and control strain (CK) zoospores, and 150 for transformant zoospores). Images were taken after 48 h, and the experiments were repeated at least three times.

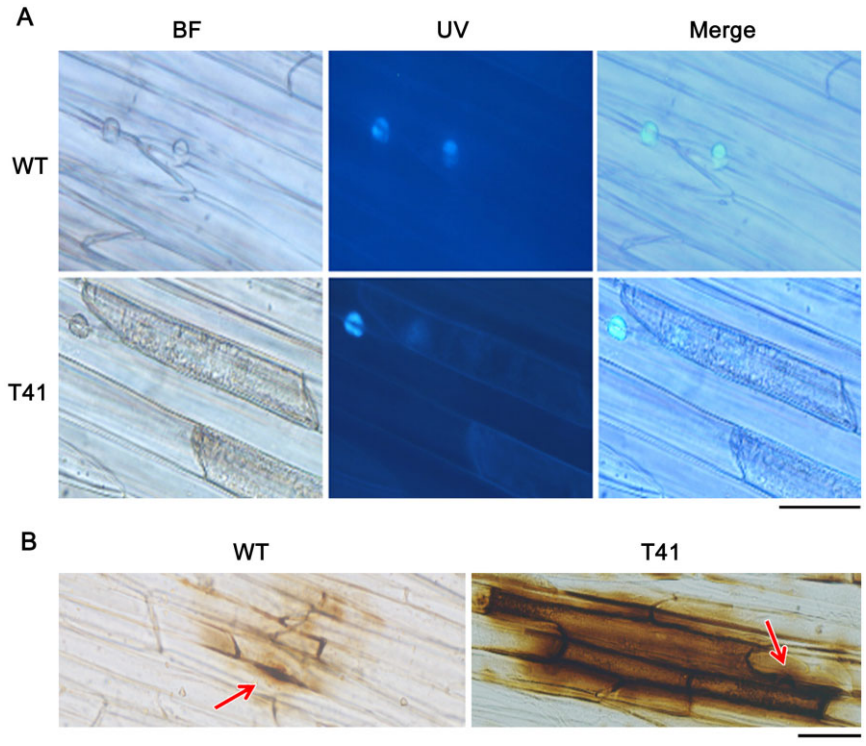

Fig. 3 *PsMPK7*-silenced transformants did not suppress host callose deposition or the reactive oxygen species (ROS) burst. (A) Etiolated soybean seedlings were infected with zoospores and stained with aniline blue after 16 h. The epidermal cells were peeled for microscopic observation. Callose in the host tissues beneath the zoospore penetration sites was seen as fluorescent solid circular shapes (see the corresponding light microscopic fields). Bar represents 50 μm. (B) 3,3'-Diaminobenzidine staining of soybean seedlings infected by *PsMPK7*-silenced transformants and the wild-type (WT) after 10 h. Epidermal cells were peeled for microscopic observation. The penetration sites are indicated by red arrows. Bar represents 50 μm.

PsMPK7-silenced transformants were not affected and remained similar to those of WT and CK (Fig. 5A).

PsMPK7 and oxidative, osmotic and salt stress tolerance

To determine whether *PsMAPK7* was a stress-associated MAPK, its expression was investigated under oxidative, osmotic and salt stress conditions using H_2O_2 (1.0, 2.0 and 3.0 mM), sorbitol (1.0 M) and NaCl (0.25 M) treatments, respectively. *PsMAPK7* expression increased significantly ($P < 0.01$) by approximately twofold in response to sorbitol and NaCl. *PsMAPK7* expression also increased in response to the various concentrations of H_2O_2 and, although not significant, the increases were positively correlated with the increasing H_2O_2 concentrations (Fig. S4, see Supporting Information).

The *PsMPK7*-silenced lines, T16, T6 and T41, and the WT and CK lines were cultured on V8 agar medium supplemented with different concentrations of H_2O_2, sorbitol, NaCl or $CaCl_2$ (Fig. 5A). All of the *PsMPK7*-silenced transformants exhibited significantly ($P < 0.01$) higher inhibition of growth rates than WT and CK at 1.0 mM H_2O_2, and did not grow at 2.0 or 3.0 mM H_2O_2, whereas the WT and CK lines survived (Fig. 5A, B). In addition, under the stresses of NaCl, $CaCl_2$ and sorbitol, the *PsMPK7* transformants showed significantly ($P < 0.01$ or $P < 0.05$) higher rates of growth inhibition than WT or CK (Fig. 5A, B). These results

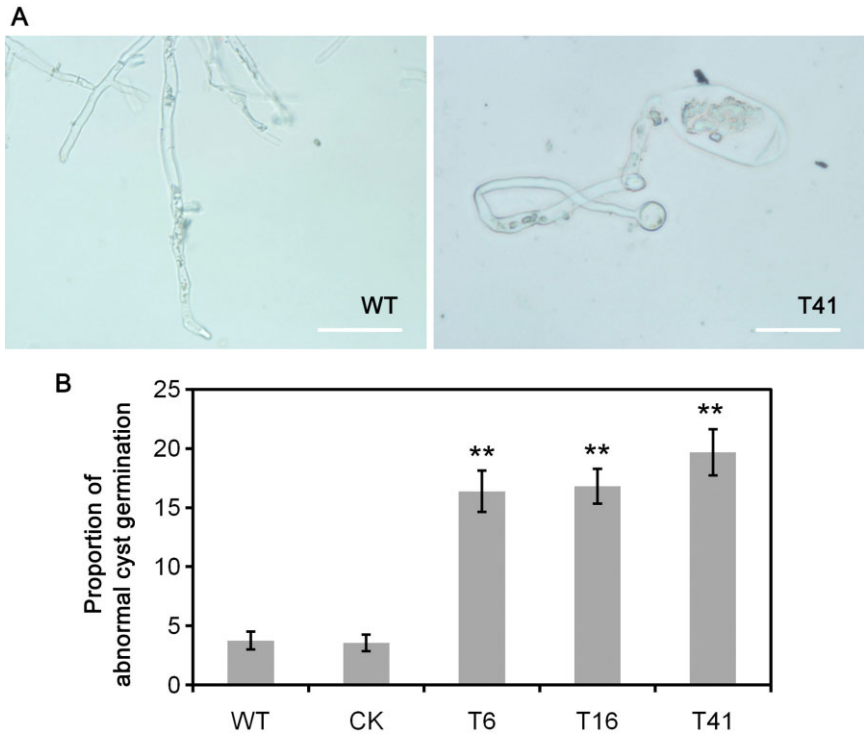

Fig. 4 Abnormal cyst germination of *PsMPK7*-silenced transformants. (A) Zoospores of the wild-type (WT0 and the *PsMPK7*-silenced transformant T41 were encysted by vortexing for 90 s, and the cysts were incubated in clarified 1% V8 liquid medium at 25 °C and stained in 0.3 mM nitroblue tetrazolium chloride (NBT) aqueous solution for 20 min after 48 h. Three independent experiments were performed and yielded similar results. Representative images are presented. Bars represent 50 μm. (B) The proportions of abnormally germinated cysts (cysts that did not grow) were counted at 48 h under a microscope in three independent experiments. CK, control strain.

indicated that the function of *PsMPK7* was related to osmoregulation and tolerance to various stresses.

Requirement of *PsMPK7* for oospore production

To analyse the involvement of Ps*MPK7* in sexual reproduction, the oospore development of the silenced transformants was examined by microscopy. As shown in Fig. 6A, oospore production was reduced markedly after growth for 30 days on lima bean agar (LBA) medium in the *PsMPK7*-silenced transformants. The average numbers of oospores in each 1-cm^2 zone adjacent to the inoculation site declined from more than 670 in WT and 660 in CK to no more than 32 in the *PsMPK7*-silenced transformants (T16, 32; T6, 23; T41, 17; $P < 0.01$; Fig. 6A, B). In addition, many more oospores produced by the transformants were abnormal (60%) than were those produced by WT and CK (both 5%; $P < 0.01$; Fig. 6A, C).

Silencing of *PsMPK7* expression decreases extracellular laccase activity

We examined the secretion of extracellular enzymes to learn more about the effects of *PsMPK7* silencing. The activity of the laccase enzyme was measured because laccase is a type of secreted extracellular protein that is widely distributed in plants and fungi. The secreted laccase activity of WT, CK and the transformants grown on solid lima bean medium was measured using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Significantly decreased laccase activity was observed for the three transformants when compared with WT and CK (Fig. S5A, see Supporting Information). Transcription levels of six predicted signal peptide-containing laccase genes (IDs: 137983, 137984, 137978, 137979, 137990 and 129781) were then measured in WT and T41 at the mycelial stage. The transcripts of all six genes were significantly reduced in T41 when compared with WT (Fig. S5B). Hence, the silencing of *PsMPK7* expression affected the transcription of laccase genes and led to the decrease in extracellular laccase activity. Whether *PsMPK7* silencing broke the secretion pathway is still unknown.

DISCUSSION

In this study, we identified and functionally characterized a putative *P. sojae* MAPK gene, *PsMPK7*. The *PsMPK7* gene was transcribed constitutively throughout the *P. sojae* life cycle, with the highest expression levels occurring during the zoospore, cyst and cyst germination stages. Silencing of *PsMPK7* expression in *P. sojae* affected cyst germination and oospore production, and failed to scavenge ROS that accumulated at the infection site. *PsMPK7*-silenced transformants lost pathogenicity to soybean, but this was not a direct result of impaired penetration ability. Gene expression profiling and colony growth rate assays indicated that *PsMPK7* is a stress-associated *MAPK* that contributes to the tolerance to various stresses.

The ability of fungi to sense and respond to environmental stress is critical for their survival. In the fission yeast *Schizosaccharomyces pombe*, the MAPK Sty1, in combination with

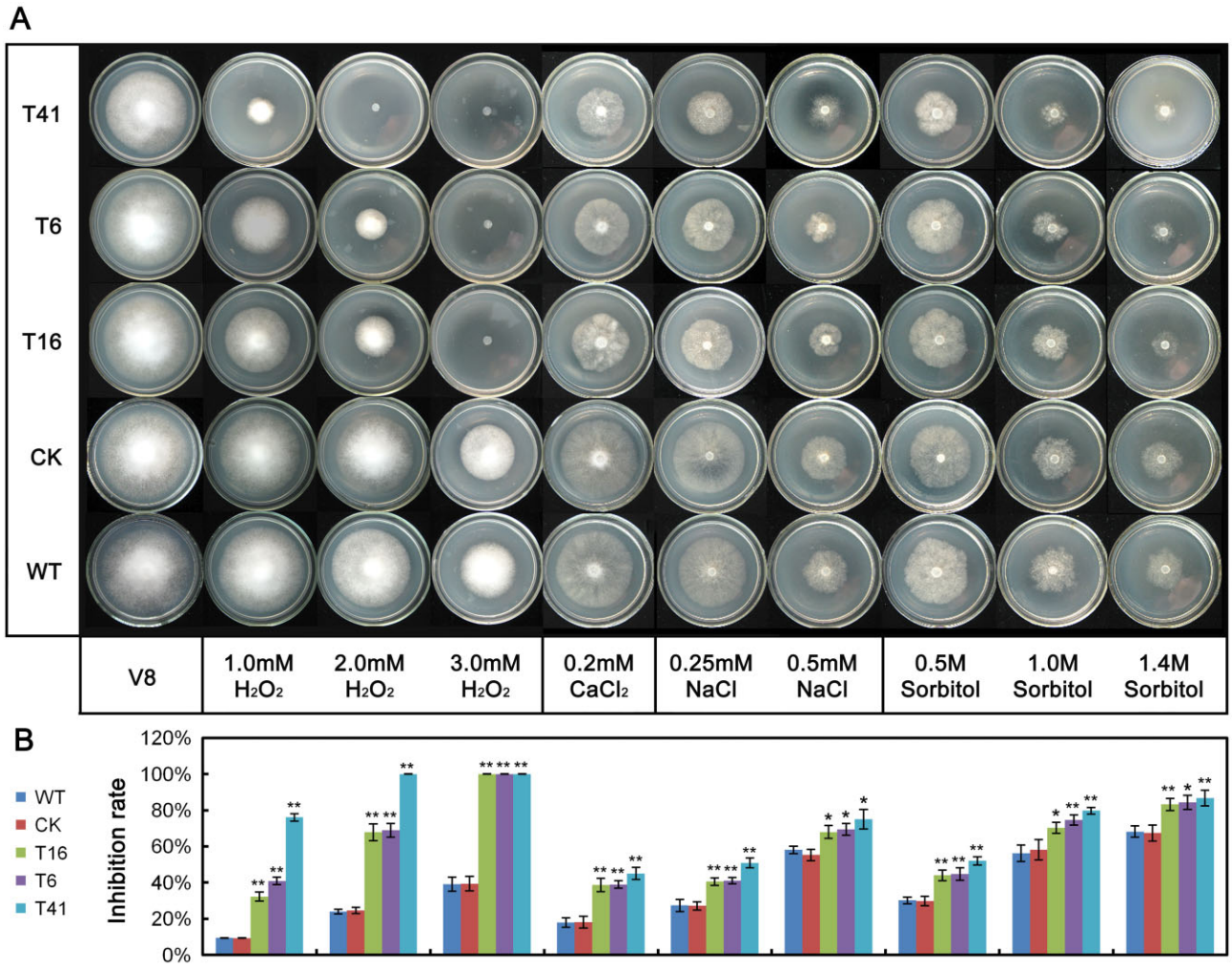

Fig. 5 Assay of mycelial growth under different stress conditions. (A) Assay of mycelial growth of wild-type (WT), control strain (CK) and *PsMPK7*-silenced transformants on V8 agar medium only or supplemented with 1, 2 or 3 mM H_2O_2, 0.2 M $CaCl_2$, 0.25 or 0.5 M NaCl and 0.5, 1.0 or 1.4 M sorbitol. (B) Colony diameters were measured in each independent biological experiment after 5.5 days of growth. Rates of growth inhibition were calculated for each treatment relative to growth on V8 agar medium only. Three independent biological experiments were performed with three replicates each. Error bars represent the standard deviation and asterisks denote significant differences between the *PsMPK7*-silenced transformants and the WT (**$P < 0.01$; *$P < 0.05$).

the transcription factor Yap1, regulates genes in response to oxidative stress (D'Autreaux and Toledano, 2007), and the Sty1 regulatory pathway is conserved amongst other fungi, such as *Colletotrichum lagenarium*, *Botrytis cinerea* and *Fusarium graminearum* (Kojima *et al*., 2004; Segmuller *et al*., 2007; Zheng *et al*., 2012). However, oomycetes are distantly related and evolutionarily distinct from fungi, which makes the identification of any Yap1 or Sty1 homologues in *P. sojae* using sequence-based bioinformatics search methods difficult. In addition, all of the 14 predicted MAPKs in *P. sojae* lack the dual phosphorylation lip sequence TGY, which is a hallmark of SAPKs in fungal and mammalian systems (Hamel *et al*., 2012). In this study, *PsMPK7*-silenced lines that exhibited increased sensitivity to oxidative, osmotic and salt stresses were very similar to the phenotype of the *Sty1* mutant in *S. pombe* (Toone and Jones, 1998), demonstrating that the sensing and response to varied environmental stresses are critical for the survival and pathogenicity of oomycete pathogens, and that one or more MAPK pathways may also function in these processes.

The Sty1 homology pathway varies significantly among different fungal pathogens for plant infection. It is essential for pathogenicity in *F. graminearum* (Zheng *et al*., 2012), *M. graminicola* (Mehrabi *et al*., 2006), *B. cinerea* (Segmuller *et al*., 2007), *Cryphonectria parasitica* (Park *et al*., 2004), *Alternaria alternata* (Lin and Chung, 2010), *Cochliobolus heterostrophus* (Igbaria *et al*., 2008) and *Alternaria brassicicola* (Joubert *et al*., 2011), whereas it is dispensable in *Magnaporthe oryzae*, *Colletotrichum lagenarium* (Kojima *et al*., 2004) and *Bipolaris oryzae* (Moriwaki

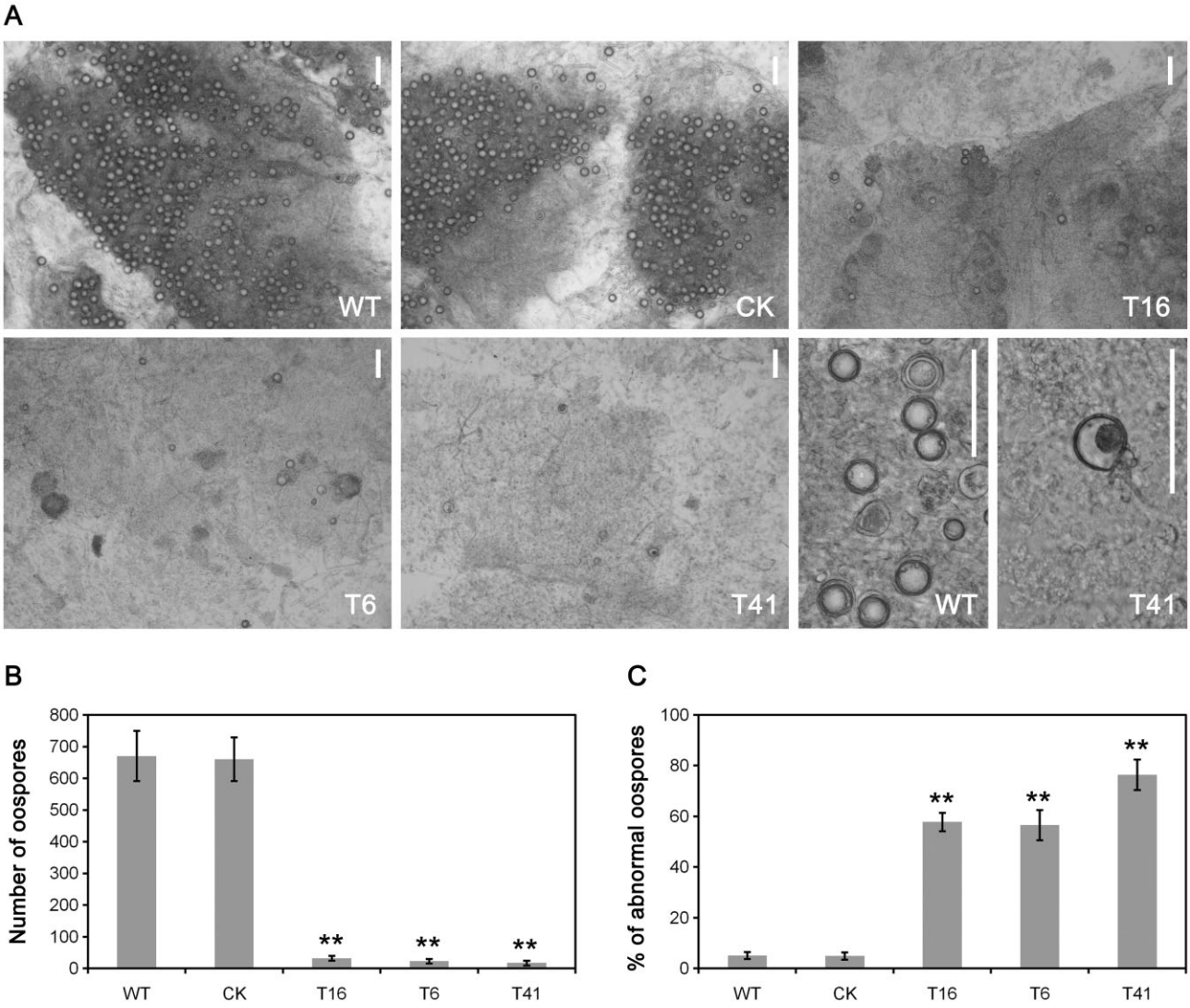

Fig. 6 Oospores of *PsMPK7*-silenced transformants. (A) Microscopic observation of oospore production for wild-type (WT), control strain (CK) and *PsMPK7*-silenced transformant strains (T6, T16 and T41). All strains were grown for 30 days on lima bean agar (LBA) medium to induce oospore formation. Bars represent 100 μm. (B) Mean values of oospores per square centimetre around the inoculation site. Data represent three independent experiments in triplicate. $^{**}P < 0.01$. (C) Abnormal oospores were counted under the microscope. The ratios of abnormal oospores to the total number of oospores were calculated. The data represent three independent experiments in triplicate. $^{**}P < 0.01$.

et al., 2006). We found that the virulence of the *PsMPK7* mutants was lost regardless of unwounding or wounding, indicating that the wound does not help the mutant to penetrate soybean tissue, and *PsMPK7* plays a critical role in pathogenesis.

During plant interactions with fungi or oomycetes, plants use many different defence mechanisms to protect themselves from the pathogens. The host plant cellular environment may act as one of the first major challenges to pathogens (Kim *et al*., 2009). Fungi or oomycetes require adaptive mechanisms to survive. We showed that the *PsMPK7*-silenced transformants were more sensitive to H_2O_2, salt and hyperosmotic stress, and DAB staining indicated that higher levels of H_2O_2 accumulated in soybean cells around the *PsMPK7*-silenced transformant infection sites, suggesting reasons why the *PsMPK7*-silenced transformants lost pathogenicity. Thus, we speculate that the loss of pathogenicity of the transformants may be caused by a lost or severely diminished ability to degrade H_2O_2 and/or reductions in other stress tolerance capabilities during infection. ROS production may function as a second messenger to induce various plant defence responses, and is essential for pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in plants (Gan *et al*., 2009; Nurnberger *et al*., 2004; Torres and Dangl, 2005; Zhang *et al*., 2009). Callose is another plant defensive compound known to be involved in cell wall appositions, and is deposited during both compatible and incompatible

interactions (Huckelhoven, 2007). However, *PsMPK7* transformants induced normal callose deposition. The silencing of *PsMPK7* expression affected the transcription of laccase genes and resulted in decreasing extracellular laccase activity. However, whether *PsMPK7* silencing broke the secretion pathway is still unknown. If so, the secretion, or even production, of effector proteins to suppress plant defence responses might also be affected. This is another possibility to explain the lost pathogenicity of *PsMPK7*-silenced transformants.

This is the first study to report that MAPK gene-silenced *Phytophthora* transformants display abnormal (possibly polar) growth during cyst germination. A nitroblue tetrazolium chloride (NBT) staining experiment demonstrated that ROS accumulation was high in the transformants (Fig. 3A), which may explain why the zoospores could not mature. In contrast, during sexual development, oospores produced by the transformants were critically reduced, similar to observations in the mutants that lack *Sty1* in *F. graminearum* (Zheng *et al*., 2012) and *Aspergillus nidulans* (Kawasaki *et al*., 2002). In addition, a large proportion of the transformant-produced oospores were abnormal. Whether the loss of pathogenicity was a direct or indirect effect of *PsMPK7* silencing remains unclear. Nevertheless, we have demonstrated that PsMPK7 is a stress-associated protein kinase in *P. sojae* that plays a role in various important physiological processes.

EXPERIMENTAL PROCEDURES

Phytophthora sojae strains and culture conditions

The *P. sojae* genome sequencing strain P6497 (Race 2) was provided by Professor Brett Tyler (Oregon State University, Corvallis, OR, USA). The WT strain and silenced transformants were grown routinely on 10% V8 medium at 25 °C in darkness (Erwin and Ribiero, 1996). To test mycelial growth, the strains were subcultured twice, and a 5-mm-diameter agar disc was removed from the edge of an actively growing culture and transferred to the centre of the plate. Oospores were obtained after growth for 30 days on LBA medium at 25 °C in darkness. For use in an extracellular laccase assay, strains were cultured on LBA medium containing 0.8 mM ABTS, which is commonly used with H_2O_2 as a substrate for laccase. To test the expression of *PsMPK7* under different stress conditions, strain blocks (5 mm × 5 mm) were placed into freshly prepared 10% V8 liquid plates for 3 days; H_2O_2, sorbitol or NaCl was added to the liquid and mycelia were collected after 10–30 min for RNA extraction. To test for stress sensitivity, strain blocks (5 mm × 5 mm) were placed onto freshly prepared 10% V8 agar plates supplemented with H_2O_2 (1–3 mM), NaCl (0.25 and 0.5 M), $CaCl_2$ (0.2 M) or sorbitol (0.5, 1.0 and 1.4 M), and cultured in darkness at 25 °C for 5.5 days. To examine the germination of zoospores, 500-μL zoospore suspensions were incubated in 1% V8 liquid medium in 1.5-mL tubes and vortexed for 90 s to induce encystment. The tubes were incubated at 25 °C in darkness for 48 h. At least 100 cysts were examined for each treatment and all treatments were performed in triplicate.

Plasmid construction and generation of *P. sojae* transformants

A partial sequence of the *PsMPK7* open reading frame (ORF) was amplified from cDNA by polymerase chain reaction (PCR) using PrimeStar polymerase (TaKaRa Bio, Otsu, Japan) and the primers PsMPK7-F (5'-GCCAAGAATGGAGCTGAGTC-3') and PsMPK7-R (5'-GCAAGTCCCAGGTCACAAAT-3'). The amplified fragment was digested with *Eco*RI and *Xba*I, and ligated in the antisense orientation into the pTOR vector to produce the plasmid pTOR-PsMPK7. The insert sequence was confirmed by sequencing. *Phytophthora sojae* was transformed with pTOR-PsMPK7 and pTOR-GFP (control, CK) using a PEG-mediated protoplast transformation strategy (McLeod *et al*., 2004).

Putative *PsMPK7*-silenced transformants were screened by quantitative reverse transcription-PCR (qRT-PCR) using the primers PsMPK7-RT-F (5'-CATGACTGTGGACCTGATGG-3') and PsMPK7-RT-R (5'-GCAAGTCCCAGGTCACAAAT-3'). The primers actinA-F (5'-ACTGCACCTTCCAGACCATC-3') and actinA-R (5'-CCACCACCTTGATCTTCATG-3') were used to detect *actinA* (Gene ID: 108986) expression, which served as a constitutively expressed endogenous control gene. Total RNA was isolated using an RNA extraction kit (OMEGA R 6834-1; Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's protocol. First-strand cDNA was synthesized from 1–5 μg of total RNA by oligo(dT) priming using an M-MLV reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. For gene expression analysis, SYBR green qRT-PCR assays were performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. A 20-μL reaction volume contained 2 μL of reverse transcription product, 10 μL of SYBR premix Ex Taq, 0.4 μL of ROX reference dye (SYBR Prime Script RT-PCR kit; TaKaRa Bio) and 0.4 μL of each primer (10 mM). Target gene expression was determined relative to *actinA* expression using the ΔΔCt method. qRT-PCR experiments were repeated in triplicate with independent RNA isolations.

Pathogenicity test

For plant inoculation, P6497 and *PsMPK7*-silenced transformants were incubated in 10% V8 broth in darkness for 2 days. Zoospores were then induced by washing with sterilized distilled water. The soybean cultivar Hefeng 47, which is compatible with *P. sojae* strain P6497, was planted in plastic pots containing vermiculite and grown in darkness for 4 days. Because almost 20% of zoospores could not mature normally, both wounded and unwounded etiolated soybean seedlings were inoculated with 100 zoospores of WT and CK and 150 zoospores of the transformants. The etiolated seedlings were then maintained in 80% humidity and darkness at 25 °C. Pathogenicity symptoms were evaluated after 2 days.

Staining

The ROS levels were stained with the oxidant-sensitive probe NBT (Sigma, Seelze, Germany), and viewed by an Olympus IX71 bright-field microscope (Olympus, Tokyo, Japan). The NBT staining was performed in three independent biological experiments, with three replicates in each test. Etiolated soybean seedlings infected with zoospores were stained with aniline blue after 16 h to measure callose deposition.

ACKNOWLEDGEMENTS

This work was supported by grants to YW from the China National Funds for Distinguished Young Scientists (31225022), China Agriculture Research System (CARS-004-PS14) and Special Fund for Agro-scientific Research in the Public Interest (201303018). We thank the reviewer for providing good suggestions with regard to gene model correction.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Correction of *PsMPK7* gene model. (A) The automatic model (*Ps129213*) was suggested to be nine introns and almost 7 kb (red blocks). According to the distribution of transcript tags (Ye *et al*., 2011) and the alignments against homologues in other close species, the new gene model was suggested to be 4613 bp in length and intronless. Three primer pairs were designed to verify this model, i.e. F1-R, F2-R and F3-R. (B) Polymerase chain reaction (PCR) products could be obtained from *Phytophthora sojae* cDNA using F2-R and F3-R, but not F1-R. The F2-R product was sequenced and perfectly matched the prediction. The primers are listed.

Fig. S2 Protein sequence alignment of PsMPK7 and its orthologues. The protein sequences of PsMPK7 orthologues were identified from available genome assemblies of *Phytophthora ramorum*, *Phytophthora infestans*, *Phytophthora capsici*, *Hyaloperonospora arabidopsidis* and *Pythium ultimum*. The alignment was generated using BioEdit software (Hall, 1999). The STKc_MAPK domain, WW domain and dual phosphorylation lip sequence were predicted using the CD-Search program at the National Center for Biotechnology Information (NCBI) website (Marchler-Bauer *et al*., 2011)

Fig. S3 *PsMPK7* gene expression. *PsMPK7* expression was profiled throughout the *Phytophthora sojae* life cycle, including vegetative mycelia (MY), sporulating hyphae (SP), zoospores (ZO), cysts (CY), cyst germination (GC) and during the early stages of infection including 1.5, 3, 6, 12 and 24 h post-inoculation (hpi) of the susceptible soybean cv. Williams. Data were obtained from Digital Gene Expression profiling (Ye *et al*., 2011).

Fig. S4 *PsMPK7* expression under hydrogen peroxide (H_2O_2), sorbitol and NaCl stress conditions. Mycelia were cultured in liquid 10% V8 medium only or supplemented with 1, 2 or 3 mM H_2O_2 for 10 min or with 1.0 M sorbitol or 0.25 M NaCl for 30 min. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays were performed using cDNAs synthesized from mycelial RNAs, and the *Phytophthora sojae actinA* gene served as a constitutively expressed endogenous control gene. The level of *PsMPK7* expression was determined relative to that of *actinA*, and the relative gene expression levels shown were determined relative to expression on V8 medium alone (value, 1.0).

Fig. S5 Analysis of extracellular laccase activity. (A) Laccase activity was detected in lima bean agar (LBA) medium supplemented with 0.8 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) after 6 days of incubation. (B) Transcription levels of six predicted signal peptide-containing laccase genes (IDs: 137983, 137984, 137978, 137979, 137990 and 129781) were measured in wild-type (WT) and T41 at the mycelial stage by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).