A Putative MAP Kinase Kinase Kinase, *MCK1*, Is Required for Cell Wall Integrity and Pathogenicity of the Rice Blast Fungus, *Magnaporthe oryzae*

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Submitted 22 August 2007. Accepted 19 January 2008.

Insertional mutagenesis of *Magnaporthe oryzae* led to the identification of *MCK1*, a pathogenicity gene predicted to encode mitogen-activated protein kinase kinase kinase (MAPKKK) homologous to *BCK1* in *Saccharomyces cerevisiae*. Targeted disruption of *MCK1* resulted in the fungus undergoing autolysis and showing hypersensitivity to cell-wall-degrading enzyme. The *mck1* produced significantly reduced numbers of conidia and developed appressoria in a slightly retarded manner compared with the wild type. Appressorium of the *mck1* mutant was unable to penetrate into plant tissues, thereby rendering the mutant nonpathogenic. Cytorrhysis assay and monitoring of lipid mobilization suggested that the appressorial wall was altered, presumably affecting the level of turgor pressure within appressorium. Furthermore, the *mck1* mutant failed to grow inside plant tissue. Complementation of the mutated gene restored its ability to cause disease symptoms, demonstrating that *MCK1* is required for fungal pathogenicity. Taken together, our results suggest that *MCK1* is an MAPKKK involved in maintaining cell wall integrity of *M. oryzae*, and that remodeling of the cell wall in response to host environments is essential for fungal pathogenesis.

Additional keywords: *Agrobacterium tumefaciens*-mediated transformation (ATMT)

Fungal cells are enclosed in a rigid cell wall that imparts shape to the cells and mediates all exchanges between the cells and environment (Cabib et al. 2001). Although the fungal cell wall is sturdy enough to endure high turgor pressure, its organization and structure should be remodeled constantly for growth and development. Therefore, in pathogenic fungi, the ability to maintain cell wall integrity is critical to establishing the disease on their hosts, because the fungal cell wall not only provides mechanical protection against a hostile host environment but also serves as a molecular sieve and reservoir for proteins involved in host–pathogen interactions (Bernard and Latge 2001). A signal transduction pathway that ensures the continu-

ous integrity of the cell wall in varying environments has been characterized in the budding yeast *Saccharomyces cerevisiae* (Levin 2005). At the core of this signal transduction pathway is a mitogen-activated protein (MAP) kinase cascade comprising Bck1, Mkk1/2, and Slk2. Several homologues of *SLK2* have been described to be required for pathogenicity as well as cell wall integrity in both human and plant pathogens (Idnurm et al. 2005; Xu 2000). In major opportunistic human pathogens, including *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, the fungal cell wall has been studied extensively as a prime target for the development of safe and effective antifungal agents (Levin 2005). Despite the importance of cell wall integrity in fungal pathogenicity, our understanding of genetic mechanisms responsible for regulating biosynthesis and remodeling of the cell wall in plant-pathogenic fungi is far from complete.

Rice blast is the most devastating disease of cultivated rice, leading to substantial yield losses; enough to feed 60 million people every year (Talbot 2003; Zeigler et al. 1994). Rice blast infection begins when conidia of *Magnaporthe oryzae* (Couch and Kohn, 2002) land on the surface of a rice leaf and germinate. Upon recognition of environmental cues such as surface hydrophobicity, the fungus differentiates a specialized infection cell called the appressorium at the tip of germ tube (Lee and Dean 1994) and produces enormous internal turgor pressure that is required to drive a narrow penetration peg into the host epidermal cell (Howard et al. 1991). Inside the host cell, the fungus develops bulbous, ramifying invasive hyphae that are sealed in a plant membrane, and progressively colonizes the plant tissues (Kankanala et al. 2007). This infection ultimately causes young seedlings to die or leads to the destruction of grain-bearing structures of adult plants (Howard and Valent 1996; Talbot 2003).

M. oryzae has been a model for investigating the genetic basis of fungal pathogenesis due to its genetic tractability and biochemical amenability (Talbot 2003). As a result, a number of important pathogenicity genes have been discovered from this fungus during the last two decades. Furthermore, recently, the genomes of both *M. oryzae* and rice (Dean et al. 2005; Goff et al. 2002; Yu et al. 2002) were fully sequenced, providing a unique opportunity to study a host–parasite interaction at genomic level. In *M. oryzae*, three distinct MAP kinases have been identified thus far: Pmk1, Osm1, and Mps1, each of which corresponds to yeast Fus3/Kss1, Hog1, and Slk2 (Xu 2000). The Pmk1 regulates appressorium formation and invasive growth

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(Xu and Hamer 1996). Mst7 and Mst11, homologues of yeast Ste7 and Ste11, respectively, recently were characterized as upstream components of Pmk1 (Zhao et al. 2005). The Osm1 is not involved in pathogenicity but regulates cellular turgor during hyperosmotic stress by accumulating arabitol in the mycelium (Dixon et al. 1999). The Mps1 plays its role in the appressorium-mediated penetration process. It is the Mps1 that was suggested to be involved in maintenance of cell wall integrity and penetration peg formation in *M. oryzae* (Xu et al. 1998). However, unlike the yeast counterparts, the study on upstream and downstream components of Mps1 is yet to be characterized.

In an attempt to identify the genes involved in pathogenicity of *M. oryzae*, we recently carried out a large-scale insertional mutagenesis using *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Rho et al. 2001) and reported 202 new pathogenicity loci through the extensive screens of mutants in this fungus (Jeon et al. 2007). Here, we describe the identification and functional analysis of one of pathogenicity genes,

which is involved in maintaining cell wall integrity. In one mutant defective in pathogenicity, T-DNA tagged a gene (*MCK1*) encoding a putative MAP kinase kinase kinase (MAPKKK). The *MCK1* is homologous to the *BCK1* gene that was isolated as extragenic suppressor of protein kinase C (*PKC*) deletion mutant in *S. cerevisiae* (Lee and Levin 1992). To elucidate the functions of *MCK1*, we investigated roles of the gene in maintaining the integrity of the fungal cell wall by targeted gene disruption and assessed the contribution of cell wall integrity to fungal pathogenesis. Our study illuminates the importance of the cell wall in fungal pathogenesis.

RESULTS

Identification of a pathogenicity gene, *MCK1*, encoding a putative MAPKKK

For a genome-wide identification and analysis of the genes involved in pathogenicity of *M. oryzae*, 21,070 mutants were

Fig. 1. Identification of a pathogenicity gene, *MCK1*. **A**, Two-week-old culture of wild-type strain KJ201 and T-DNA insertion mutant 413B5 on complete media. **B**, Pathogenicity of KJ201 and 413B5 on rice plants. **C**, Genomic location of T-DNA insertion in 413B5. The numbers below the open reading frame (ORF) indicate the relative distance from start codon of the ORF. RB, right border region of T-DNA; pTrpC, *Aspergillus nidulans* TrpC promoter; HPH, hygromycin phosphotransferase; LB, left border region of T-DNA. **D**, Phylogenetic and domain architectural analysis of Mck1.

generated through ATMT and screened for their ability to cause disease on rice plants (Jeon et al. 2007). Among the pathogenicity-defective mutants, one mutant, 413B5, showed pleiotropic phenotypes ranging from autolysis of mycelia and reduced conidiation to loss of pathogenicity (Fig. 1A and B; Table 1). To identify the genomic location of T-DNA insertion in the mutant, genomic sequences flanking the borders of T-DNA were amplified using thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) and the amplified products were sequenced. Through a BLAST search against *M. oryzae* genome, we were able to locate the T-DNA insertion at the region 15 bp downstream of stop codon of MGG00883.5, an annotated gene which we named *MCK1* (Fig. 1C). The *MCK1* has five exons encoding 1,515 amino acids. A BLASTP search against GenBank suggested that the protein encoded by *MCK1* is an MAPKKK, 54.33, 40.95, and 39.22% identical to Bck1, Ste11, and Ssk2 of *S. cerevisiae*, respectively. Phylogenetic analysis indicated that Mck1 is closely related to Bck1, a MAPKKK involved in maintaining cell wall integrity of *S. cerevisiae*, and less related to the known MAPKKs and MAPKs (Fig. 1D).

Targeted disruption of *MCK1*.

To determine whether the T-DNA insertion is linked to the phenotypes of 413B5 and to characterize the role of *MCK1*, targeted disruption of *MCK1* was carried out. The disruption construct was generated by modified double-joint PCR (Yu et al. 2004), through which the flanking sequences of a target region spanning approximately 2.5 kb were amplified and fused to hygromycin cassette. The resulting PCR product was directly transformed into a protoplast of wild-type strain KJ201 (Fig. 2A). The hygromycin-resistant mutants produced by protoplast transformation were screened for the integration of disruption construct by PCR. Genomic DNAs from 12 mutants produced the expected 2.4-kb fragment in PCR (data not shown). Single integration of the disruption construct was confirmed in three mutants by Southern blot analysis (Fig. 2B). KJ201 had a 2.7-kb *XhoI* fragment, and MP1-2, MP1-30, and MP1-31 did not have a 2.7-kb but a 4.7-kb *XhoI* fragment, indicating that, by homologous recombination, the target region, including the sequences encoding kinase domain, was replaced by hygromycin cassette. Loss of kinase domain was further confirmed by reverse-transcription (RT)-PCR analysis, wherein the kinase domain-encoding part of the transcript was not detected in the targeted disruption mutant, MP1-30 (Fig. 2C).

MCK1 is required for maintenance of cell wall integrity.

Growth of the *mck1* was indistinguishable from that of wild-type strain KJ201 on a V8 and oatmeal agar plate (Table 1). However, like the T-DNA mutant, the *mck1* mutant comparatively lacks aerial hypha and underwent progressive autolysis of mycelia after incubation on oatmeal agar plate for at least

5 days, establishing the linkage between T-DNA insertion and observed phenotypic changes. (Fig. 3A, left panels). Autolysis begins at the center of the mycelium and radiates outward, and has a tendency to be more intense in *mck1* than in 413B5.

Fig. 2. Targeted disruption of *MCK1*. **A**, Diagrammatic scheme of the targeted disruption. Disruption construct was generated by double-joint polymerase chain reaction (PCR). The resulting PCR product was used directly in protoplast transformation. By homologous recombination, a part of *MCK1* including the sequences encoding the kinase domain and short flanking regions was replaced by *hph* cassette. Arrowheads indicate the location of primers used in reverse-transcription PCR. A bold line below the disruption construct represents the sequences used as a probe in Southern blot analysis. **B**, Southern blot analysis of mutants disrupted in *MCK1*. Genomic DNA was digested with *XhoI* and probed with flanking sequences of *HPH*. **C**, Expression analysis of *MCK1* in KJ201 and *mck1*, using actin as control. Gene disruption resulted in the loss of the transcript harboring the protein kinase domain in *mck1*.

Table 1. Comparison of mycelial growth, conidiation, germination, and appressorium formation among strains

Phenotypes	413B5	<i>mck1</i>	MC1-1	KJ201
Growth (cm) ^a	3.5 ± 0.2	3.3 ± 0.2	3.2 ± 0.2	3.3 ± 0.1
Conidiation (×10 ⁵ /ml) ^b	0.8 ± 0.3	0.6 ± 0.3	15.2 ± 1.1	17.1 ± 0.5
Germination (%) ^c	99.4 ± 0.6	99.4 ± 1.0	97.5 ± 2.9	98.4 ± 1.2
Appressorium (%) ^d				
4 hpi	13.2 ± 0.5	4.2 ± 2.3	49.4 ± 8.0	40.5 ± 2.6
6 hpi	62.9 ± 16.2	38.7 ± 21.6	83.3 ± 10.7	80.9 ± 7.7
8 hpi	82.6 ± 7.6	64.7 ± 13.5	95.2 ± 0.8	93.5 ± 3.8

^a Growth was measured as the diameter of mycelium at 5 days postinoculation.
^b Conidiation was measured as the number of conidia from the culture flooded with 5 ml of sterilized distilled water.
^c Germination was measured as the percentage ratio of germinating conidia to total conidia.
^d On inductive surface, appressorium formation was measured as the percentage ratio of appressorium-forming conidia to germinating conidia; hpi = hours postinoculation.

When the mycelia were examined by scanning electron microscopy (SEM), KJ201 showed a network of dense aerial hyphae, whereas the lysis zone of *mck1* mycelium was devoid of such a network and flooded with substances presumed to be

Fig. 3. Comparison of cell wall integrity between KJ201, *mck1*, and 413B5. **A**, Autolysis phenotype of *mck1* and 413B5 on oatmeal agar plates. The regions demarcated by rectangles (left panels) were examined by scanning electron microscopy ($\times 1,000$) (right panels). Insets in the right panels are the magnified view of hyphae of each strain ($\times 5,000$). Scale bar = 10 μ m. **B**, Protoplast production by cell-wall-degrading enzyme. Photographs were taken 1 h postincubation. Upper panels show the mycelia of KJ201 and *mck1* treated with cell-wall-degrading enzyme. The protoplast production was quantified in the lower panel at regular time intervals.

cell lysates (Fig. 3A, right panels). A magnified view of hyphal surfaces revealed that hyphae of *mck1* displayed coarse, porous surface in marked contrast to smooth hypha of KJ201 (Fig. 3A, insets), suggesting the defect in cell wall integrity.

To test cell wall integrity, an equal amount of mycelia from *mck1* and KJ201 was treated with cell-wall-degrading enzyme (Sigma-Aldrich, St. Louis). The *mck1* was hypersensitive to the enzyme treatment, producing protoplasts at a faster rate than KJ201. The mycelia of *mck1* started to release protoplasts within 10 min after enzyme treatment (200 mg ml⁻¹) and were turned mostly into protoplasts after 60 min at 25°C (Fig. 3B). This result is reminiscent of what was observed in the deletion mutant of *MPS1* (Xu et al. 1998), a previously studied MAPK in *M. oryzae* as a homologue of yeast *SLT2*, suggesting that *MCK1* is an upstream component of *MPS1* and is required for the maintenance of cell wall integrity. The *mck1*, like the *mps1*, did not show autolysis in a temperature-dependent manner. However, unlike the *mps1*, the autolysis phenotype of the *mck1* was not suppressed by addition of 1 M sorbitol to culture medium (data not shown).

***MCK1* is essential for conidiogenesis of *M. oryzae*.**

In the *mck1*, the ability to produce conidia (conidiation) is considerably impaired (Table 1). Conidiation of the mutant is only approximately 5% of that of the wild type. To explain this discrepancy, we compared conidiophore development of the wild type and mutant. The *mck1* developed conidiophores much less frequently than the wild type at 12 h postincubation (Fig. 4). Moreover, in many cases, only a single conidium was differentiated from each conidiophore of *mck1*, in contrast to the conidiophore of the wild type producing three to five conidia in a sympodial pattern. However, the amount of aerial hyphae was indistinguishable between KJ201 and *mck1*. This observation shows that *MCK1* is required for efficient conidiogenesis but not for aerial hyphae development. The time-dependent nature of the autolysis phenotype suggests that *MCK1* is involved in sustaining the aerial structures of the fungus. Thus, it is logical to think that a defect in conidiophore development and differentiation of conidia from the conidiophore, and autolysis of aerial structures, contribute, in combination, to the markedly reduced conidiation of *mck1*. Furthermore, when agar block was cut out of the region that had experienced complete autolysis and moved onto fresh media, hyphal growth originating from the agar block was observed within a few days (data not shown), indicating that fungal hyphae inside the agar block survived autolysis.

***MCK1* is indispensable for pathogenicity of *M. oryzae*.**

To investigate the role of *MCK1* in pathogenesis, conidial suspensions were sprayed onto rice seedlings of susceptible cv. Nagdongbye. Our pathogenicity assay consistently showed that *mck1* is nonpathogenic (Fig. 5A). The pathogenicity defect of *mck1* could be restored to the wild-type level in MC1-1 strain produced by the introduction of kinase domain using knock-in strategy (see Materials and Methods). This result demonstrates that *MCK1* is essential for fungal pathogenicity. To determine why *mck1* is unable to cause disease on its host, we first examined infection-related developments. Conidia produced by the *mck1* have normal morphology and germination rate, but appressorium formation was delayed by 2 to 3 h compared with wild-type KJ201 and knock-in complement MC1-1 (Table 1). Retardation of appressorium formation was not complemented by addition of cAMP (data not shown). In addition, a calcofluor staining pattern of appressoria showed no differences between the wild-type and *mck1* (data not shown). We concluded that conidial germination and appressorium formation were not significantly affected by *mck1* mu-

tation. To test if appressoria of *mck1* is functional, penetration and invasive growth were monitored using onion epidermis (Chida and Sisler 1987). Wild-type appressoria penetrated into epidermic cells and developed ramifying invasive hyphae (Fig.

5B, left panels), whereas mutant appressoria failed to penetrate plant cells (Fig 5B, right panels). These results show that *MCK1* is required for the fungus to breach the cuticle layer of the host plant.

Fig. 4. Development of aerial structures. Upper panels show conidiophore development. Arrows indicate the conidia borne on a conidiophore. Lower panels show scanning electron microscopic images of conidiophore and aerial hyphae. Scale bar = 50 μ m.

Fig. 5. Pathogenicity of KJ201, *mck1*, and MC1-1 (complement). **A.** Rice plants were spray inoculated with conidial suspensions (5×10^4 spores ml^{-1}). **B.** Penetration of onion epidermis and invasive growth. Conidial suspensions (2×10^4 spores ml^{-1}) were dropped on onion epidermis, and appressorium-mediated penetration was monitored under differential interference contrast (DIC) microscope 48 h postinoculation. A, appressorium; C, conidium; IH, invasive hyphae. Scale bar = 10 μ m. **C.** Rice leaves were wound inoculated using a 22-gauge syringe with conidial suspensions (5×10^4 spores ml^{-1}). Arrowheads indicate the site of inoculation.

Next, we examined the invasive growth of *mck1* by injecting the conidia of *mck1* into plant tissues using a 22-gauge syringe. This allowed direct entry of the fungus into host tissues through a wound site without appressorium. The wild-type strain developed large disease lesions but *mck1* was unable to develop disease lesions, indicating that the mutant lost its ability to grow inside the host plant. Based on these results, we hypothesized that inability of the mutant to proliferate within host tissue is attributable to its increased sensitivity to chemicals and proteins produced by host plants as a defense mechanism against the pathogen. To test this idea, we compared the growth of the mutant with that of the wild type in the medium containing lysing enzyme (LE) and hydrogen peroxide (H₂O₂) separately or in combination (Table 2). In the presence of either LE or H₂O₂, hyphal extension of *mck1* was slower than that of the wild type, and the combination of LE and H₂O₂ almost completely inhibited the growth of *mck1*. Furthermore, addition of ascorbic acid (10 mM or higher) as a scavenger of reactive oxygen species during wound inoculation enabled the mutant to develop disease lesion on rice plants (data not shown). Our data suggest that maintaining cell wall integrity plays pivotal roles in protecting the fungus against host defenses during invasive growth.

Estimation of turgor pressure suggests structural alteration in the appressorial wall of *mck1*.

To elucidate the nature of the defect in appressorium-mediated penetration of *mck1*, we measured the internal concentration of solute and turgor pressure within appressorium using an incipient cytorrhysis assay (Howard et al. 1991). In this assay, appressoria were allowed to form on a hydrophobic inductive surface for 48 h and incubated with glycerol solutions of varying concentration to quantify the proportion of collapsed appressoria. We expected that the proportion of collapsed appressoria would be either similar between the wild type and mutant or higher in the mutant, the latter of which indicates lower turgor pressure within mutant appressoria. Strangely, *mck1* appressoria tended to collapse less frequently than wild-type appressoria over the whole range of glycerol concentrations tested (Fig. 6A).

Appressoria generate turgor pressure by accumulating a high concentration of glycerol. A major source of appressorial glycerol is the lipid droplets that are mobilized from conidia to incipient appressoria in which rapid degradation of lipid bodies occurs at the onset of turgor generation (Thines et al. 2000). To delve into the lower cytorrhysis rate of the mutant, we were determined to examine the lipid mobilization. When we monitored the mobilization of lipid droplets, the wild-type strain translocated the lipid droplets fully to the incipient appressoria within 24 h and degraded them within 48 h. However, *mck1* showed slight retardation in translocation of the lipid droplets to appressoria (Fig. 6B), indicating that turgor pressure within the mutant appressoria is not as high as that within wild-type appressoria.

Next, we evaluated the porosity of an appressorial wall using a solute exclusion technique (Money, 1990), where appressoria are incubated in solutions of polyethylene glycols (PEGs) of different average molecular weights and screened

for cytorrhysis and plasmolysis. Cytorrhysis occurs when a solute molecule is excluded from the pores in the cell wall, whereas plasmolysis is the evidence of permeation. To test the effect of molecular size only, molar concentration of each PEG species was adjusted to generate an external osmotic pressure of 4MPa (Money 1989). For the wild type, the proportion of appressoria showing cytorrhysis remained dominant (ratio of plasmolysis to cytorrhysis = 0.18 and 0.07 in PEG 1000 and 8000, respectively). For the mutant appressoria, however, dominant cellular response shifted from plasmolysis to cytorrhysis (ratio of plasmolysis to cytorrhysis = 2.09 and 0.29 in PEG 1000 and 8000, respectively) as the diameter of solute species applied increased. This result indicates that the appressorial walls of the wild type and mutant differ significantly in porosity. Considering the role of *MCK1* in maintaining the integrity of the cell wall, our results strongly suggest that the cell wall structure of *mck1* appressoria is altered in a way that the solutes can diffuse through the pore of cell wall.

DISCUSSION

In our previous study, we had employed a forward genetics approach to discover pathogenicity genes in the rice blast fungus *M. oryzae* (Jeon et al. 2007). A large-scale insertional mutagenesis and extensive screens of the resulting mutants for phenotypes led us to identify one mutant, 413B5, displaying drastic phenotypic changes such as autolysis in vegetative culture and loss of pathogenicity. TAIL-PCR and sequencing revealed that one T-DNA insertion in the mutant is at 15 bp downstream of the stop codon of the locus MGG00883 (*MCK1*). Although T-DNA insertion did not occur within the open reading frame (ORF) of *MCK1*, we were unable to detect *MCK1* mRNA in our RT-PCR analysis for 413B5. It was reported previously in the study using transposon as a mutagen that downstream insertion in *MAC1*, the adenylate cyclase gene, results in premature termination of transcription and, consequently, dramatic reduction in mRNA level (Hamer et al. 2001). Based on our data, we conclude that downstream T-DNA insertion in *MCK1* generated an allele with markedly reduced gene expression and function.

Our analysis of sequence homology and phylogeny indicated that Mck1 is a MAPKKK closely related to Bck1, which is an integral component of the MAP kinase pathway regulating cell wall integrity of *S. cerevisiae*. To date, two MAP kinase pathways have been demonstrated to be required for pathogenicity of *M. oryzae*. These pathways involve Pmk1 and Mps1 MAP kinases, which are the homologues of yeast Fus3/Kss1 and Slt2, respectively (Xu and Hamer 1996; Xu et al. 1998). The *mck1*, like the *mps1* mutant, shows an autolysis phenotype and hypersensitivity to the cell-wall-degrading enzyme, suggesting that Mck1 is the MAPKKK that leads to the activation of Mps1. Hypersensitivity to LE also was observed in the deletion mutants of Mst7 and Mst11, but these mutants did not display autolysis (Zhao et al. 2005). It is conceivable that some of the cell-wall-related genes are dependent on the activation of both pathways for their transcription. To scrutinize the hypersensitivity to a cell-wall-degrading enzyme, we checked the expression of genes possibly involved in cell wall synthesis, such as chitin synthases and glucan synthase, using RT-PCR. However, no significant difference was detected between the mutant and wild type (data not shown). This may suggest that *MCK1* is involved in regulating the composition of the cell wall rather than its content.

In the deletion mutant of yeast *BCK1*, autolysis of cells was temperature sensitive and complemented by the addition of 1 M sorbitol to the media (Lee and Levin 1992; Martin et al. 1993). The autolysis phenotype of both the *mps1* and *mck1* is

Table 2. Comparison of growth under in planta mimicking condition^a

Strain	MM	MM+H ₂ O ₂	MM+LE	MM+H ₂ O ₂ +LE
<i>mck1</i>	1.5 ± 0.0	0.3 ± 0.2	2.0 ± 0.0	0.1 ± 0.0
KJ201	1.6 ± 0.1	1.0 ± 0.1	2.3 ± 0.0	1.0 ± 0.3

^a Growth was measured 6 days postinoculation as the diameter (cm) of mycelium. MM = minimal media, H₂O₂ = hydrogen peroxide (0.1 mM), and LE = lysing enzyme (2 mg ml⁻¹).

not temperature dependent but age dependent. However, autolysis was complemented by 1 M sorbitol only in the *mps1*, not in the *mck1*, suggesting that Mck1 may have additional interaction partners other than the one leading to the phosphorylation of Mps1.

In plant-pathogenic fungi, asexual spores (conidia) are the major source of primary inoculum and dissemination. In light of this, the ability to produce conidia is critical to an epidemic of the disease. Conidia of *M. oryzae* are produced by an expansion and swelling of the apex of the conidiophore in a sympodial pattern (Lau and Hamer 1998). In our analysis of early conidial ontogeny, the *mck1* showed a much lower number of conidiophores than the wild type. Autolysis of the aerial struc-

ture normally occurs in 5-day-old cultures on oatmeal agar plates. Therefore, we could rule out the possibility that autolysis decreases the number of conidiophores in the early stage of aerial structure development because conidiophore development was monitored for 24 h. In addition, *mck1* differentiated, in most cases, only one conidium from each conidiophore. A similar phenotype was observed in the study of the deletion mutant of *MPG1*, which encodes a class of fungal hydrophobin required for interaction with the hydrophobic surface and full virulence of *M. oryzae* (Talbot et al. 1996). In a filamentous basidiomycete fungus, *Schizophyllum commune*, it was shown that the gene encoding fungal hydrophobin, *SC3*, functions not only at hydrophilic–hydrophobic interfaces but also

Fig. 6. Estimation of turgor pressure within appressorium. **A**, Cytorrhysis assay using various concentrations of glycerol (1 to 5 M). For each glycerol concentration, at least 100 appressoria were observed and the number of collapsed appressoria was counted from two independent experiments. **B**, Cellular distribution of lipid droplets in KJ201 and *mck1*. Conidia of two strains were allowed to germinate and develop appressoria on plastic coverslips. At regular time intervals, two strains were stained with Nile Red, and developmental stages and epifluorescence were observed under differential interference contrast microscope. Scale bar = 20 μ m.

affects cell wall composition (van Wetter et al. 2000). These suggest the possibility that Mpg1 has roles in Mck1-mediated maintenance of cell wall integrity, which is required for efficient conidiation.

The role of *MCK1* in fungal pathogenicity was tested on rice leaves by spray inoculation of conidial suspensions. Rice leaves challenged with conidia of *mck1* did not develop any disease lesions but complementation of *mck1* restored the ability of the mutant to cause disease on rice to the wild-type level, indicating that *MCK1* is essential for pathogenicity of *M. oryzae*. In the assay using onion epidermis, it was shown that *mck1* develops appressorium at the tip of the germ tube but is unable to penetrate into plant tissue.

The cytorrhysis assay and cell wall pore measurement in appressoria of *mck1* suggested the altered structure of appressorial wall, indicating that Mck1-mediated maintenance of cell wall integrity plays an important role in appressorium-mediated host penetration. It is highly likely that the altered appressorial wall of *mck1* modifies the pore size of the cell wall, allowing the passage of solute molecules through large pores. This may account for the lower collapse rate of *mck1* appressoria during cytorrhysis assay. This pore-size-dependent diffusion of solute would impair the accumulation of turgor pressure within the appressorium considerably. In previous studies using the melanin-deficient mutant, the role of the melanin layer in reducing the pore size of the appressorial wall has been demonstrated nicely (Chumley and Valent 1989; Howard et al. 1991). However, melanin layer establishment cannot be separated from cell wall formation. Therefore, we reason that a defective cell wall would entail the uneven deposition of melanin, which can be resolved only at ultramicroscopic level, making the melanization of mutant appressoria appear normal.

It was suggested that formation of a penetration peg is regulated by Mst12 and essential for plant cells to initiate localized defense responses (Park et al. 2004). It appears that Mck1 is not involved in regulation of penetration hyphae formation. However, maintenance of cell wall integrity also may be required for the elaboration of penetration hyphae.

During the penetration and invasive growth, fungal pathogens encounter proteins and chemicals such as chitinase, pathogenesis-related proteins, and reactive oxygen species produced by host plants as defense responses (Hammond-Kosack and Jones 1996). For successful infection and colonization, the pathogen should incapacitate such defense responses by strengthening the cell wall or detoxifying the oxidative burst. Wound-inoculation experiments showed that the *mck1* lost its ability to grow inside plant tissue. Addition of LE or H₂O₂ to culture medium suppressed hyphal extension of *mck1* more severely than that of the wild type (Table 2), suggesting that Mck1 is involved in physically counteracting plant defenses through the remodeling of the cell wall.

In summary, these results suggest that Mck1 is an upstream component of Mps1 and that it is involved in the maintenance of cell wall integrity. Considering that *M. oryzae* has to undergo morphological transitions for reproduction and infection of its host, Mck1-mediated maintenance of cell wall integrity has profound implications for the pathogenic development of the fungus. The role of Mck1 may be of particular importance for the generation of turgor pressure, elaboration of penetration hyphae, and invasive growth. Further elucidation of genes and mechanisms underlying cell wall integrity and remodeling would shed light on the role of the fungal cell wall during the interaction between pathogen and host.

MATERIALS AND METHODS

Strains and culture conditions.

M. oryzae strain KJ201 was provided by the National Institute of Crop Science Institute (Suwon, Korea) and was used as the wild type throughout this work. The wild-type strain and all the mutants generated in this study were cultured on V8 (4% V8 juice) or oatmeal (50 g of oatmeal per liter) agar plates at 25°C under continuous fluorescent light to promote conidiation. Selection of hygromycin-resistant mutants was performed using TB3 media supplemented with hygromycin B at 200 µl ml⁻¹. Fungal mycelia were harvested from 3-day-old culture in liquid complete media (0.6% yeast extract, 0.6% casamino acid, and 1% glucose) and used for genomic DNA and total RNA extractions.

Multiple sequence alignment and phylogenetic analysis.

Amino acid sequences were aligned using ClustalW 1.83 (Thompson et al. 1994). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) with a bootstrap method using 1,000 repetitions and viewed in Tree-ViewX.

Isolation and manipulation of DNA and RNA.

Genomic DNA isolation was performed using two separate methods depending on the purpose. Genomic DNA for general experiments was prepared by the standard method (Sambrook et al. 1989). Genomic DNA of mutants for PCR screening was isolated as follows. Fungal mycelia on TB3 media was taken and ground in a 1.5-ml e-tube with 500 µl of extraction buffer (100 mM Tris-HCl, 10 mM EDTA, and 1 M KCl). Cell debris was removed by centrifugation at 5,000 rpm for 15 min and genomic DNA was pelleted by isopropyl alcohol. The pellet was washed with 70% alcohol and dissolved in sterilized, distilled water. Then, 2 µl of each DNA sample was used for PCR reactions, with a volume up to 20 µl containing 1 µl of deoxyribonucleoside triphosphates (2.5 mM dNTP mix), 2 µl of 10× PCR buffer, 1 µl of 100 nM each primer, and 1 unit of *Taq* polymerase. A Perkin-Elmer 9600 DNA Thermal Cycler was employed for 30 cycles of PCR reaction. Restriction enzyme digestion, agarose gel separation, cloning, and DNA gel blotting were performed following the standard protocols (Sambrook et al. 1989). A hygromycin probe of 1.4 kb was prepared by digestion of pCB1004 (Carroll et al. 1994) with *Hpa*I. Hybridization was carried out in a solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, and denatured salmon sperm DNA at 100 µl ml⁻¹, at 65°C. Blots were exposed to a phosphorimager analyzer (BAS-2040; Fuji Photo Film Co., Ltd., Tokyo) and visualized by phosphorimager analyzer software.

Total RNA was isolated from the frozen fungal mycelia using an Easy-Spin RNA extraction kit (iNtRON Biotechnology, Seoul, Korea). For RT-PCR, 3 µg of total RNA was

Table 3. Polymerase chain reaction primers used in this study

Name	Sequences (5' → 3') ^a
MCK5'F	TCAACTCCAAAATCATCAACAT
MCK5'R	<u>GCACAGGTACACTTGT</u> <u>TTAGAGAGTACACGCTGA</u> GAGGATAAC
MCK3'F	<u>CCTTCAATATCATCTTCTGTCGAGCTCGCTGTATA</u> TTGGGACTA
MCK3'R	TTCCCTTTTGTGATTGACTGC
HPHF	CGACAGAAGATGATATTGAAGG
HPHR	CTCTAAACAAGTGTACCTGTGC
MRT'F	CGATGGCCTGTTTGTATTTC
MRT'R	GATAAGAAGCGCATGAAGGA
TUBF	TCGACAGCAATGGAGTTTACAAC
TUBR	AGCACCAGACTGACCGAAGAC

^aUnderlined sequences are homologous to each end of *HPH*.

reverse-transcribed into first-strand cDNA with oligo dT primer using the SuperScript first-strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) according to the manufacturer's instruction. PCR was performed in 20 µl of reaction mixture containing 1 µl of cDNA from each sample, 1 µl of deoxyribonucleoside triphosphates (2.5 mM dNTP mix), 2 µl of 10× PCR buffer, 1 µl of 100 nM each primer, and 1 unit of *Taq* polymerase. In all, 25 cycles of RT-PCR were run on a Perkin-Elmer 9600 DNA thermal cycler.

Amplification of flanking sequences by TAIL-PCR.

TAIL-PCR was carried out as previously described (Liu and Whittier 1995), with modifications. Nested, T-DNA-specific primers were used together with arbitrary degenerate primers (AD primers), which are designed to differ in their annealing temperatures. Alternating cycles of high and low annealing temperature yielded specific products edged by a T-DNA-specific primer on one side and an AD primer on the other. Further specificity was obtained through subsequent rounds of TAIL-PCR (secondary and tertiary PCR) using nested T-DNA-specific primers. The reaction mixture from tertiary PCR was purified to exclude the remnant of primers and nucleotides, and directly sequenced with a specific primer designed for tertiary PCR.

Targeted disruption and complementation of *MCK1*.

Targeted disruption was performed using modified double-joint PCR (Yu et al. 2004). The region of approximately 2.5 kb, including putative kinase domain and short 3' flanking sequences, was replaced by an approximately 2.0-kb *hygB* cassette. Both 5' and 3' flanking regions of target sequences were amplified and fused to the *hygB* cassette using specific primer pairs with a 23-bp sequence tail in 5' reverse and 3' forward primer (Table 3, underlined sequences). An approximately 1.0-kb fragment of the 5' flanking region was amplified with primer pair MCK5'F and MCK5'R. An approximately 1.3-kb fragment of the 3' flanking region was amplified with primer pair MCK3'F and MCK3'R (Table 3). After fusion with the *hph* cassette, the nested primer pair amplified a final transformation construct of approximately 4.2 kb in size. Fungal protoplasts were directly transformed with double-joint PCR product following purification. Complementation of the *mck1* mutant was carried out by knock-in experiment. The kinase domain-containing fragment of *MCK1* (approximately 4.8 kb) was amplified in PCR using MCK5'F and MCK3'R as a pair of primers, and this fragment was co-transformed with a geneticin resistance gene into the protoplast of *mck1* mutant.

Vegetative growth, conidiation, and conidiophore development.

Vegetative growth was measured on complete media, oatmeal agar, and V8 juice agar plates on days 3 and 5 with three replicates. The ability to produce conidia was measured by counting the number of conidia from 5-day-old V8 agar plate. Conidia were collected by flooding the plate with 5 ml of sterilized distilled water. The number of conidia was counted using a hemacytometer under a microscope. Conidiophore development was monitored as previously described (Lau and Hamer 1998).

Protoplast production assay.

M. oryzae mycelia were cultured in complete media for 2 days and harvested by centrifugation for 10 min at 5,000 rpm. Mycelia were washed twice and resuspended with 20% sucrose. LE (Sigma-Aldrich) was added to a mycelial suspension (2 mg ml⁻¹). The lysis was stopped after 30, 60, and 90 min of incubation by placing the reaction tube in ice. The protoplasts were separated from the mycelia by filtration through Mira-

cloth. Protoplast release and cell wall degradation were examined with a light microscope and counted using a hemacytometer with three replicates.

Appressorium formation, turgor, and penetration assay.

Conidial germination and appressorium formation were measured on a plastic coverslip. Conidia were harvested from 8- to 10-day-old culture on an oatmeal agar plate with sterilized distilled water. A conidial suspension of 40 µl was pipetted onto a plastic coverslip following adjustment of its concentration to approximately 2×10^4 spores/ml. Drops were placed in a moistened box and incubated at 25°C. After 8 h of incubation, the percentage of conidia germinating and germinated conidia forming appressoria was determined by microscopic examination of at least 100 conidia per replicate in at least three independent experiments, with three replicates per experiment. Lipid droplets were stained with Nile Red and examined by epifluorescent microscopy as previously described (Thines et al. 2000). Appressorium turgor was measured by incipient cytorrhysis assay using a 1- to 5-molar concentration of glycerol solution as previously described (Foster et al. 2003). The onion penetration assay was performed as previously described (Xu et al. 1998). Onion epidermal strips were isolated and 15 µl of conidial suspension was inoculated onto the adaxial surface. After 48 h of incubation, onion epidermal strips were fixed with lactophenol (lactic acid/phenol/glycerol/water = 1:1:1:1) and infection hyphae and host cell autofluorescence were observed under differential interference contrast microscope.

Pathogenicity assay.

Conidia were harvested from an 8- to 10-day-old culture on an oatmeal agar plate with sterilized distilled water. Conidial suspension was adjusted to 5×10^4 spores/ml in concentration and spray inoculated on susceptible rice seedlings (cv. Nagdong) of three- to four-leaf stage. Inoculated plants were kept in dew chamber for 24 h and then moved to a greenhouse.

Cryoscanning electron microscopy.

Agar plugs taken from oatmeal cultures were mounted using two-sided adhesive carbon tape to a metal stub and plunged into liquid nitrogen for cryofixation for 20 s. Frozen specimens were placed in a cryo-chamber of a cryo-transfer system (CT1500; Oxford Instruments, Oxon, U.K.) maintained at -170°C and transferred to a cold specimen stage of a scanning electron microscope (JSM-5410LV; JEOL, Tokyo). Sublimation of superficial frozen water was performed by heating and maintaining the cold specimen stage at -60°C for 10 min, and the process was monitored with the electron microscope at an accelerating voltage of 5 kV. The specimens were sputter-coated with gold (approximately 30 nm in thickness) in the cryo-chamber, and observed with the electron microscope at 20 kV.

ACKNOWLEDGMENTS

This research was partially supported by a grant from Biogreen 21 project (20050401034629) funded by the Rural Development Administration, by a grant from the Crop Functional Genomics Center (CG1141) of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, and by Korean Research Foundation Grant (KRF-2006-005-J04701) to Y. H. Lee. J. Jeon, J. Goh, S. Yoo, M. Chi, and J. Choi gratefully acknowledge the graduate fellowship provided by the Ministry of Education through the BK21 program. We are grateful to Yangseon Kim for her valuable comments and suggestions on the manuscript.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Broad Institute's *Magnaporthe grisea* database:
www.broad.mit.edu/annotation/fungi/magnaporthe/
 National Center for Biotechnology Information's GenBank website:
www.ncbi.nlm.nih.gov/Genbank/
 TreeViewX program:
darwin.zoology.gla.ac.uk/~rpage/treeviewx/index.html