Two PAK Kinase Genes, *CHM1* and *MST20*, Have Distinct Functions in *Magnaporthe grisea*

Lei Li, Chaoyang Xue, Kenneth Bruno, Marie Nishimura, and Jin-Rong Xu

Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, U.S.A.

Submitted 1 August 2003. Accepted 11 December 2003.

In the rice blast fungus *Magnaporthe grisea*, the Pmk1 mitogen-activated protein (MAP) kinase is essential for appressorium formation and infectious growth. *PMK1* is homologous to yeast Fus3 and Kss1 MAP kinases that are known to be regulated by the Ste20 PAK kinase for activating the pheromone response and filamentation pathways. In this study, we isolated and characterized two PAK genes, *CHM1* and *MST20*, in *M. grisea*. Mutants disrupted in *MST20* were reduced in aerial hyphae growth and conidiation, but normal in growth rate, appressorium formation, penetration, and plant infection. In *chm1* deletion mutants, growth, conidiation, and appressorium formation were reduced significantly. Even though appressoria formed by *chm1* mutants were defective in penetration, *chm1* mutants were able to grow invasively on rice leaves and colonize through wounds. The *chm1* mutants were altered in conidiogenesis and produced conidia with abnormal morphology. Hyphae of *chm1* mutants had normal septation, but the length of hyphal compartments was reduced. On nutritionally poor oatmeal agar, *chm1* mutants were unstable and produced sectors that differed from original *chm1* mutants in growth rate, conidiation, or colony morphology. However, none of the monoconidial cultures derived from these spontaneous sectors were normal in appressorial penetration and fungal pathogenesis. These data suggest that *MST20* is dispensable for plant infection in *M. grisea*, but *CHM1* plays a critical role in appressorium formation and penetration. Both *mst20* and *chm1* deletion mutants were phenotypically different from the *pmk1* mutant that is defective in appressorium formation and infectious hyphae growth. It is likely that *MST20* and *CHM1* individually play no critical role in activating the *PMK1* MAP kinase pathway during appressorium formation and infectious hyphae growth. However, *CHM1* appears to be essential for appressorial penetration and *CHM1* and *MST20* may have redundant functions in *M. grisea*.

Additional keywords: Pyricularia grisea.

Rice blast, caused by *Magnaporthe grisea* (Hebert) Barr (anamorph *Pyricularia grisea* Sacc.), is one of the most severe diseases of rice throughout the world (Valent and Chumley 1991). *M. grisea* also causes disease on many other grass species, including economically important crops such as barley, wheat, and millet. It forms specialized infection structures known as appressoria for plant penetration. The fungus gener-

ates enormous turgor pressure in appressoria to penetrate the underlying plant surface (deJong et al. 1997; Howard et al. 1991). Once inside plants, inter- and intracellular growth of bulbous, lobbed invasive or infectious hyphae eventually results in lesion formation on infected leaves.

M. grisea forms appressoria on artificial hydrophobic surfaces that mimic rice leaf surfaces. The cAMP signaling pathway and a mitogen-activated protein (MAP) kinase gene *PMK1* have been shown to regulate surface recognition and appressorial maturation, respectively (Mitchell and Dean 1995; Xu and Hamer 1996). *PMK1* also is essential for appressorium penetration and infectious hyphal growth (Xu and Hamer 1996). The *pmk1* deletion mutants are nonpathogenic and fail to cause blast lesions on compatible rice plants. Recent studies in several phytopathogenic fungi, including *Ustilago maydis* (Brachmann et al. 2003; Mayorga and Gold 1999), *Fusarium oxysporum* (Di Pietro et al. 2001), *Fusarium graminearum* (Jenczmionka et al. 2003), *Botrytis cinerea* (Zheng et al. 2000), and *Claviceps purpurea* (Mey et al. 2002), have indicated that the *PMK1* pathway may be well conserved in fungal pathogens for regulating plant infection processes (Xu 2000). In all the appressorium-forming fungal pathogens examined to date, including *Colletotrichum lagenarium*, *Cochliobolus gloeosporioides*, *Cochliobolus heterostrophus*, and *Pyrenophora teres*, the *PMK1* homologs are essential for appressorium formation (Ruiz-Roldan et al. 2001; Xu 2000).

PMK1 is homologous to *Saccharomyces cerevisiae* *FUS3/KSS1*, and it can functionally complement the mating defect in a yeast *fus3 kss1* double mutant (Xu and Hamer 1996). In yeast, *FUS3* and *KSS1* are partially redundant MAP kinases functioning downstream from the MAP kinase kinase (MAPKK) Ste7 and MAPKK kinase (MAPKKK) Ste11 for regulating mating responses (Dohlman and Thorner 2001; Gustin et al. 1998). When pheromone-receptor binding results in the dissociation of an inhibitory G α subunit Gpa1 from stimulatory G $\beta\gamma$ subunits, Ste20 interacts with the liberated G β Ste4 and functions as a MAPKKK kinase (Dan et al. 2001) to activate the downstream MAP kinase cascade. In addition to playing an important role in mating responses, Ste20 and a few other elements of the pheromone-response pathway, including Ste11, Ste7, and Kss1, also are involved in regulating invasive growth and pseudohyphal development (often referred to as filamentation) in *S. cerevisiae* (Breitkreutz and Tyers 2002; Eby et al. 1998; Roberts and Fink 1994).

Ste20 is a member of the p21-activated kinase (PAK) family (Dan et al. 2001). PAK kinases have been implicated in various aspects of cytoskeletal reorganization regulated by Rho GTPases such as Rac and Cdc42. In *S. cerevisiae*, there are two other PAK kinases, Cla4 and Skm1. Ste20 and Cla4, but not Skm1, act as effectors of the Cdc42 GTPase and have diverse and partially redundant functions in organization of the actin

Corresponding author: Jin-Rong Xu; 915 West State Street, Lilly Hall, Purdue University; Telephone: 765-496-6918; Fax: 765-494-0363; E-mail: jinrong@purdue.edu

cytoskeleton during the cell cycle and morphogenesis (Eby et al. 1998; Holly and Blumer 1998). The *cla4 ste20* double mutant is not viable and fails to maintain septin rings at the bud neck and undergo cytokinesis. Whereas Ste20 mediates signaling of pheromone response and filamentation, Cla4 is involved primarily in budding and cytokinesis (Cvrckova et al. 1995). Skm1 does not play a redundant role with Ste20 or Cla4 in yeast and appears to be dispensable for growth and differentiation under laboratory conditions (Martin et al. 1997). In fission yeast *Schizosaccharomyces pombe*, Shk1 (Pak1) and Shk2 (Pak2) are two PAK kinases homologous to *Saccharomyces cerevisiae* Ste20 and Cla4, respectively. Pak1 is essential for viability and required for the establishment of proper cell polarity, mating, and cell cycle control (Marcus et al. 1995; Qyang et al. 2002). Pak2 is not essential for mating in *Schizosaccharomyces pombe*, but it interacts with Cdc42 to signal through the Mkh1p-Pek1p-Spm1p pathway for regulating normal cell morphology and growth (Merla and Johnson 2001).

To understand the role of PAK kinases in activating the *PMK1* MAP kinase pathway, in this study we isolated and characterized two PAK kinases in *M. grisea*. These two PAK kinase genes are homologous to yeast Cla4 and Ste20 and named *CHM1* and *MST20*, respectively. The *mst20* mutants were reduced in conidiation and aerial hyphae but had no

obvious defect in conidium morphology, appressorium formation, and plant infection. Deletion of *CHM1* had pleiotropic effects on growth, differentiation, and plant infection in *M. grisea*. The *chm1* mutants were reduced significantly in vegetative growth, conidiation, and appressorium formation. Appressoria formed by *chm1* mutants were nonfunctional and failed to penetrate intact rice leaves, but *chm1* mutants were able to infect and colonize plant tissue through wounds. Because *mst20* and *chm1* deletion mutants, in contrast to *pmk1* mutants, formed appressoria and were able to infect through wounds, it is likely that *MST20* and *CHM1* individually play no critical role in activating the *PMK1* MAP kinase pathway during appressorium formation and infectious hyphae growth. However, *CHM1* appears to be essential for appressorial penetration and *CHM1* and *MST20* may have redundant functions in *M. grisea*.

RESULTS

CHM1 and *MST20* are two PAK kinases closely related to Cla4 and Ste20 in yeast.

A polymerase chain reaction (PCR)-based strategy was used to isolate PAK kinases from *M. grisea* strain Guy11. After sequencing corresponding cosmid and cDNA clones, the *CLA4* homolog in *M. grisea* was found to encode an 856-amino acid (aa) protein that is 43% identical to the protein kinases Cla4 of *Saccharomyces cerevisiae*. It was named *CHM1* (Cla4 homolog in *M. grisea* 1; GenBank accession number AY057371). The other PAK kinase gene was named *MST20* (*M. grisea* *STE20* homolog; GenBank accession number AY332225). *MST20* encodes a 914-aa protein with 47% identity to Ste20 of *Saccharomyces cerevisiae*. In the recently available *M. grisea* 70-15 genome sequence, there is a predicted open reading frame identical with *CHM1* but no *MST20* in predicted genes. Only a portion of the *MST20* gene was found in contig 2.1205 of the 70-15 genome sequence.

The predicted proteins of *CHM1* and *MST20* contained a conserved C-terminal serine/threonine catalytic kinase domain and a p21-Rho-binding domain (PBD) in its N-terminal non-catalytic region (Fig. 1A), typical structural features of PAK kinases. Similarly to Cla4 and Cla4-like PAK kinases, *CHM1* had a pleckstrin homology (PH) domain that was absent in *MST20* (Fig. 1A). PAK kinases homologous to *CHM1* and *MST20* were found in recently available *Neurospora crassa* and *Fusarium graminearum* genome sequences. Phylogenetic analysis with PAK kinases from *Saccharomyces cerevisiae* and filamentous fungi clearly separated *CHM1* and *MST20* homologs into two different clades (Fig. 1B). *CHM1* and *MST20* have the highest degree of identity with the *Fusarium graminearum* homolog of Cla4 (79% identity) and *N. crassa* homolog of Ste20 (67% identity), respectively. *MST20* has the C-terminus Gβ binding site (SHLSPLVRAAR) that is conserved among Ste20 homologs from fungi and mammals (Leeuw et al. 1998). The underlined residues are identical among Mst20, Ste20, and their homolog in *N. crassa*.

MST20 is dispensable for appressorium formation and plant infection in *M. grisea*.

To determine the function of *MST20*, a gene replacement vector, pCX32, was constructed by replacing a 3.5-kb fragment of the *MST20* gene with the hygromycin phosphotransferase (*hph*) gene (Fig. 2A). After transforming Guy11 with pCX32, 50 hygromycin-resistant transformants were isolated and screened by PCR with primers P3 and P5 (Fig. 2A). Two putative *mst20* deletion mutants, ms45 and ms85, were identified. When Southern blots of DNAs digested with *Pst*I were hybridized with a *Pst*I fragment deleted in pCX32 (Fig. 2A,

Fig. 1. Structures and phylogenetic relationships of *CHM1* and *MST20*. **A**, Structures of *MST20* and *CHM1* PAK kinases. The pleckstrin homology (PH), p21-Rho-binding domain (PBD), and kinase catalytic domain (kinase) were indicated with hatched, filled, and open boxes, respectively. *MST20* had a putative Gβ-interacting site (Gb) at the C-terminus. **B**, Phylogenetic comparison of fungal PAK kinases. The amino acid sequences of PAK kinases from *Magnaporthe grisea* (Chm1 and Mst20), *Saccharomyces cerevisiae* (Cla4, Ste20, and Skm1), *Ashbya gossypii* (AgCla4), *Fusarium graminearum* (FgCla4 and FgSte20), and *Neurospora crassa* (NcCla4 and NcSte20), were analyzed by the Clustal X (Thompson et al. 1997).

probe 1), the wild-type Guy11 had a 2.1-kb band (Fig. 2B). There was no detectable signal in ms45 and ms85 (Fig. 2B). When the same blot was stripped and rehybridized with a 1.4-kb *NcoI* *KpnI* fragment from pCX32 (Fig. 2A, probe 2), Guy11 had a 1.0-kb band that was absent in both *mst20* deletion mutants (Fig. 2B). Instead, ms45 and ms85 had a 2.5-kb band diagnostic of the gene replacement event. These data indicate that ms45 and ms85 are true *mst20* mutants resulting from gene replacement.

Both *mst20* mutants had a normal growth rate on a variety of media tested, including oatmeal agar and complete medium (CM) or 5× YEG (Zheng et al. 2000). However, colonies formed by *mst20* mutants produced significantly fewer aerial

Fig. 2. *MST20* knockout construct and mutants. **A**, Restriction map of the *MST20* genomic region and knockout construct. Thick arrows indicate orientations of the *MST20* and hygromycin phosphotransferase (*hph*) genes. The restriction enzymes are *ApaI* (A), *BamHI* (B), *KpnI* (K), *NcoI* (N), *PstI* (P), *XhoI* (X), and *XbaI* (Xb). The *XbaI* and *KpnI* sites introduced during the cloning processes are marked by *. The *MST20* knockout construct pCX32 was constructed by replacing the *BamHI* *XhoI* 1.6-kb fragment of *MST20* with the *hph* gene. Dotted lines indicate the flanking sequences of *MST20* used for constructing pCX32. P3, P5, P7, and P8 are polymerase chain reaction primers used for screening *mst20* knockout transformants. **B**, Southern blot analyses of *mst20* knockout mutants. Genomic DNAs of the wild-type strain (Guy11) and the *mst20* knockout mutants (ms45 and ms85) were digested with *PstI*. The blot on the left was hybridized with a 2.1-kb *PstI* fragment from pCX31 (probe 1). On the right was the same blot stripped and re-hybridized with a 1.4-kb fragment released from pCX32 with *NcoI* and *KpnI* (probe 2). The size of hybridization bands was labeled on the left (in kilobases). **C**, Colony morphology of the wild-type strain (Guy11) and an *mst20* knockout mutant (ms85). Oatmeal cultures were photographed after incubating at room temperature for 2 weeks.

hyphae and appeared to be less pigmented than those of Guy11 under the same incubation conditions (Fig. 2C). Although conidium morphology was normal in *mst20* mutants, conidiation was reduced approximately 95% in comparison with that of Guy11 (Table 1). Both *mst20* mutants formed melanized appressoria that could penetrate onion epidermal cells as efficiently as Guy11. Leaves sprayed with *mst20* mutants developed typical blast lesions (data not shown). No obvious defect in infection-related morphogenesis was observed in *mst20* mutants, indicating that *MST20* is dispensable for appressorium formation, penetration, and infectious growth. These processes are known to be regulated by *PMK1*; therefore, it is likely that *MST20* does not play an essential role in activating the *PMK1* MAP kinase cascade in *M. grisea*. Both *mst20* mutants were female sterile. However, the ectopic transformant ms39 also was female sterile, indicating that female fertility may have been lost during transformation. When the wild-type *MST20* allele was reintroduced into ms85, female fertility was not restored in the resulting transformants, indicating that loss of female fertility in the *mst20* mutants may not be directly related to deletion of *MST20*.

The *chm1* gene replacement mutant had pleiotropic phenotypes in growth and conidiation.

The *CHM1* knockout vector pJX74 was constructed by replacing a 2.2-kb fragment of *CHM1* with the *hph* gene (Fig. 3A) and transformed into Guy11 protoplasts. Two putative *chm1* gene replacement transformants, MC3 and MC55, were identified by screening with primers C4 and H2 (Fig. 3A). When Southern blots of genomic DNAs digested with *EcoRI* were hybridized with the 5.6-kb *EcoRI* fragment from pNX41, MC3 and MC55 had a 4.5-kb band, whereas the wild-type strain Guy11 had a 5.6-kb band (Fig. 3B). In a randomly selected ectopic transformant, MC52, both 5.6- and 4.5-kb bands were observed. When the same blot was rehybridized with the *hph* gene from pCB1003, all the transformants, but not Guy11, had the 4.5-kb band (data not shown). These data indicated that MC3 and MC55 were true gene replacement mutants of *CHM1*. These two *chm1* mutants had the same phenotypes described below.

The *chm1* deletion mutants produced typical grayish *M. grisea* mycelia, but their growth rate was significantly reduced when measured in race-tube cultures (Table 1). On oatmeal agar, MC55 grew less than 2 mm/day on average. The growth rate of Guy11 was 3.3 mm/day under the same incubation conditions. On CM, a similar reduction in growth rate was observed in the *chm1* mutant, although both Guy11 and MC55 grew slightly faster on CM than on oatmeal agar (Table 1).

In addition to the growth rate, conidiation also was reduced significantly in the *chm1* deletion mutants. On average, 2-week-old oatmeal cultures of Guy11 produced approximately 10,000 times more conidia than the *chm1* mutants (Table 1). We observed that *chm1* mutants produced fewer conidiophores than

Table 1. Growth rate and conidiation

Strain	Growth rate (mm/day) ^a		Conidiation
	Oatmeal agar	Complete medium	(×10 ⁵ conidia per plate)
Guy11 (wild-type)	3.3 ± 0.3	3.8 ± 0.0	44.27 ± 6.47
MC55 (<i>chm1</i>)	1.8 ± 0.1	1.9 ± 0.2	0.04 ± 0.01
CC3 (<i>Chm1</i> ⁺)	2.7 ± 0.6	3.3 ± 0.0	39.80 ± 6.30
RCH1 (sector)	1.7 ± 0.0	2.1 ± 0.0	5.52 ± 1.30
ms85 (<i>mst20</i>)	3.2 ± 0.1	3.6 ± 0.2	2.80 ± 1.20

^a Growth rate was measured on race tubes after 35 days. Average growth rates and standard errors (mean ± standard error) were calculated from at least three independent measurements.

Guy11. The average number of conidiophores formed by 2-week-old oatmeal cultures was $1.0 \pm 0.6/\text{mm}^2$ in MC55 and $19.5 \pm 6.5/\text{mm}^2$ in Guy11. Conidium morphology also was altered in the *chm1* mutants. Most of conidia produced by MC3 and MC55 were irregular in shape and often two-celled, in contrast to three-celled pyriform conidia formed by Guy11 (Fig. 4). When examined under the scanning electronic microscope, a single conidium often was found to be terminally born on branched conidiophores in MC3 (Fig. 4). The wild-type strain produced conidia sympodially on unbranched conidiophores. The conidiophores of *chm1* mutants were approximately twice as thick as those of Guy11 (Fig. 4). The constriction at the base of wild-type conidia where conidia are attached to the conidiophores was absent or incompletely formed in *chm1* mutants (Fig. 4).

The *chm1* mutant was defective in appressorium formation and penetration.

Although conidia from *chm1* mutants had altered morphology, over 95% of them germinated after 24 h of incubation at room temperature. However, appressorium formation was reduced significantly in *chm1* mutants. While over 90% of the Guy11 germ tubes produced appressoria on plastic coverslips by 24 h, less than 5% of germ tubes formed appressoria in MC3 and MC55 under the same conditions. Even after prolonged incubation (over 72 h), no increase in appressorium formation was observed (data not shown). Addition of 10 mM cAMP or 2.5 mM 3-isobutyl-1-methylxanthin (IBMX) to conidial suspensions of MC55 had no stimulatory effect on

appressorium formation (data not shown), suggesting that the reduction in appressorium formation in *chm1* mutants cannot be suppressed with exogenous cAMP and is not likely related to defects in surface recognition. It is possible that *chm1* mutants are defective in specific cytoskeleton reorganization associated with appressorium formation.

Appressoria formed by *chm1* mutants on plastic coverslips and onion epidermal cells appeared to be melanized and normal in morphology (Fig. 5). However, these appressoria were defective in plant penetration. Although the majority of appressoria formed by the wild-type strain Guy11 and the *mst20* mutant ms85 penetrated and produced typical bulbous infectious hyphae in the onion epidermal cells by 48 h after inoculation, no penetration or infectious hyphae was observed in *chm1* mutants (Fig. 5) even after prolonged incubation (up to 3 days), suggesting that *chm1* mutants were defective in appressorial penetration.

CHM1 is defective in infecting intact leaves but dispensable for colonizing through wounds.

When 2-week-old rice CO-39 seedlings were sprayed with conidia from MC55, no lesions developed on inoculated leaves up to 7 days post inoculation (Fig. 6A). Rice leaves sprayed with Guy11 and the ectopic transformant MC52 developed typical rice blast lesions under the same conditions (Fig. 6A). Similarly, no lesions were observed on leaves of 8-day-old seedlings of barley cv. Golden Promise sprayed with conidia from *chm1* mutants (data not shown). These results indicate that MC55 is nonpathogenic and *CHM1* is essential for infecting healthy plants.

Appressoria formed by *chm1* mutants were defective in penetration; therefore, we used both injection inoculation and abrasion infection to determine whether *chm1* mutants were able to infect rice plants through wounds. In the injection infection assays, leaves inoculated with Guy11 developed typical spreading blast lesions in and around the injection sites (Fig. 6B). In contrast, wound sites injected with the *pmk1* mutant nn78 developed very limited plant cell necrosis. Injections with MC55 resulted in large areas of necrosis at the injection sites (Fig. 6B). However, the *chm1* mutants did not form any lesions outside injection sites because of their defects in appressorial penetration (Fig. 6B). On rice leaves abraded with diato-

Fig. 3. Gene replacement of *CHM1*. **A**, The *CHM1* genomic region and gene replacement construct. Thick arrows indicate orientations of the *CHM1* and hygromycin phosphotransferase (*hph*) genes. The restriction enzymes are *Bam*HI (B), *Eco*RI (E), *Not*I (N), *Sac*I (S), and *Xho*I (X). The *Eco*RI site derived from cloning vector pCB1003 is marked by *. Dotted lines indicate the flanking sequences of *CHM1* cloned on the gene replacement vector pJX74. C1, C2, C4, and H2 are primers used for generating and screening *chm1* knock-out mutants. **B**, Southern blot of wild-type (Guy11), ectopically inserted transformant (MC52), *chm1* gene replacement transformants (MC3 and MC55), and a *CHM1* complementation strain of MC3 (CC3). All DNA samples were digested with *Eco*RI. The blot was hybridized with the *Eco*RI 5.6-kb fragment from pNX41. The sizes of the wild-type band and diagnostic band resulting from gene replacement were 5.6 and 4.5 kb, respectively.

Fig. 4. Conidium and conidiophore morphology. The wild-type strain (Guy11) produced typical three-celled pyriform conidia. The majority of conidia produced by the *chm1* deletion mutant (MC55) were two-celled and irregularly shaped. When examined under a scanning electronic microscope, Guy11 formed multiple conidia sympodially on conidiophores. In MC55, conidia were born terminally on branched conidiophores. C = conidium, CP = conidiophore, and BA = basal appendage where conidia attach to conidiophores. Bars represent 10 μm .

maceous earth and inoculated with the *chm1* mutant, blast lesions were visible 5 days after inoculation (Fig. 6C). No blast lesions developed on abraded leaves inoculated with conidia from the *pmk1* or *mst12* mutants (Fig. 6C). In addition, conidiation was observed in the necrotic area at the injection sites and lesions formed abraded leaves caused by the *chm1* mutant after incubating on water agar for 2 to 3 days (data not shown). These data indicate that, although *CHM1* is required for appressorial penetration and infecting intact leaves, it is dispensable for infection through wounds and infectious hyphae growth inside plant tissue.

***CHM1* is required for ascospore viability.**

To confirm that all the phenotypes described above are caused by the disruption of the *CHM1* gene, we introduced a wild-type *CHM1* allele into the *chm1* mutant MC55 by co-transformation of pNX41 (Fig. 3A) with pAC905. All five zeocin-resistant transformants with an ectopically integrated wild-type *CHM1* gene, including CC3 (Fig. 3B), grew as fast as Guy11 and produced abundant three-celled pyriform conidia (Table 1). Appressorium formation and plant penetration in CC3 also were restored to the wild-type level (data not shown). Thus, *CHM1* complemented all the defects observed in *chm1* mutants and deletion of *CHM1* is responsible for all the phenotypes described above. We also transformed *N. crassa* cosmid clone H071G3 containing the *NcCLA4* gene into MC55 by co-transformation with pAC905. Two resulting transformants with H071G3 integrated in the genome were phenotypically similar to CC3 (data not shown), indicating that *NcCLA4* functionally complements the *chm1* mutant.

To further confirm that all the phenotypes described above are caused by the disruption of the *CHM1* gene, we crossed MC55 (*MAT1-2*) with 2539 (*MAT1-1*). Fertile perithecia were formed by MC55, although the number of perithecia was reduced in comparison with Guy11. However, none of the monoconidial isolates derived from over 70 asci analyzed was the *chm1* deletion mutant. We also analyzed 20 asci from a cross between

CC3 and 2539. Southern blot analysis indicated that eight progeny carried both the *chm1* deletion and the ectopically integrated *CHM1* gene, but none of the progeny examined had the *chm1* deletion alone without the ectopic *CHM1* allele. Failure to isolate *chm1* progeny may be related to its reduction in vegetative growth and conidiation, but it also is possible that deletion of *CHM1* had detrimental effects on ascospore viability.

Hyphal growth was affected by deletion of *CHM1*.

The *chm1* deletion mutants still displayed normal polarized growth at hyphal tips and grew by hyphal extension and branching. The hyphal tip cells of *chm1* mutants had regular morphology and contained a single nucleus as normally observed in Guy11 (Fig. 7). The *AgCla4* gene is required for septum formation in *Ashbya gossypii* (Ayad-Durieux et al. 2000); therefore, we also examined septation in *chm1* mutants by staining with Calcofluor. Septa were observed in conidia, germ tubes, and vegetative hyphae grown in solid or liquid media (Figs. 4 and 7). Like the wild-type strain Guy11, *chm1* mutants had one nucleus in each hyphal compartment (Fig. 7), suggesting that cytokinesis was normal in *chm1* mutants. These data indicate that *CHM1* is dispensable for septum formation and maintaining hyphal tip growth in *M. grisea*. However, the average length of hyphal compartments (distance between two septa) was reduced from $33.3 \pm 3.5 \mu\text{m}$ (mean $\pm$ standard error) in Guy11 to $16.84 \pm 2.9 \mu\text{m}$ in MC55. The width of MC55 hyphae ($2.6 \pm 0.5 \mu\text{m}$) was similar to that of Guy11 ($2.5 \pm 0.5 \mu\text{m}$). The *chm1* mutants appeared to branch more frequently than Guy11, but this phenotype could be a consequence of the shortened hyphal compartments.

Spontaneous mutations in the *chm1* mutants.

When cultured on oatmeal agar plates, *chm1* mutants were unstable, and various sectors became visible in most *chm1* colonies older than 7 days (Fig. 8A). However, *chm1* mutants

Fig. 5. Appressoria formed by *chm1* mutants were nonfunctional for penetration. Panels on the left are appressoria formed on Teflon membranes by the wild-type strain Guy11, the *chm1* deletion mutant MC3, and the *mst20* deletion mutant ms85. Panels on the right are appressoria formed by Guy11, MC3, and ms85 on onion epidermal cells. A = appressorium, C = conidium, IF = infectious hyphae. Bar represents 10 μm .

Fig. 6. Infection assays with *chm1* mutants. **A**, Rice leaves sprayed with *Magnaporthe grisea* conidia. The wild-type (Guy11) and one *Chm1*⁺ complemented transformant (CC3) caused numerous lesions on CO-39 seedlings. Leaves inoculated with 0.25% gelatin (Gelatin) or conidia from the *chm1* mutants (MC3 and MC55) and *pmk1* mutant (nn78) had no lesions. **B**, Leaves of rice CO39 seedlings inoculated by injection with conidia from the wild-type (Guy11), *chm1* mutant (CH55), and *pmk1* mutant (nn78). **C**, Abraded leaves of rice CO39 seedlings inoculated with conidia from Guy11, CH55, and nn78. Typical leaves were photographed 6 days after inoculation.

appeared to be stable and rarely produced sectors when cultured on CM or TB3 (Hou et al. 2002) plates. The instability of *chm1* mutants may be related to nutritional conditions because supplementation of oatmeal agar with 2% yeast extract and 2% glucose reduced the sectoring frequency of *chm1* mutants to the same level as that when they were cultured on CM or TB3 plates (data not shown). In *M. grisea*, the *mac1* mutant deleted from the adenylate cyclase gene is known to produce abundant spontaneous mutations, which can be suppressed with exogenous cAMP (Adachi and Hamer 1998). In oatmeal agar plates supplemented with 10 mM cAMP or 2.5 mM IMBX, *chm1* mutants still formed abundant sectors (data not shown), indicating that exogenous cAMP could not suppress sectoring in *chm1* mutants.

We isolated single spores from 39 sectors from MC3 and MC55 cultures. Most of the resulting monoconidial strains were stable and grew without forming sectors on oatmeal agar plates. Many of them, including S0, S3, S5, S7, and RCH1, were increased in conidiation and appressorium formation, and often produced three-celled conidia. However, as the original *chm1* mutants, none of them were able to penetrate onion epidermal cells and infect healthy rice leaves (data not shown). Interestingly, some of these spontaneous mutants displayed unique defects in appressorium morphogenesis. In S0 and S5, the majority of germlings (>75%) formed 'elongated' appressoria on hydrophobic surfaces (Fig. 8B). It appeared that germ-tube tips of S0 and S5 were differentiated into appressoria without much swelling. These appressoria were melanized but failed to penetrate onion epidermal cells. In S3, S7, and RCH1, over 90% of the conidia produced twisted germ tubes on hydrophobic but not on hydrophilic surfaces (Fig. 8B). These germlings curled clockwise and failed to differentiate into appressoria. This phenomenon was not observed in vegetative hyphae of these sectors grown on oatmeal agar plates or in liquid CM. It is possible that these mutants had different cytoskeleton defects associated with appressorium morphogenesis.

DISCUSSION

PAK kinases are conserved in eukaryotic cells and involved in various cellular signaling and developmental processes as effectors of Rho-family GTPases, including Rho, Rac, and Cdc42 (Dan et al. 2001; Daniels and Bokoch 1999). *Chm1* and

Mst20 have typical structural components of PAK kinases, with a conserved catalytic domain and a PBD or Cdc42-Rac interactive binding (CRIB) domain. In *Saccharomyces cerevisiae*, the PAK kinase *Ste20* plays critical roles in activating the pheromone and filamentation pathways via the *Fus3* and *Kss1* MAP kinases (Breitkreutz and Tyers 2002; Eby et al. 1998). *Pmk1* is the only ortholog of yeast *Fus3* and *Kss1* in *M. grisea* and it is essential for appressorium formation and infectious hyphae growth (Xu and Hamer 1996). To our surprise, *MST20* is dispensable for appressorium formation, penetration, and infectious growth. Similarly to *pmk1* mutants, mutants of *M. grisea* deleted from the *Ste7* or *Ste11* homolog failed to form appressoria and were unable to infect and colonize healthy or wounded rice leaves (J.-R. Xu, unpublished data). However, *mst20* deletion mutants had no obvious defect in appressorium formation and lesion development, indicating that the *PMK1* MAP kinase cascade is activated by upstream elements other than *MST20*.

In *Saccharomyces cerevisiae*, the interaction of Cdc42 with *Ste20* mediates signaling in the haploid invasive growth and pheromone response (Ash et al. 2003; Lamson et al. 2002). Recently, the Cdc42 homolog has been characterized in *M. grisea*. Mutants deleted from *MgCDC42* are viable and still form melanized appressoria that are capable of penetrating onion epidermal cells and cause lesions on rice seedlings (S.-C. Wu, personal communication). Deletion of *MST20* fails to produce any phenotype similar to that of *pmk1* mutants and *MgCDC42* appears to be dispensable for appressorium formation; therefore, it is unlikely that the function of *MST20* closely parallels that of *Ste20* in *Saccharomyces cerevisiae*.

CEK1, *CST20*, and *HST7* in *Candida albicans* are functional homologs of the *Saccharomyces cerevisiae* *FUS3/KSS1*, *STE20*, and *STE7* genes, respectively (Kohler and Fink 1996; Leberer et al. 1997; Liu 2002). Homozygous mutants deleted from *CEK1*, *CST20*, or *HST7* genes are significantly attenuated in virulence (Csank et al. 1998; Liu 2002), and have defects in switching from unicellular budding to invasive hyphal growth on certain media. However, hyphal development is not

Fig. 7. Septation and hyphal branching in the *chm1* mutant. Vegetative hyphae of the wild-type strain (Guy11) and the *chm1* mutant (MC3) were stained with Calcofluor and Hoechst 33258 and examined under phase contrast (DIC) and UV light (UV). N = nucleus, S = septum. Bars represent 10 μ m.

Fig. 8. Instability of *chm1* mutants. **A**, Sectors were produced *chm1* deletion mutants MC3 and MC55 grown on oatmeal agar plates (MC3-O and MC55-O). No or only a rare sector was visible in *chm1* mutants when cultured on complete medium (MC3-CM). Photos were taken after incubating at room temperature for 12 days after inoculation. **B**, Conidia and appressoria produced by spontaneous mutants S5 and RCH1. Germ tubes of RCH1 usually curled clockwise (view from the germination site on conidium) and failed to form any appressoria. Appressoria formed by sector S5 were melanized but appeared to be elongated without swelling.

impaired in some other media and still induced by serum in those mutants (Csank et al. 1998; Kohler and Fink 1996; Leberer et al. 1997). Interestingly, mating tests between *Candida albicans* strains with altered mating type loci have indicated that *CEK1* and *HST7* are essential but *CST20* is dispensable for mating (Magee et al. 2002). Therefore, multiple signaling pathways regulating mating, hyphal development, and infection processes must exist in *Candida albicans*. Similar regulatory mechanisms probably exist in *M. grisea*. In *U. maydis*, *don3* encodes a Ste20-like protein kinase that controls the separation of mother and daughter cells (Weinzierl et al. 2002). However, similar to *mst20* mutants in *M. grisea*, the *don3* deletion mutant has no defect in filamentous growth of the dikaryon and plant infection (Weinzierl et al. 2002). In another basidiomycete, *Cryptococcus neoformans*, three PAK kinase genes have been characterized, including *MAT α* - and *MAT α* -specific *STE20 α* and *STE20 α* , and *MAT* locus-unlinked *PAK1* (Wang et al. 2002). Only *PAK1* contains the PH domain, and, structurally, it is more similar to *Saccharomyces cerevisiae* *STE20* than *STE20 α* or *STE20 α* . The *pak1* deletion mutants of serotype A and serotype D are attenuated for virulence, but deletion of *STE20 α* reduces virulence only in serotype A but not serotype D. Interestingly, capsule size and viability at elevated temperature, two known virulence factors in *Cryptococcus neoformans*, are reduced only in the serotype A *ste20 α* mutant but not in the *pak1* mutants (Wang et al. 2002). To determine whether the mating-type locus had any effect on the function of *CHM1* in *M. grisea*, we also isolated a *chm1* deletion mutant in the wild-type strain 70-15 (*MAT1-1*). The phenotype of the *chm1* mutant of 70-15 was similar to that of CH55 (data not shown).

In *Candida albicans*, *CaCla4* is critical for virulence. The homozygous *cacla4* deletion mutant is reduced in colonization of kidneys in infected mice (Leberer et al. 1997). In *M. grisea*, *chm1* deletion mutants were pleiotropic and defective in several steps of infection-related morphogenesis. Conidia formed by *chm1* mutants had abnormal morphology and were reduced significantly in appressorium formation. In contrast to the *magB* and *mpg1* mutants of *M. grisea* that are known to be reduced in appressorium formation (Liu and Dean 1997; Talbot et al. 1993), *chm1* mutants did not respond to exogenous cAMP and were defective in appressorial penetration. The *chm1* mutants were unable to infect intact healthy rice leaves but were capable of colonizing plant tissues through wounds (Fig. 5). This phenotype of *chm1* mutants is similar to that of the *cpkA*, and *pls1* (*punchless*) mutants that are known to be defective in appressorial penetration in *M. grisea* (Clergeot et al. 2001; Mitchell and Dean 1995). Preliminary data with the cytorrhysis assay (Howard et al. 1991) indicated that there was no significant difference in appressorium turgor between the *chm1* mutant and the wild-type strain Guy11. It is possible that appressoria formed by the *chm1* mutant are defective in ultra-structure or penetration peg formation.

Both *chm1* and *mst20* mutants were able to form appressoria and colonize plant tissue through wounds; therefore, we conclude that *CHM1* and *MST20* individually are not essential for appressorium formation and infectious growth, two major processes regulated by *PMK1*. *CHM1* apparently plays a major role in conidiation, appressorium formation, and plant penetration, but *MST20* also is involved in conidiation and aerial hyphal growth. These two genes may have some redundant functions in fungal growth and infection-related morphogenesis. The *chm1* ascospores appear to be not viable; therefore, we attempted to isolate *chm1 mst20* double mutants by transforming the *CHM1* gene replacement construct pJX74 into the *mst20* mutant ms85. After screening over 450 transformants derived from six independent transformations, no *chm1*

mst20 double mutant was identified, indicating that deletion of both *CHM1* and *MST20* may be lethal in *M. grisea*. In *Saccharomyces cerevisiae*, the *ste20 cla4* double mutant is not viable. In *Cryptococcus neoformans*, *ste20 α pak1* and *ste20 α pak1* double mutants are synthetically lethal, indicating that these PAK kinases share an essential function in fungal growth (Wang et al. 2002).

CaCla4 also is essential for hyphal development on synthetic media and in infected mice in *Candida albicans* (Leberer et al. 1997). In another yeast, *Yarrowia lipolytica*, deletion of *CLA4* completely eliminates its ability to grow in a hyphal form and invade agar (Szabo 2001). The *chm1* mutants of *M. grisea* were viable and had no defect in maintenance of polarized growth of the hyphal tip. However, the growth rate and the average length of hyphal compartment (distance between two septa) were reduced in the *chm1* mutants. It appears that *chm1* mutants were reduced in the hyphal extension rate and branched more frequently than Guy11 (Fig. 7). This phenotype of *chm1* mutants is similar to what has been observed in the *Ashbya gossypii* mutant deleted from the *CLA4* homolog (AgCla4). *A. gossypii* is a filamentous fungus closely related to *Saccharomyces cerevisiae*. The AgCla4 deletion mutant is blocked in the slow-growing phase and produces short filaments with premature branchings (Ayad-Durieux et al. 2000). Whereas the *agcla4* mutant is defective in septation (Ayad-Durieux et al. 2000), hyphae of *chm1* mutants had evenly distributed septa and contained one nucleus in each compartment (Fig. 7).

In addition to reduction in conidiophore development, *chm1* mutants produced conidia terminally instead of sympodially on branched conidiophores. The constriction at the attachment site between conidia and conidiophores was incomplete in *chm1* mutants (Fig. 4) and *chm1* conidia had wider appendages. Thus, *CHM1* apparently regulates conidiophore and conidium development in *M. grisea*. In *Saccharomyces cerevisiae*, *CLA4* is required for the correct localization and assembly of the septin ring, which is involved in maintaining the neck constriction (Schmidt et al. 2003). In *Cryptococcus neoformans*, the *ste20 α* mutant forms elongated buds that fail to separate from often abnormally wide mother-bud necks (Wang et al. 2002).

Interestingly, *chm1* mutants were unstable and produced various sectors on oatmeal plates (Fig. 8). PAK kinases are known to have a diverse range of downstream effectors (Dan et al. 2001; Daniels and Bokoch 1999), and spontaneous mutants of MC3 and MC55 may harbor mutations in these downstream targets of *CHM1*. However, sectors rarely were formed when *chm1* mutants were cultured on nutritionally rich media. Although it is not clear how nutritional conditions affect instability, one possibility is that the MAP kinase pathway involved in nutrient sensing may be affected in *chm1* mutants. In *Shizosaccharomyces pombe*, it has been shown that *PAK2* functions above the *SPM1* MAP kinase pathway (Merla and Johnson 2001). *SPM1* is homologous to the *SLT2* MAP kinase in *Saccharomyces cerevisiae* that is involved in nutrient sensing and cytoskeleton reorganization (Gustin et al. 1998). In *M. grisea*, deletion of the *SLT2* homolog *MPS1* also significantly reduced conidiation. Similarly to *chm1* mutants, *mps1* mutants are defective in appressorial penetration, but are able to colonize wounded rice leaves (Xu et al. 1998). The *M. grisea* *mac1* (adenylate cyclase) and *mgb1* (G β subunit of heterotrimeric G-protein) deletion mutants are known to produce frequent spontaneous mutations on oatmeal agar (Adachi and Hamer 1998; Nishimura et al. in press). One of the *mac1* suppressor mutants has a point mutation in the regulatory subunit of PKA *SUM1* (Adachi and Hamer 1998). Several spontaneous mutants isolated from the *mgb1* mutant formed elongated appressoria

(M. Nishimura and J.-R. Xu, *unpublished data*) similar to what were observed in S0 and S5 (Fig. 8B). *MGB1* has the highly conserved HIKE motif (Alberti 1999) that functions as a protein-binding site for PH domain, a motif found in *CHM1*.

Although various spontaneous mutants of MC3 and MC55 had some of the phenotypes associated with *CHM1* deletion partially suppressed, such as growth rate, conidiation, conidium morphology, and appressorium formation, none of them was normal in appressorial penetration and infection on healthy plant leaves. These data indicate that some morphological defects of the *chm1* mutant, but not its failure in appressorial penetration, can revert due to various spontaneous mutations. In *M. grisea*, appressorial penetration is a complicated process involving appressorium pore formation, development of the penetration peg, and differentiation of primary infectious hyphae. *CHM1* may be involved in regulating different steps of appressorial penetration by associating with different downstream targets.

The Rho family GTPases are known to play critical roles in regulating cytoskeletal rearrangements that are important for many cellular processes such as adhesion, cell division, and differentiation (Dan et al. 2001; Daniels and Bokoch 1999; Eby et al. 1998). Different members of the Rho GTPases are activated to regulate various cytoskeletal changes associated with different differentiation processes. In the *M. grisea* genome sequence currently available, *CHM1* and *MST20* are the only proteins containing the PBD domain, but there are several (>6) putative Rho GTPases. Therefore, it is likely that multiple upstream Rho GTPases may converge their signaling at *CHM1* or *MST20* in *M. grisea*. Data presented in this study indicated that *CHM1*, but not *MST20*, plays major roles in cytoskeleton reorganizations that are associated with conidiogenesis and infection-related morphogenesis. Most of the phenotypes observed in *chm1* deletion mutants, including production of short hyphal compartments and abnormal conidia, reduction in appressorium formation, and failure in appressorial penetration, may be associated with or caused by defects in proper regulation of cytoskeletal rearrangements. Production of twisted germ tubes and elongated appressoria by some spontaneous mutants also likely resulted from defects in cytoskeletal rearrangements during appressorium formation. We are in the process of constructing and testing different GFP-tubulin and GFP-actin constructs in *M. grisea* to examine cytoskeletal defects in the *chm1* mutant and its sectors.

MATERIALS AND METHODS

Stains and growth media.

Wild-type strains Guy11 (*MAT1-2*) and 2539 (*MAT1-1*), and all transformants were cultured on oatmeal agar plates at 25°C under fluorescent light for conidiation and stored on desiccated Whatman #1 filter paper at -20°C. Media were supplemented with hygromycin B (Calbiochem, La Jolla, CA, U.S.A.) at 250 µg/ml or zeocin (Invitrogen, Carlsbad, CA, U.S.A.) at 100 µg/ml for selecting hygromycin-resistant and zeocin-resistant transformants, respectively. Monoconidial cultures were isolated for transformants as previously described (Xu and Hamer 1996). Genetic crosses and progeny isolations were performed as described (Crawford et al. 1986). Growth rate was determined with race-tube cultures grown on oatmeal agar after incubation at room temperature.

Isolating the *CHM1* and *MST20* genes.

Two degenerate PCR primers PK1F (GTNGCNATHAAR CARATGAA) and PK2R (GCRCARAANCCRAARTC) were designed according to the amino acid residues VAIKQMN and DFGFCA that are conserved among the PAK kinases from

Saccharomyces cerevisiae, *Candida albicans*, and *Shizosaccharomyces pombe* (Leberer et al. 1997). Primers PK1F and PK2R were used to amplify PAK genes from Guy11 genomic DNA by a PCR reaction consisting of 30 cycles of 39 s at 94°C, 30 s at 48°C, and 1 min at 72°C, followed by 5 min at 72°C. PCR products amplified with these two primers were cloned in the pGEM-T Easy vector (Promega Corp., Madison, WI, U.S.A.) and sequenced. One PCR clone, pJX137, contained a 351-bp insert that was homologous to *Saccharomyces cerevisiae* *STE20*. Two PCR clones, pJX136 and pJX138, contained a 363-bp insert that was homologous to *Saccharomyces cerevisiae* *CLA4*. Clones pJX137 and pJX138 were used to screen a Guy11 pMOcosX cosmid genomic library and a λZAPII cDNA library constructed with RNA isolated from a nitrogen-starved Guy11 culture (Xu and Hamer 1996). Corresponding cDNA and genomic clones were isolated and sequenced with the ABI Prism BigDye Terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, U.S.A.). Motif and localization searches were performed with Pfam programs.

MST20 gene replacement.

Cosmid clones 6I24 and 8M5 containing the *MST20* gene were sequenced by primer walking. A 4.2-kb fragment containing the *MST20* gene was amplified with primers P7 (5'-ATTCTAGATTGAGCATCGACAGCCGTGTAGACG-3') and P8 (5'-AGGGTACCACATGAGTTATCCAAATATCAA-3') and cloned between the *Xba*I and *Kpn*I sites on pBCKS+ (Stratagene) as pCX31. To generate the *MST20* gene-replacement construct pCX32, pCX31 was digested with *Bam*HI and *Xho*I, then ligated with the *hph* gene released from pADH5 (Park et al. 2002). After linearizing with *Xba*I, pCX32 was transformed into Guy11 protoplasts as described (Xu and Hamer 1996). Hygromycin-resistant transformants were screened with primers P3 (5'-CGCAGCTTCACCAAGATT-3') and P5 (5'-AATCCTGGCATCATAGTAGA-3') that were located within the deleted portion of the *MST20* gene. Gene-replacement mutants were confirmed further by Southern blot analyses.

CHM1 gene replacement.

The *Hpa*I 1.4-kb fragment containing the *hph* gene was released from pCB1003 and cloned in the filled *Xba*I site of pBCKS+. The resulting construct was digested with *Eco*RI and *Bam*HI and ligated with an *Eco*RI *Bam*HI 1.6-kb fragment containing the *CHM1* upstream sequence (Fig. 3A) to generate pJX58. A 1.8-kb fragment containing the *CHM1* downstream flanking sequence (Fig. 3A) was amplified and cloned into the *Nor*I site of pJX58 to create the *CHM1* gene replacement vector pJX74. Plasmid DNAs of pJX74 were digested with *Eco*RI and transformed into Guy11. Hygromycin-resistant transformants were screened by PCR with primers C4 (5'-GAAC CACCGTCAACAAAC-3') and H2 (5'-CGGTCGGCATC TACTCTATT-3'). The sequence of primer C4 is located outside of the upstream *CHM1* flanking sequence cloned on pJX74 (Fig. 3A). Only transformants with homologous recombination between pJX74 and the endogenous *CHM1* yielded a 2.9-kb band with primers C4 and H2. Putative *chm1* knockout mutants identified in the PCR screen were further confirmed by Southern blot analysis.

For complementation, pNX41 containing a 5.6-kb fragment of *CHM1* (Fig. 3A) and pAC905 containing the bleomycin/zeocin resistance gene were co-transformed into MC3 and MC55 as previously described (Zheng et al. 2000). Zeocin-resistant transformants were screened by PCR with primers C1 (5'-CAGGTGCGCATCAAGCAA-3') and C2 (5'-CACTCGG ACTCAGTGACAT-3') that are located in the *CHM1* region

deleted in pJX74 (Fig. 3A). Transformants yielding a 1,020-bp PCR product with primers C1 and C2 were confirmed further by Southern analyses.

Assaying appressorium formation and penetration.

Conidia were harvested from 10-day-old oatmeal agar cultures and resuspended in sterile distilled water to 5×10^4 conidia per ml. Drops of the conidial suspension (50 μ l) were placed on plastic microscope cover slips (Fisher Scientific Co., Pittsburgh, PA, U.S.A.) and incubated in a moist chamber at room temperature. Conidial germination and appressorium formation were examined at 0.5, 2, 12, and 24 h after incubation. Appressorial penetration on onion epidermal strips was assayed as previously described (Xu et al. 1997). Appressorium formation and infectious hyphal growth were examined after 48 to 72 h of incubation at room temperature. Photographs were taken with a Nikon Eclipse E800 epifluorescence microscope. Germinating conidia and vegetative hyphae were stained with Calcofluor at 10 μ g/ml and Hoechst 33258 at 100 ng/ml as described (Harris et al. 1994) and examined by epifluorescence microscopy. Conidia were resuspended in 10 mM cAMP and tested for cAMP responsiveness as described (Lee and Dean 1993).

Plant infection assays.

Conidia were collected from oatmeal agar cultures and resuspended to 5×10^5 conidia/ml in 0.25% gelatin solution. Two-week-old seedlings of rice cv. CO39 and 8-day-old seedlings of barley cv. Golden Promise were used for infection assays. Plant incubation, spray inoculation, and lesion examination were performed as described (Valent and Chumley 1991; Xu et al. 1997). For injection inoculation, conidial suspensions were injected into leaf sheaths of rice seedlings with a 26G_{1/2} needle. Lesion formation and necrosis around the inoculation sites were examined when wounded leaves had unfolded 5 to 7 days after injection. For abrasive inoculation, the second and third leaves of 2-week-old rice seedlings were powdered with diatomaceous earth (Sigma-Aldrich, St. Louis). A drop of conidial suspension (30 μ l) then was applied onto each leaf and rubbed gently with diatomaceous earth. To reisolate *chm1* mutants from infected leaves, wound inoculation sites were excised, surface sterilized, and incubated on 4% water agar as previously described (Xu and Hamer 1996) to induce conidiation.

ACKNOWLEDGMENTS

We thank L. Dunkle and C. Woloshuk for critical reading of this manuscript, D. Sherman for assistance in microphotographs, and X. Zhao for rice infections. This work was supported by grants (to J.-R. Xu) from the United States Department of Agriculture National Research Initiative and the National Science Foundation.

LITERATURE CITATIONS

Adachi, K., and Hamer, J. E. 1998. Divergent cAMP signaling pathways regulate growth and pathogenesis in the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 10:1361-1373.

Alberti, S. 1999. HIKE, a candidate protein binding site for PH domains, is a major regulatory region of G-beta proteins. *Proteins* 35:360-363.

Ash, J., Wu, C. L., Larocque, R., Jamal, M., Stevens, W., Osborne, M., Thomas, D. Y., and Whiteway, M. 2003. Genetic analysis of the interaction between Cdc42p and the CRIB domain of Ste20p in *Saccharomyces cerevisiae*. *Genetics* 163:9-20.

Ayad-Durieux, Y., Knechtel, P., Goff, S., Dietrich, F., and Philippsen, P. 2000. A PAK-like protein kinase is required for maturation of young hyphae and septation in the filamentous ascomycete *Ashbya gossypii*. *J. Cell Sci.* 113:4563-4575.

Brachmann, A., Schirawski, J., Muller, P., and Kahmann, R. 2003. An unusual MAP kinase is required for efficient penetration of the plant sur-

face by *Ustilago maydis*. *EMBO (Eur. Mol. Biol. Organ.) J.* 22:2199-2210.

Breitkreutz, A., and Tyers, M. 2002. MAPK signaling specificity: It takes two to tango. *Trends Cell Biol.* 12:254-257.

Clergeot, P. H., Gourgues, M., Cots, J., Laurans, F., Latorse, M. P., Pepin, R., Tharreau, D., Notteghem, J. L., and Lebrun, M. H. 2001. PLS1, a gene encoding a tetraspanin-like protein, is required for penetration of rice leaf by the fungal pathogen *Magnaporthe grisea*. *Proc. Natl. Acad. Sci. U.S.A.* 98:6963-6968.

Crawford, M. S., Chumley, F. G., Weaver, C. G., and Valent, B. 1986. Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. *Genetics* 114:1111-1129.

Csank, C., Schroppel, K., Leberer, E., Marcus, D., Mohamed, O., Meloche, S., Thomas, D. Y., and Whiteway, M. 1998. Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. *Infect. Immun.* 66:2713-2721.

Cvrckova, F., Devirgilio, C., Manser, E., Pringle, J. R., and Nasmyth, K. 1995. Ste20-like protein-kinases are required for normal localization of cell-growth and for cytokinesis in budding yeast. *Genes Dev.* 9:1817-1830.

Dan, I., Watanabe, N. M., and Kusumi, A. 2001. The Ste20 group kinases as regulators of MAP kinase cascades. *Trends Cell Biol.* 11:220-230.

Daniels, R. H., and Bokoch, G. M. 1999. p21-activated protein kinase: A crucial component of morphological signaling. *Trends Biochem. Sci.* 24:350-355.

deJong, J. C., McCormack, B. J., Smirnov, N., and Talbot, N. J. 1997. Glycerol generates turgor in rice blast. *Nature* 389:244-245.

Di Pietro, A., Garcia-Maceira, F. I., Meglec, E., and Roncero, M. I. G. 2001. A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. *Mol. Microbiol.* 39:1140-1152.

Dohlman, H. G., and Thorner, J. W. 2001. Regulation of G protein-initiated signal transduction in yeast: Paradigms and principles. *Annu. Rev. Biochem.* 70:703-754.

Eby, J. J., Holly, S. P., van Drogen, F., Grishin, A. V., Peter, M., Drubin, D. G., and Blumer, K. J. 1998. Actin cytoskeleton organization regulated by the PAK family of protein kinases. *Curr. Biol.* 8:967-970.

Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. 1998. MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 62:1264-1300.

Harris, S. D., Morrell, J. L., and Hamer, J. E. 1994. Identification and characterization of *Aspergillus nidulans* mutants defective in cytokinesis. *Genetics* 136:517-532.

Holly, S. P., and Blumer, K. J. 1998. PAK-like kinases Ste20 and Cla4 control actin polarity in budding yeast. *Mol. Biol. Cell* 9:1648.

Hou, Z., Xue, C., Katan, T., Peng, Y., Kistler, H. C., and Xu, J. R. 2002. A MAP kinase gene (*MGV1*) for hyphal growth and plant infection in *Fusarium graminearum*. *Mol. Plant-Microbe Interact.* 15:1119-1127.

Howard, R. J., Ferrari, M. A., Roach, D. H., and Money, N. P. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc. Natl. Acad. Sci. U.S.A.* 88:11281-11284.

Jenczmionka, N. J., Maier, F. J., Losch, A. P., and Schafer, W. 2003. Mating, conidiation and pathogenicity of *Fusarium graminearum*, the main causal agent of the head-blight disease of wheat, are regulated by the MAP kinase *gpmk1*. *Curr. Genet.* 43:87-95.

Kohler, J. R., and Fink, G. R. 1996. *Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. *Proc. Natl. Acad. Sci. U.S.A.* 93:13223-13228.

Lamson, R. E., Winters, M. J., and Pryciak, P. M. 2002. Cdc42 regulation of kinase activity and signaling by the yeast p21-activated kinase Ste20. *Mol. Cell. Biol.* 22:2939-2951.

Leberer, E., Ziegelbauer, K., Schmidt, A., Marcus, D., Dignard, D., Ash, J., Johnson, L., and Thomas, D. Y. 1997. Virulence and hyphal formation of *Candida albicans* require the Ste20p-like protein kinase CaCla4p. *Curr. Biol.* 7:539-546.

Lee, Y. H., and Dean, R. A. 1993. cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *Plant Cell* 5:693-700.

Leeuw, T., Wu, C. L., Schrag, J. D., Whiteway, M., Thomas, D. Y., and Leberer, E. 1998. Interaction of a G-protein beta-subunit with a conserved sequence in Ste20/PAK family protein kinases. *Nature* 391:191-195.

Liu, H. P. 2002. Co-regulation of pathogenesis with dimorphism and phenotypic switching in *Candida albicans*, a commensal and a pathogen. *Int. J. Med. Microbiol.* 292:299-311.

Liu, S. H., and Dean, R. A. 1997. G protein alpha subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 10:1075-1086.

- Magee, B. B., Legrand, M., Alarco, A. M., Raymond, M., and Magee, R. T. 2002. Many of the genes required for mating in *Saccharomyces cerevisiae* are also required for mating in *Candida albicans*. *Mol. Microbiol.* 46:1345-1351.
- Marcus, S., Polverino, A., Chang, E., Robbins, D., Cobb, M. H., and Wigler, M. H. 1995. Shk1, a homolog of the *Saccharomyces cerevisiae* Ste20 and mammalian p65(PAK) protein kinases, is a component of a Ras/Cdc42 signaling module in the fission yeast *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. U.S.A.* 92:6180-6184.
- Martin, H., Mendoza, A., RodriguezPachon, J. M., Molina, M., and Nombela, C. 1997. Characterization of *SKM1*, a *Saccharomyces cerevisiae* gene encoding a novel Ste20/PAK-like protein kinase. *Mol. Microbiol.* 23:431-444.
- Mayorga, M. E., and Gold, S. E. 1999. A MAP kinase encoded by the *ubc3* gene of *Ustilago maydis* is required for filamentous growth and full virulence. *Mol. Microbiol.* 34:485-497.
- Merla, A., and Johnson, C. 2001. The *Schizosaccharomyces pombe* Cdc42p GTPase signals through Pak2p and the Mkh1p-Pek1p-Spm1p MAP kinase pathway. *Curr. Genet.* 39:205-209.
- Mey, G., Oeser, B., Lebrun, M. H., and Tudzynski, P. 2002. The biotrophic, non-appressorium-forming grass pathogen *Claviceps purpurea* needs a Fus3/Pmk1 homologous mitogen-activated protein kinase for colonization of rye ovarian tissue. *Mol. Plant-Microbe Interact.* 15:303-312.
- Mitchell, T. K., and Dean, R. A. 1995. The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. *Plant Cell* 7:1869-1878.
- Nishimura, M., Park, G., and Xu, J. R. 2004. The G-beta subunit *MGB1* is involved in regulating multiple steps of infection-related morphogenesis in *Magnaporthe grisea*. *Mol. Microbiol.* 50:231-243.
- Park, G., Xue, C. Y., Zheng, L., Lam, S., and Xu, J. R. 2002. *MST12* regulates infectious growth but not appressorium formation in the rice blast fungus *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 15:183-192.
- Qyang, Y. B., Yang, P. R., Du, H. Y., Lai, H., Kim, H. W., and Marcus, S. 2002. The p21-activated kinase, Shk1, is required for proper regulation of microtubule dynamics in the fission yeast, *Schizosaccharomyces pombe*. *Mol. Microbiol.* 44:325-334.
- Roberts, R. L., and Fink, G. R. 1994. Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type -mating and invasive growth. *Genes Dev.* 8:2974-2985.
- Ruiz-Roldan, M. C., Maier, F. J., and Schafer, W. 2001. PTK1, a mitogen-activated-protein kinase gene, is required for conidiation, appressorium formation, and pathogenicity of *Pyrenophora teres* on barley. *Mol. Plant-Microbe Interact.* 14:116-125.
- Schmidt, M., Varma, A., Drgon, T., Bowers, B., and Cabib, E. 2003. Septins, under Cla4p regulation, and the chitin ring are required for neck integrity in budding yeast. *Mol. Biol. Cell* 14:2128-2141.
- Szabo, R. 2001. Cla4 protein kinase is essential for filament formation and invasive growth of *Yarrowia lipolytica*. *Mol. Genet. Genom.* 265:172-179.
- Talbot, N. J., Ebbole, D. J., and Hamer, J. E. 1993. Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 5:1575-1590.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL-X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882.
- Valent, B., and Chumley, F. G. 1991. Molecular genetic analysis of the rice blast fungus *Magnaporthe grisea*. *Annu. Rev. Phytopathol.* 29:443-467.
- Wang, P., Nichols, C. B., Lengeler, M. B., Cardenas, M. E., Cox, G. M., Perfect, J. R., and Heitman, J. 2002. Mating-type-specific and non-specific PAK kinases play shared and divergent roles in *Cryptococcus neoformans*. *Eukaryotic Cell* 1:257-272.
- Weinzierl, G., Leveleki, L., Hassel, A., Kost, G., Wanner, G., and Bolker, M. 2002. Regulation of cell separation in the dimorphic fungus *Ustilago maydis*. *Mol. Microbiol.* 45:219-231.
- Xu, J. R. 2000. MAP kinases in fungal pathogens. *Fungal Genet. Biol.* 31:137-152.
- Xu, J. R., and Hamer, J. E. 1996. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev.* 10:2696-2706.
- Xu, J. R., Staiger, C. J., and Hamer, J. E. 1998. Inactivation of the mitogen-activated protein kinase *MPS1* from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. *Proc. Natl. Acad. Sci. U.S.A.* 95:12713-12718.
- Xu, J. R., Urban, M., Sweigard, J. A., and Hamer, J. E. 1997. The *CPKA* gene of *Magnaporthe grisea* is essential for appressorial penetration. *Mol. Plant-Microbe Interact.* 10:187-194.
- Zheng, L., Campbell, M., Murphy, J., Lam, S., and Xu, J. R. 2000. The *BMP1* gene is essential for pathogenicity in the gray mold fungus *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* 13:724-732.

AUTHOR-RECOMMENDED INTERNET RESOURCES

The Broad Institute Center for Genome Research *Magnaporthe grisea* database: www-genome.wi.mit.edu/annotation/fungi/magnaporthe
 The Sanger Institute Protein families database of alignment and hidden Markov models: www.sanger.ac.uk/Software/Pfam/index.shtml