The *Colletotrichum lagenarium* MAP Kinase Gene *CMK1* Regulates Diverse Aspects of Fungal Pathogenesis

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The infection process of *Colletotrichum lagenarium*, the causal agent of cucumber anthracnose disease, involves several key steps: germination; formation of melanized appressoria; appressorial penetration; and subsequent invasive growth in host plants. Here we report that the *C. lagenarium* *CMK1* gene encoding a mitogen-activated protein (MAP) kinase plays a central role in these infection steps. *CMK1* can complement appressorium formation of the *Pmk1* MAP kinase mutant of *Magnaporthe grisea*. Deletion of *CMK1* causes reduction of conidiation and complete lack of pathogenicity to the host plant. Surprisingly, in contrast to *M. grisea* *pmk1* mutants, conidia of *cmk1* mutants fail to germinate on both host plant and glass surfaces, demonstrating that the *CMK1* MAP kinase regulates conidial germination. However, addition of yeast extract rescues germination, indicating the presence of a *CMK1*-independent pathway for regulation of conidial germination. Germinating conidia of *cmk1* mutants fail to form appressoria and the mutants are unable to grow invasively in the host plant. This strongly suggests that MAP kinase signaling pathways have general significance for infection structure formation and pathogenic growth in phytopathogenic fungi. Furthermore, three melanin genes show no or slight expression in the *cmk1* mutant when conidia fail to germinate, suggesting that *CMK1* plays a role in gene expression required for appressorial melanization.

Additional keywords: *PKS1*, *SCD1*, *THR1*.

Anthracnose fungi belonging to the genus *Colletotrichum* cause severe and destructive diseases in numerous crops. Anthracnose is a disease of the foliage, stems, or fruits that typically appears as dark-colored spots or slightly sunken lesions

(Agrios 1988). *Colletotrichum lagenarium* is the causal agent of anthracnose of cucumber. Symptoms appear first on the leaves as small, water-soaked, yellowish areas that turn to brown. Infections are initiated by conidial attachment. The conidia then germinate and form infection structures called appressoria. Appressoria contain a pigmented melanin layer that is essential for penetration into host plants (Kubo et al. 1982). Melanized appressoria form infection pegs, leading to cuticular penetration (Perpetua et al. 1994). Following penetration, thick, infectious hyphae grow and extend inside the plant cells. To study appressorial melanization of *C. lagenarium*, we isolated and characterized three genes, *PKS1*, *SCD1*, and *THR1*, encoding major enzymes for melanin biosynthesis (Takano et al. 1995; Perpetua et al. 1996; Kubo et al. 1996). These melanin genes are transcribed at an early stage of appressorium formation, suggesting that transcription of melanin genes is linked to regulation mechanisms for pre-penetration morphogenesis that include germination and/or appressorium formation (Takano et al. 1997b).

In many fungal pathogens, including *C. lagenarium*, pre-penetration morphogenesis is triggered by perception of physical and/or chemical signals. Appressorium formation of the rust fungus *Uromyces appendiculatus* is induced by certain topographical features (Hoch et al. 1987). In the rice blast fungus *Magnaporthe grisea*, surface hydrophobicity and wax components stimulate appressorium formation (Lee and Dean 1994; Uchiyama and Okuyama 1990; Gilbert and Dean 1996). Also, the avocado pathogen *C. gloeosporioides* requires both hard surface contact and host surface wax for germination and appressorium formation (Podila et al. 1993; Flaishman et al. 1995). Pharmacological analysis suggests that the perception of environmental signals initiates intracellular signal transduction pathways in these fungi, then promotes infection-related morphogenesis (Lee and Dean 1993; Flaishman et al. 1995; Kim et al. 1998). In *M. grisea*, molecular genetic analysis demonstrated that cyclic AMP (cAMP) and mitogen-activated protein (MAP) kinase signal transduction pathways play important roles in pathogenesis (Dean 1997; Hamer et al. 1997).

The MAP kinase cascades generally consist of three conserved kinases, a MEK kinase (MAPKKK), a MEK

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(MAPKK), and a MAP kinase, that function sequentially and mediate appropriate cellular responses to distinct environmental inputs (Nishida and Gotoh 1993; Blumer and Johnson 1994). In *Saccharomyces cerevisiae*, the MAP kinase cascades regulate diverse responses such as mating, glycerol synthesis, and cell wall biosynthesis (Herskowitz 1995; Hunter and Plowman 1997). There are a few reports about MAP kinase modules in filamentous fungi (Dickman and Yarden 1999). In the rice blast fungus, two MAP kinase (Pmk1 and Mps1) pathways play pivotal roles in fungal pathogenicity. Pmk1 is functionally related to the yeast Fus3 MAP kinase, which is essential for mating responses (Elion et al. 1990). The inactivation of the Pmk1 MAP kinase resulted in a defect in both appressorium formation and invasive growth in plants but showed no other effect on development, including mating reaction (Xu and Hamer 1996). Another MAP kinase, Mps1, related to the yeast Slr2 (Mpk1) kinase, is required for plant penetration (Lee et al. 1993; Xu et al. 1998). These results suggest that MAP kinase pathways are functionally conserved but play diverse roles in different organisms.

Here we report on the identification of the *C. lagenarium* MAP kinase gene designated *CMK1* (*Colletotrichum* MAP Kinase 1) related to *M. grisea* *PMK1*. Inactivation of *CMK1* resulted in complete loss of pathogenicity to cucumber leaves. In contrast to the *M. grisea* *pmk1* mutant, *CMK1*-disrupted mutants failed to germinate on the host plant and on artificial surfaces. Further analysis of the *cmk1* mutants revealed that *CMK1* regulates diverse steps in pathogenesis, including appressorium formation. The conserved roles of *CMK1* and *PMK1* in appressorium formation suggests a generalized signaling pathway for the formation of fungal infection structures.

RESULTS

Isolation of the MAP kinase gene *CMK1*.

A polymerase chain reaction (PCR)-based screen was used to isolate putative MAP kinase homologs from a wild-type strain of *C. lagenarium*. Degenerate oligonucleotide primers were designed from conserved amino acid sequences of the MAP kinases in other organisms. cDNA synthesized from mRNA of conidia at the pre-penetration stage was used as a template for PCR screening. We isolated two typical MAP kinase genes by the screening. The DNA sequence of a 96-bp PCR product in clone pRCMK1 showed a high degree of identity with the *M. grisea* *PMK1* and *S. cerevisiae* *FUS3* MAP kinase genes. The other clone showed a high similarity with the *S. cerevisiae* *HOG1* MAP kinase gene (data not shown). The insert DNA of pRCMK1 was used as a probe to isolate cDNA and genomic clones containing a *C. lagenarium* MAP kinase gene designated *CMK1*. *CMK1* contained one open reading frame composed of 355 codons and three introns (Fig. 1, upper panel). The deduced amino acid sequence of *CMK1* shared significant homology (96% identity) with the *M. grisea* Pmk1 MAP kinase. Also, *CMK1* showed 59% identity with *S. cerevisiae* Fus3 and Kss1, and 55% with human Erk1 (Fig. 1, upper panel). The amino acid sequence of *CMK1* contains all 11 conserved protein kinase domains (Hanks and Quinn 1991). In addition, *CMK1* contains the sequence TEY, a site for threonine and tyrosine phosphorylation required for kinase activation (Fig. 1, upper panel).

It has been shown that *M. grisea* *PMK1* knockout mutants fail to form appressoria (Xu and Hamer 1996). We investigated whether *C. lagenarium* *CMK1* complements the appressorium formation defect of the *pmk1* mutant nn78. The 7.5-kb *PstI*-*ApaI* genomic DNA fragment that contains a 3.5-kb 5' upstream region and the full coding sequence of *CMK1* was introduced into a transformation vector, pCB1531 (Sweigard et al. 1997), carrying a bialaphos-resistant gene bar as a selective marker, which resulted in plasmid pCBCMK1. *M. grisea* *pmk1* mutant nn78 was transformed with pCBCMK1 or the control plasmid pCB1531. Bialaphos-resistant transformants carrying pCBCMK1 were selected, and the introduction of the *CMK1* fragment was confirmed by DNA gel blot analysis (data not shown). Two independent transformants, nnCM1 and nnCM2, were examined for appressorium formation on a hydrophobic surface. Their conidia efficiently formed melanized appressoria similar to those of the wild-type strain Guy11, whereas conidia of pCB1531-introduced transformant nnCO1 germinated but failed to form appressoria, the same as the recipient *pmk1* strain nn78 (Fig. 1, lower panel). From three independent experiments, we obtained identical results (data not shown). Thus, we conclude that *C. lagenarium* *CMK1* encodes a MAP kinase related to *M. grisea* Pmk1, and could complement infection-structure formation of the *pmk1* mutant.

CMK1 is essential for pathogenicity.

To determine the function of *CMK1* in *C. lagenarium*, a gene disruption strategy was employed. A gene disruption vector, pCMK1GD, containing a hygromycin-resistant gene cassette flanked by 5' and 3' ends of the *CMK1* gene was constructed (Fig. 2A). pCMK1GD was introduced into the wild-type strain 104-T and transformants were selected on medium containing hygromycin. Among 30 hygromycin-resistant transformants, four transformants were confirmed to have gene replacement events by DNA gel blot analysis (Fig. 2B). Genomic DNA was digested with *HindIII* and probed with the 2.0-kb PCR fragment containing *CMK1*. The result showed that the wild-type strain 104-T and the transformants DCM2 and DCM4 contained a common 3.9-kb *HindIII* fragment. DCM2 and DCM4 also each contained an additional band, indicating that pCMK1GD was introduced through ectopic integration. The transformants DCM1, DCM3, DCM7, and DCM17 did not contain a 3.9-kb *HindIII* fragment, but contained a common 5.3-kb *HindIII* fragment, which was consistent with the expected length for the gene replacement event (Fig. 2B).

The $\Delta cmk1::Hph$ strains (DCM1, DCM3, DCM7, and DCM17) showed mycelial growth rates similar to that of the wild type 104-T on PDA (potato dextrose agar) plates (Table 1). However, conidiation was significantly reduced in the $\Delta cmk1::Hph$ strains on PDA plates, compared with the wild-type strain (Table 1). We also tested conidium attachment of the *cmk1* mutants to a glass surface. Conidium attachment of the $\Delta cmk1$ strains on a glass surface was reduced, compared with that of the control strains. About 20% of conidia of the $\Delta cmk1$ strains remained attached whereas more than 80% of conidia of the control strains attached to a glass surface after shaking (Table 1).

Infection assays were performed to assess the pathogenicity of the *cmk1* mutants on host cucumber leaves. Conidial suspensions from the $\Delta cmk1::Hph$ strains, the wild type, and the

CMK1	1	-----MSRANAP-NPSGSRKISFNVSEQYDIQDVVGEAGYGVVCSAI
Pmk1	1	-----MSRANPPSNSSGSRKISFNVSEQYDIQDVVGEAGYGVVCSAI
Fus3	1	-----MPKRIIVNINSSDFTQLKSLLGEGAGYGVVCSAI
Kss1	1	-----MARTITFDIPSOYKLVDLIGEGAGYGVVCSAI
Erk1	1	MAAAAAQGGGGGEPRRTGEGVGPVGEVEMVKGQPDVGVFRYTQLQYIIGEGAGYGVVCSAI
II III IV		
CMK1	42	HKPSGQKVAIKKITPPFDHSMFCLRTLREMKLLRYF-NHENIISILDIQKPRSSETFNEVY
Pmk1	43	HKPSGQKVAIKKITPPFDHSMFCLRTLREMKLLRYF-NHENIISILDIQKPRSSETFNEVY
Fus3	32	HKPGEIVAIKKITPPFDKPLFALRTLREIKLLKHF-KHENIISILDIQKPRSSETFNEVY
Kss1	32	HKPSGQKVAIKKITPPFSKKLFVTRTREIKLLRYFHEHENIISILDKVRPVSIDKLNNAVY
Erk1	61	DHVRKTRVAIKKITSPFHHQTYCQRTLREIQILLRF-RHENVIGIRDLKASTLEAMRDVY
V VI		
CMK1	101	LIQELMETDMHRVIRTQD-----LSDDHCQYFIYQTLRALKAMHSANVLHRDLKPSNLLL
Pmk1	102	LIQELMETDMHRVIRTQD-----LSYDHCQYFIYQTLRALKAMHSANVLHRDLKPSNLLL
Fus3	91	LIQELMETDMHRVISTQ-----MLSDDHICQYFIYQTLRALKAMHSANVLHRDLKPSNLLL
Kss1	92	LVEELMETDQKVINNOMSGFSTLSDDHVQYFTYQTLRALKAMHSANVLHRDLKPSNLLL
Erk1	120	IVQDLMETDLYKDKTSQ-----LSNDHICQYFIYQTLRALKAMHSANVLHRDLKPSNLLL
VII VIII		
CMK1	156	NANCDLVKVCDFGLARSAAS-----QEDNSG-FMTEYVATRWYRAPEIMLTFFKEYTKAID
Pmk1	157	NANCDLVKVCDFGLARSAAS-----QENNSG-FMTEYVATRWYRAPEIMLTFFKEYTKAID
Fus3	146	NSNCDLVKVCDFGLARIIIDESAADNSEPTGQSGMTEYVATRWYRAPEIMLTSAKYBRAMD
Kss1	152	NSNCDLVKVCDFGLARCLAS-SSDSRETLVG-FMTEYVATRWYRAPEIMLTFOEYTTAMD
Erk1	175	NTTCDLVKVCDFGLARITADP-----FHDHGTG-FMTEYVATRWYRAPEIMLTNSKGYTKSID
IX X		
CMK1	209	VWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTPT-MEDYYGIKSRRAREYIRSLPF
Pmk1	210	VWSVGCILAEMLSGKPLFPFGKDYHHQLTLILDVLGTPT-MEDYYGIKSRRAREYIRSLPF
Fus3	206	VWSCGCI LAEFLRNPIFPFGKDYHHQLLLIFGIGTPHSDNDLRCIESPRAREYIRSLPF
Kss1	209	IWSCGCI LAEMVSGKPLFPFGKDYHHQLWLILEVLGTPT-FEDFNQIKSRRAREYIRSLPF
Erk1	228	IWSVGCILAEMLSNRPIFPFGKHYLDQLNHLGLGSPS-QEDLNCIINMKARNYLQSLPS
XI		
CMK1	268	KKKVPFRTLFPKT--SDLA LD LLEKLLAFNPVKRITVEDAL KHPYLEPYHDPDDEPTAPP
Pmk1	269	KKKVPFRTLFPQD--FGSRLD LLEKLLAFNPVKRITVEAL KHPYLEPYHDPDDEPTAPP
Fus3	266	YPAAPLEKMTFR--VNPCKGIDLLQRLMLVFPDAKRITAKKAL EHPYLOTYHDPDDEPECEP
Kss1	268	KPPPLPWEVWSKTDLNPDMDIDLLDKMLQFNPDKRISAAAL EHPYLAHYHDPDDEPEYFP
Erk1	287	KTKVAWAKLFPKS--DSKALD LLDRLMLTFNPNKRITVEALAHHPYLEQYQYDPTDEP----
CMK1	326	--IPEEFFDFD-----KHKDNLKSKEQLKQLIYQEI MR-----
Pmk1	327	--IPEEFFDFD-----KHKDNLKSKEQLKQFIYQEI MR-----
Fus3	324	--IPEEFFDFD-----HHKEALTTKDLKKKLINWEI FS-----
Kss1	328	LNLDDEFWKLDNKIMRPEEEEEVPIEMLKDMYDEIMKTME-----
Erk1	341	--WAEFPETEA-----MELDDLPKERLKELEIQETARFQPGVLEAP

Fig. 1. Structural and functional characterization of *Colletotrichum lagenarium* mitogen-activated protein (MAP) kinase gene *CMK1*. **Upper panel**, Amino acid sequence alignment of *CMK1* with *Magnaporthe grisea* *Pmk1*, *Saccharomyces cerevisiae* *Fus3* and *Kss1*, and human *Erk1*. Sequences are aligned with the CLUSTAL W program (Thompson et al. 1994). Identical amino acids are indicated as white letters on black background. Similar residues are shown on a gray background. Gaps introduced for the alignment are indicated by hyphens. The protein kinase domains are indicated by Roman numerals above sequences. Sequence TEY required for kinase activation is marked by asterisks. GenBank accession number for the *CMK1* gene is AF174649. GenBank accession numbers for *Pmk1*, *Fus3*, *Kss1*, and *Erk1* are U70134, M31132, M26398, and X60188, respectively. **Lower panel**, Complementation of appressorium formation of *M. grisea* *pmk1* mutant nn78 by introduction of *CMK1*. Conidial suspensions of tested strains were placed on a hydrophobic surface of GelBond film, and incubated at 24°C for 12 h. **A**, *M. grisea* wild-type strain Guy-11; **B**, *pmk1* mutant nn78; **C**, *CMK1*-introduced transformant of nn78 (nnCM1); **D**, pCB1531-introduced transformant of nn78 (nnCO1). A, appressorium; C, conidium; G, germ tube. Bar = 20 μm.

ectopic-integration transformants were spotted on detached cucumber leaves and incubated for 7 days. The ectopic-integration transformant DCM2 formed yellowish lesions in all spotted regions the same as the wild type. In contrast, the *Δcmk1* strain DCM1 never formed any lesions on cucumber leaves (Fig. 3). Identical results were observed for all the *Δcmk1* strains examined (data not shown). After 2 weeks of incubation, yellowish lesions caused by both wild type and ectopic transformants spread and turned brown. In the leaves inoculated by the *cmk1* mutants, not even small lesions were observed (data not shown). To determine whether loss of pathogenicity in the *cmk1* mutants could be complemented by reintroduction of *CMK1*, pCBCMK1 containing the *CMK1* gene with the bar gene was transformed into the *cmk1* mutant DCM1. Bialaphos-resistant transformants were selected and DNA gel blot analysis confirmed introduction of the *CMK1* gene in two independent transformants, RCM1 and RCM2 (data not shown). The transformants RCM1 and RCM2 were tested for their pathogenicity on cucumber leaves. We found that they formed lesions similar to those of the wild-type strain (Fig. 3), indicating the reintroduction of *CMK1* com-

plemented pathogenicity of the *CMK1* knockout transformant DCM1. We conclude that *CMK1* is essential for pathogenicity of *C. lagenarium*.

Failure of conidial germination in *cmk1* mutants.

To investigate the deficient steps in the pathogenicity of the *Δcmk1::Hph* strains, we examined the behavior of the *Δcmk1* strains on the lower surface of host cucumber cotyledons by microscopic observation. In the wild type, most conidia germinated at 6 h post inoculation and formed darkly melanized appressoria within 12 h (Fig. 4). In contrast, none of the *Δcmk1* strains germinated on host leaves after 12 h (Fig. 4). The germination frequency of conidia of the *Δcmk1* strains was below 1% after 24 h. Furthermore, the very rare germinating conidia of the *Δcmk1* strains did not form appressoria on host leaves (data not shown). These results suggest that loss of pathogenicity in the *Δcmk1* strains is mainly due to the deficiency of the pre-penetration process, i.e., both conidial germination and appressorium formation.

To evaluate whether disruption of *CMK1* resulted in inherent defects in conidial germination, germination of the *cmk1* mutants was assayed in vitro. When wild-type conidia were suspended in water and incubated on hard, artificial surfaces,

Fig. 2. Disruption of the *Colletotricum lagenarium* *CMK1* gene. **A**, The *CMK1* locus and gene-replacement vector pCMK1GD. pCMK1GD contains a hygromycin-resistant gene (*HPH*) cassette flanked by border sequences from *CMK1*. By homologous recombination through double crossing over, the major region of *CMK1* was replaced by the *HPH* cassette. Restriction enzyme sites are indicated as follows: A, *Apa*I; H, *Hind*III; X, *Xho*I. **B**, DNA gel blot analysis of *CMK1* gene replacement transformants. Genomic DNAs were extracted from (lane 1) wild-type strain 104-T, (lanes 2–5) *CMK1* gene replacement transformants DCM1, DCM3, DCM7, and DCM17, and (lanes 6 and 7) ectopic transformants DCM2 and DCM4. All genomic DNA samples were digested with *Hind*III. Blot was probed with a 2-kb polymerase chain reaction product containing *CMK1*.

Table 1. Characteristics of *cmk1* mutant strains

Strain ^a	Vegetative growth ^b (mm)	Conidia ^c (×10 ⁶)	Conidium attachment ^d (%)
104-T	31.8 ± 0.2	16.2 ± 3.3	87.1 ± 0.8
DCM1	30.7 ± 0.9	0.6 ± 0.2	22.4 ± 2.6
DCM3	31.5 ± 0.8	0.7 ± 0.1	17.4 ± 0.6
DCM7	31.7 ± 1.0	0.6 ± 0.1	24.3 ± 4.3
DCM17	31.0 ± 0.4	0.6 ± 0.2	18.8 ± 0.3
DCM2	30.2 ± 0.5	17.7 ± 6.9	89.8 ± 7.6
DCM4	30.3 ± 0.5	15.6 ± 2.8	88.2 ± 3.5

^a 104-T is the wild-type strain. DCM1, DCM3, DCM7, and DCM17 are *CMK1* disrupted transformants. DCM2 and DCM4 are ectopic integration transformants.

^b Strains were grown on potato dextrose agar at 24°C for 7 days, and colony diameter was measured.

^c Conidia were collected from 7-day-old cultures and counted.

^d Conidial suspensions were placed on glass petri dishes. At 1 h post incubation, all conidia in a marked area were counted. Glass dishes were shaken with a reciprocal shaker (200 rpm, 5 min; Toyko Rikakikai, Tokyo), and rinsed with water. Conidia remaining in the marked area were counted. Each value represents mean ± SD from three independent experiments.

Fig. 3. *CMK1* is required for pathogenicity on cucumber leaves. Conidial suspensions of tested strains were spotted on right half of detached cucumber leaves. On left half of leaves, the wild-type strain 104-T was inoculated as a positive control. Shown are leaves inoculated with following strains: leaf A, ectopic integration transformant DCM2; leaf B, *Δcmk1::Hph* transformant DCM1; leaf C, *CMK1*-introduced transformant of DCM1, RCM1.

conidia started to germinate and form appressoria. The frequency of conidial germination is significantly affected by differences in surface properties of hard surfaces such as glass and plastic. Conidia can efficiently germinate on glass petri dishes after 6 h. However, only 20 to 30% conidial germination is observed after 24 h incubation on plastic petri dishes (Table 2). These results suggest that contact of conidia with the glass surface induces germination more efficiently, compared with the plastic surface. Germinating conidia of the wild type can efficiently differentiate into appressoria on both glass and plastic surfaces (Fig. 4).

Germination of the $\Delta cmk1::Hph$ strains was investigated on both glass and plastic surfaces. The $\Delta cmk1$ strains hardly germinate on glass surfaces. Only 1 to 2% of conidia germinated after 24 h whereas most of the wild-type conidia germinated after 6 h (Table 2 and Fig. 4). Germination was still poor after 48 h (data not shown). Also, conidia of the $\Delta cmk1$ strains never germinated on plastic surfaces. We conclude that the failure of germination is an inherent defect in the $\Delta cmk1$ strains.

Rescue of germination in *cmk1* mutants by addition of yeast extract.

In addition to contact stimuli on glass surfaces, chemical compounds also trigger conidial germination of *C. lagenarium*. It was previously found that yeast extract induced conidial germination of the *C. lagenarium* wild-type strain (Y. Takano, unpublished result). Yeast extract enabled wild-type conidia to germinate efficiently on a non-inducible plastic surface. By addition of 0.1% yeast extract, most conidia (>95%) completed germination and appressorium formation on a plas-

tic surface after 6 h (Table 2). In the presence of yeast extract, secondary germination and branching of germ tubes occurred, and these secondary germ tubes frequently formed appressorium-like structures after 12 h (Fig. 4).

We investigated the effect of yeast extract on germination of the $\Delta cmk1::Hph$ strains. Yeast extract completely rescued conidial germination of the $\Delta cmk1$ strains, although germination was significantly delayed, compared with the control strains (Table 2). At 6 h post incubation on a glass surface, the germination rate of the $\Delta cmk1$ strains was only 1 to 2%

Table 2. Conidial germination in *cmk1* mutants

Strain	Water		Yeast extract	
	Glass	Plastic	Glass	Plastic
6 h				
104-T	92.6 ± 4.0 ^a	20.2 ± 9.1	98.4 ± 1.1	96.7 ± 0.7
DCM1	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 1.2	0.6 ± 0.9
DCM3	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 1.0	0.3 ± 0.4
DCM2	90.1 ± 1.8	22.1 ± 5.0	97.4 ± 1.8	98.0 ± 0.6
12 h				
104-T	98.2 ± 1.1	27.8 ± 4.1	98.6 ± 0.5	98.2 ± 1.0
DCM1	0.5 ± 0.4	0.0 ± 0.0	62.8 ± 6.6	16.9 ± 5.4
DCM3	0.2 ± 0.3	0.0 ± 0.0	74.2 ± 12.6	24.8 ± 15.2
DCM2	97.4 ± 2.4	22.5 ± 1.2	98.8 ± 0.1	98.7 ± 0.1
24 h				
104-T	98.3 ± 0.8	28.3 ± 6.1	99.4 ± 0.5	99.5 ± 0.4
DCM1	1.0 ± 0.8	0.0 ± 0.0	94.2 ± 0.7	68.2 ± 5.6
DCM3	1.1 ± 0.8	0.0 ± 0.0	94.8 ± 2.4	69.3 ± 7.2
DCM2	98.8 ± 0.5	20.5 ± 1.6	99.0 ± 0.2	98.7 ± 0.1

^a For each time point, at least 200 conidia were examined to calculate percentage of germination. The mean and SD were calculated from three independent experiments.

Fig. 4. Conidial germination and appressorium formation of *cmk1* mutants. Conidia of the wild-type 104-T and $\Delta cmk1$ strain DCM1 were incubated on host cucumber cotyledons and a glass surface at 24°C for 12 h. The transformant DCM1 hardly germinated on glass and host surfaces. Following addition of 0.1% yeast extract, DCM1 could germinate but failed to form appressoria whereas the wild type formed appressoria. A, appressorium; C, conidium; G, germ tube. Bar = 20 μ m.

whereas most of the wild-type conidia had completed germination. However, the frequency of germination of the mutants eventually reached around 94% by 24 h. On plastic, conidia of *cmk1* mutants could germinate if 0.1% yeast extract was added. The frequency of germination on plastic was approximately 70% at 24 h, which was relatively low compared with that on the glass surface (Table 2). Other complex nutrients, tryptone and peptone, also rescued germination of the $\Delta cmk1$ strains. However, the efficiency of germination in the presence of these nutrients was relatively low compared with that with yeast extract (data not shown).

***CMK1* is required for both appressorium formation and invasive growth in plants.**

The germinating conidia of the wild-type strain formed darkly melanized appressoria following glass surface germination (Fig. 4). When germination of the *cmk1* mutants was rescued by the addition of yeast extract, germinating conidia still failed to form appressoria on either glass or plastic surfaces, unlike the wild type (Fig. 4). Germ tubes of the *cmk1* mutants curled and hooked repeatedly and continued to elongate (Fig. 4). No clear melanization was observed in these germinating conidia. These results demonstrate that *CMK1* is required for appressorium formation. We conclude that the inability of *cmk1* mutants to cause symptoms occurs due to a failure of *cmk1* mutants to germinate efficiently and form appressoria.

To assess invasive growth ability of the *cmk1* mutants, we performed inoculation assays through wound sites. For a positive control strain retaining invasive growth ability, we used the albino mutant 79215, which carries a mutation of the melanin gene *PKS1* and cannot penetrate because of a lack of melanization in appressoria. Conidia of the ectopic transformant DCM2, the *pks1* mutant 79215, and the $\Delta cmk1$ strain DCM1 were incubated in yeast extract at 32°C for 16 h. These conditions allowed all strains to form vegetative hyphae but not appressoria (Takano et al. 1997b). The morphology of the germinating conidia was similar for all strains examined (data not shown). The germinated conidia were harvested, and suspensions of conidia were inoculated on wounded sites of leaves. In addition, preincubated conidia that did not start germination were inoculated on intact leaves to test an ability of appressorial penetration in each strain. Following incubation, the *pks1* mutant 79215 only formed lesions on wounded plants whereas DCM2 formed lesions on both intact and wounded plants (Fig. 5). In contrast, even when inoculated on wounded plants, the $\Delta cmk1$ strain DCM1 did not develop lesions, but caused tiny, yellow spots that were identical to those produced in the water control (Fig. 5). Microscopic examination revealed that the $\Delta cmk1$ strain hardly extended vegetative hyphae inside plants (data not shown). These results indicate that *CMK1* is required for invasive growth in cucumber leaves.

***CMK1* is involved in expression of melanin genes *PKS1*, *SCD1*, and *THR1* for appressorial melanization.**

The $\Delta cmk1::Hph$ strains formed melanized colonies like the wild type, indicating that they could produce melanin during vegetative growth. During pre-penetration morphogenesis of the wild-type conidia, melanization is confined to appressoria. The *cmk1* mutants also failed to synthesize melanin as judged

by the lack of pigment in conidia or elongated germ tubes. To test if melanin biosynthesis genes were expressed in the *cmk1* mutants, we examined the expression of three genes, *PKS1*, *SCD1*, and *THR1*, encoding major melanin biosynthetic enzymes in the $\Delta cmk1$ strain DCM1. First, we investigated the expression of the melanin genes during mycelial melanization. Mycelial melanization in the wild type can be induced rapidly by transferring mycelia to media of high osmolarity (Takano et al. 1995). Mycelial melanization in *cmk1* mutants was induced as rapidly as in the wild type (data not shown). RNA blot analysis showed that the transcripts of *PKS1*, *SCD1*, and *THR1* accumulated after the induction of melanin biosynthesis in both wild type and DCM1, indicating that the melanin biosynthesis genes were expressed normally in the *cmk1* mutant during mycelial melanization (Fig. 6A). During pre-penetration events in the wild type, *PKS1*, *SCD1*, and *THR1* transcripts were not detected in preincubated conidia, but accumulated at 3 h post incubation in both water and yeast extract solution when approximately 50 to 70% of conidia germinated (Takano et al. 1997b and Fig. 6B). In contrast, when DCM1 was incubated in water where conidia could not germinate, the transcripts of the two melanin genes *PKS1* and *SCD1* were not detected at 3 h. On the other hand, the *THR1* transcript was detected as two bands at 3 h in DCM1, whereas the accumulation level of transcripts was significantly reduced compared with that of the wild type. After long incubation (at 16 h), neither the *PKS1*, *SCD1*, nor *THR1* transcripts were detected in DCM1 (Fig. 6B). In the case of incubation in yeast extract solution, the expression pattern at 3 h was similar to the case of incubation in water. However, at 16 h, when 70 to 80% of the DCM1 conidia germinated, three melanin genes were highly expressed (Fig. 6B).

DISCUSSION

We have identified and characterized the MAP kinase gene *CMK1* to assess roles of MAP kinase pathways in fungal pathogenesis of *C. lagenarium*. *CMK1* complemented appressorium formation defects of the *pmk1* mutant of *M. grisea*, indicating that *CMK1* could be functional in *M. grisea*. The gene disruption analysis of *CMK1* in *C. lagenarium* demonstrated that *CMK1* is essential for pathogenicity. This suggests

Fig. 5. Invasive growth of *cmk1* mutants in cucumber plants. Right halves of detached cucumber leaves were wounded by scratching with a needle. Before inoculation on wounded leaves, conidia of each strain were incubated in 0.1% yeast extract solution at 32°C for 16 h. This incubation induced conidial germination without appressorium formation. After incubation at 32°C, conidia were recovered, and a suspension of germinating conidia was spotted on wounded sites of the right half leaves. To assess appressorial penetration ability, preincubated conidia of each strain were spotted on the left half leaves without wounding treatment. Inoculated leaves were incubated at 24°C for 7 days. A, water control; B, ectopic integration transformant DCM2; C, $\Delta cmk1::Hph$ transformant DCM1; D, the *pks1* mutant 79215.

that Fus3/Kss1-related MAP kinases may play a pivotal role in fungal phytopathogenicity. Further analysis revealed that *cmk1* mutants have defects in both appressorium formation and pathogenic growth, in common with the *M. grisea pmk1* mutants. However, unlike the *pmk1* mutants, the *cmk1* mutants fail to undergo conidial germination on hard surfaces.

Signal transduction pathways regulating conidial germination.

Yeast extract efficiently induces germination of the wild-type conidia on a non-inducible plastic surface, suggesting that yeast extract induces germination independent of surface

signals. Yeast extract also rescued germination of the $\Delta cmk1::Hph$ strains. On the other hand, an inhibitory effect of yeast extract on appressorium formation has been reported in *M. grisea* and *C. gloeosporioides* (Beckerman and Ebbole 1996; Podila et al. 1993). In *M. grisea*, yeast extract inhibits appressorium formation in a mating type-specific manner whereas other complex nutrients, peptone and tryptone, show no effect. In contrast, yeast extract induces germination without inhibition of appressorium formation in *C. lagenarium*. Furthermore, peptone and tryptone also induced germination although yeast extract was most effective. Thus, it is conceivable that general complex nutrients could induce conidial germination of *C. lagenarium*, but yeast extract contains additional, effective compounds for induction of germination.

The finding that the *cmk1* mutants hardly germinated on a glass surface indicates a failure to respond to glass surface signals. Also, restoration of germination by yeast extract in the *cmk1* mutants was not complete; germination of the *cmk1* mutants was significantly delayed, compared with the wild type. These observations suggest that the CMK1 MAP kinase pathway regulates germination triggered by both glass surface contact and nutrient signals. However, restoration of germination in the *cmk1* mutants strongly suggests the presence of a bypass pathway that can induce germination independent of the CMK1 pathway.

In addition, disruption of *CMK1* caused reduction of conidiation, whereas the *M. grisea pmk1* mutants produce conidia normally. In contrast, disruption of the *Neurospora crassa nrc-1* gene, encoding a MAP kinase kinase kinase related to yeast Ste11, resulted in constitutive conidiation (Kothe and Free 1998). These results suggest that the Fus3/Kss1-related MAP kinase pathways might have distinct roles for conidiation among filamentous fungi. Also, conidia of the *cmk1* mutants showed reduced ability to attach to a glass surface. In *Colletotrichum graminicola*, extracellular materials have been shown to be involved in conidium attachment (Mercure et al. 1995). We speculate that synthesis or secretion of some extracellular materials might be impaired by disruption of *CMK1*. In *M. grisea*, it has been reported that a mutant showing a defect in conidium attachment could germinate efficiently (Lau and Hamer 1998), implying that conidium attachment is dispensable for conidial germination. However, at present, we cannot exclude the possibility that failure of germination in the *cmk1* mutants is partially due to weak attachment of conidia.

CMK1 MAP kinase pathway for fungal pathogenesis.

When germinating conidia of the $\Delta cmk1$ strains were incubated in yeast extract solution, they developed elongating germ tubes but never formed appressoria. This phenotype is similar to the phenotype of the *pmk1* mutant of *M. grisea*. In *M. grisea*, it has been proposed that the Pmk1 MAP kinase pathway acts cooperatively with a cAMP signaling pathway for appressorium formation because addition of cAMP could induce the early step of appressorium formation in *pmk1* mutants (Xu and Hamer 1996). To study the roles of the cAMP signaling pathway and the relationship between the CMK1 MAP kinase and the cAMP signaling pathway for fungal pathogenicity, we have recently cloned a gene encoding adenylate cyclase of *C. lagenarium* (Y. Takano and K. Komeda, unpublished result). Gene disruption analysis of the *C. lagenarium* adenylate cyclase gene is now in progress.

Fig. 6. Expression of three melanin genes, *PKS1*, *SCD1*, and *THR1*, in the *cmk1* mutant. **A**, Expression of melanin genes during mycelial melanization of the wild-type strain 104-T and the *cmk1* mutant DCM1. For mycelial melanization, mycelia grown in liquid medium was transferred to high osmotic solution. RNA samples were isolated from mycelia before melanin induction (at 0 h), and at 8 and 24 h after the start of melanin induction. Approximately 4 μ g of total RNA was loaded into each lane. **B**, Expression of melanin genes during incubation of the *cmk1* mutant conidia. Conidia were incubated on glass dishes in water (W) or in 0.1% yeast extract solution (Y). For the wild type, RNA samples were isolated from conidia at 0 and 3 h after the start of incubation. For the *cmk1* mutant DCM1, RNA samples were isolated from conidia at 0, 3, and 16 h. Approximately 2 μ g of total RNA was loaded into each lane. Blots were hybridized with 32 P-labeled in vitro transcripts complementary to mRNA of the *PKS1*, *SCD1*, *THR1*, and *TUB1* (*Colletotrichum lagenarium* β -tubulin gene, an internal control). The rRNA stained with ethidium bromide is also shown.

CMK1 is also required for invasive growth in a host plant although *CMK1* is dispensable for vegetative growth, which is consistent with the phenotype of the *pmk1* mutants. These results indicate that fungal invasive growth in plants is a stage quite distinct from the vegetative growth stage. At present, little is known about invasive growth inside plants in these plant fungal pathogens. Interestingly, recent reports demonstrate that *S. cerevisiae* Kss1 MAP kinase is involved in invasive growth into solid medium (Cook et al. 1997; Madhani et al. 1997). There is a possibility that invasive growth of both yeast and plant fungal pathogens is regulated by similar mechanisms.

Although *CMK1* is dispensable for vegetative growth stage on PDA, the *CMK1* gene was expressed in vegetative mycelia as well as in conidia (data not shown). We speculate that *CMK1* may be expressed constantly in each developmental stage. It is necessary to investigate whether activation of *CMK1* MAP kinase is regulated by phosphorylation and subsequent translocation to the nucleus, as shown in mammalian MAP kinases (Lenormand et al. 1993).

Regulation of melanin biosynthetic genes during appressorium formation.

In the $\Delta cmk1$ strain DCM1, expression of three melanin genes, *PKS1*, *SCD1*, and *THR1*, was induced normally during mycelial melanization. In contrast, during conidial incubation, expression of melanin genes in DCM1 was quite distinct from that of the wild type. In the case of incubation where DCM1 could not germinate, the expression of two melanin genes, *PKS1* and *SCD1*, was not induced. In the presence of yeast extract, transcripts of these melanin genes accumulated at 16 h when conidia of the DCM1 strain started to germinate, although none of the germinating conidia of DCM1 formed appressoria. These results strongly suggested that the transcription of *PKS1* and *SCD1* was tightly linked to the conidial germination process, but not to subsequent appressorium formation. Interestingly, the *THR1* transcript was detected at 3 h post incubation in both water and yeast extract, and disappeared when conidia could not germinate. When conidia germinated, the *THR1* transcript accumulated the same as those of *PKS1* and *SCD1*. We speculate that more early events, for example, attachment of conidia on a hard surface, partially induce transcription of *THR1* whereas the germination process induces high expression of *THR1*. Previously, we found that three melanin genes were transcribed in germinating conidia at 32°C although appressoria were not formed and melanization was not observed (Takano et al. 1997b). We speculated that post-transcriptional regulation was involved in appressorial melanization, and that this regulation did not function in the germinating conidia at 32°C. For DCM1, melanization was not observed in germinating conidia at 24°C although melanin genes were highly expressed, similar to germinating conidia of the wild type at 32°C. These findings suggest that the putative post-transcriptional regulation did not function in germinating conidia of the $\Delta cmk1$ strain.

We present evidence that the MAP kinase *CMK1* plays crucial roles in the fungal pathogenesis of *C. lagenarium*. Our interest is how *CMK1* MAP kinase regulates the multiple steps required for pathogenesis. Future studies should identify downstream components of the *CMK1* MAP kinase pathway. These studies will contribute to understanding the relationship

between the fungal signaling pathway and pathogenesis in more detail, and offer novel targets for strategies to protect plants from fungal infections.

MATERIALS AND METHODS

Fungal strains and culture conditions.

The *Colletotrichum lagenarium* (Pass.) Ellis & Halst. (syn. *C. orbiculare* (Berk. & Mont.) Arx) strain 104-T (stock culture of the laboratory of Plant Pathology, Kyoto University) was used as the wild-type strain. All *C. lagenarium* cultures were maintained on PDA media (3.9% [wt/vol] PDA; Difco, Detroit, MI) at 24°C in the dark. Conidia were obtained by gently scraping cultures incubated for 7 days. For conidiation of *M. grisea*, strains were grown at 24°C on oatmeal sucrose plates (5% oatmeal, 1% sucrose, 2% agar) for 14 days, followed by placing the plates under a fluorescent lamp for 3 days after removing the aerial mycelia. Conidia formed on plate were recovered with a brush, and suspended in sterilized water.

CMK1 cloning.

A nested PCR was performed to isolate the *CMK1* gene with cDNA synthesized from polyadenylated RNA prepared from conidia at 2 h after the start of incubation for appressorium formation. Three degenerated primers—primer HRD, 5'-CAYMGNGAY-NTNAARCC-3' (HRDLKP), primer CDF, 5'-GGACGGATCCTGYGAYTTYGGN-YTNGC-3' (CDFGLA), and primer WYR, 5'-CCCAGAATTCTCNGGNGCNCRTA-CCA-3' (WYRAPE)—were designed based on the conserved amino acids in *S. cerevisiae*, *M. grisea*, and human MAP kinases. The primer CDF contained a terminal *Bam*HI site, whereas the primer WYR contained a terminal *Eco*RI site. The first PCR was performed with primers HRD and WYR with Ex *Taq* polymerase (Takara, Ohtsu, Japan). Nested PCR was performed with primers CDF and WYR. PCR conditions were as follows: 30 cycles (94°C, 1 min; 52°C, 2 min; 72°C, 2 min), with a final extension at 72°C for 5 min. Nested PCR products were digested with *Bam*HI and *Eco*RI and cloned into pBlueScript II KS⁻ (Stratagene, La Jolla, CA), and sequenced. Clone pRCMK1 having homology with *M. grisea* *PMK1* was used as a probe to screen both cDNA and genomic library of *C. lagenarium* (Kubo et al. 1991). The cDNA library was constructed with polyadenylated RNA isolated from conidia, at an early stage of pre-penetration process, that were incubated in water on glass petri dishes for 2 h.

C. lagenarium and *M. grisea* transformation.

Preparation of protoplasts and transformation of *C. lagenarium* were performed according to the method described previously (Kubo et al. 1991). Hygromycin- and bialaphos-resistant transformants were selected on regeneration medium containing 100 µg of hygromycin B per ml (Wako Pure Chemicals, Osaka, Japan) and 250 µg of bialaphos per ml (kindly provided by Hiroyuki Anzai, Meiji Seika Kaisha), respectively. The protocols for *C. lagenarium* were also applied to preparation of protoplasts and transformation of *M. grisea*.

Genomic DNA blot analysis.

Total DNA of *C. lagenarium* and *M. grisea* strains was isolated from mycelia as described previously (Takano et al.

1997a). DNA probes were labeled with [α - 32 P]dCTP (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) with the BcaBEST labeling kit (Takara, Ohtsu, Japan). Hybridization was carried out with hybridization buffer (50% [vol/vol] formamide, 5 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% [wt/vol] sodium dodecyl sulfate, 5% [wt/vol] sodium dextran sulfate) at 42°C for 16 h. The membranes were washed at 42°C with 1 $\times$ SSC once for 1 min and twice for 30 min.

RNA blot analysis.

Total RNA was isolated from vegetative mycelia, melanized mycelia, preincubated conidia, and conidia incubated on glass as described previously (Takano et al. 1997b). For preparation of vegetative mycelia, agar blocks from colonies grown on PDA were inoculated into potato-sucrose liquid medium containing 0.2% yeast extract (Nacalai Tesque, Kyoto, Japan) and incubated at 26°C in a reciprocal shaker for 72 h. For induction of melanin biosynthesis, the mycelia were transferred to 1.2 M sucrose solution and incubated in the shaker (Takano et al. 1995), and then melanized mycelia were recovered. Conidia (about 5 $\times$ 10⁵ conidia per ml) suspended in water or in 0.1% yeast extract solution (Nacalai Tesque) were poured into 9-cm-diameter glass petri dishes (Iuchi, Osaka, Japan) and incubated at 24°C. Conidia were harvested at 3 and 16 h after the start of incubation. To prepare RNA from conidia before the start of incubation (at 0 h), conidia harvested from cultures were directly subjected to RNA extraction. RNA probes were prepared with T3, T7, or SP6 RNA polymerase by in vitro transcription in the presence of [α - 32 P]UTP (Amersham Pharmacia Biotech). The DNA region used for in vitro transcription of *PKS1*, *SCD1*, *THR1*, and *TUB1* was described previously (Takano et al. 1997b). RNA was blotted and hybridized mainly following procedures described by Perpetua et al. (1996).

Construction of the gene replacement vector pCMK1GD.

The 2.2-kb *SalI* fragment containing the *HPH* gene, derived from plasmid pSH75 (Kimura and Tsume 1993), was introduced into the *SalI* site of pBlueScript KS⁻, and the resultant plasmid was designated pSHPH. The 2.9-kb *HindIII* fragment containing the 3' flanking region of *CMK1* was inserted into the *HindIII* site of pSHPH to produce plasmid pSHF3. The 1.7-kb fragment containing the 5' flanking region of *CMK1* was amplified by PCR with primers CM1APS1 (5'-ACGCAAGGGGCCCTCGAAGTCT-ATGGCGTCA-3') and CM1XHAS1 (5'-AACCTGCTCGAGCTTACCTGGATCAGG-TA-3'). The primer CM1APS1 contained a terminal *ApaI* site, whereas the primer CM1XHAS1 contained a terminal *XhoI* site. PCR was performed with *Pfu* DNA polymerase (Stratagene) for high fidelity of DNA synthesis. The amplified fragment was digested with *ApaI* and *XhoI*, and introduced into the *ApaI*-*XhoI* sites of pSHF3 to give the plasmid pCMK1GD.

Pathogenicity tests.

The inoculation assay on cucumber (*Cucumis sativus* L. 'Suyo') was performed as described (Kubo et al. 1982; Takano et al. 1997a). Twenty microliters of conidial suspension (about 5 $\times$ 10⁵ conidia per ml) was spotted on the surface of cucumber leaves. After inoculation, the leaves were

placed in humid petri dishes and incubated at 24°C for 7 days. For microscopic observation, 20 μ l of conidial suspension was spotted onto the lower epidermis of cucumber cotyledons. Following incubation, the epidermal layers were peeled off, stained with lactophenol aniline blue, and observed under light microscopy at 3 days post incubation.

In the case of inoculation through wounded sites, germinating conidia were inoculated instead of preincubated conidia. Conidia were incubated in 0.1% yeast extract solution at 32°C for 16 h. Conidia of all strains germinated and formed elongated mycelia without appressorium formation. These germinating conidia were harvested as a suspension, and spotted on the wounded sites (1 to 2 mm²) produced by scratching with 26G_{1/2} needles.

In vitro assay for conidial attachment, germination, and appressorium formation.

For *M. grisea*, conidia were harvested from 17-day-old cultures grown on oatmeal agar, suspended in water, and adjusted to a concentration of 10⁵ conidia per ml. For *C. lagenarium*, conidia were harvested from 7-day-old cultures on PDA plates. For the conidial attachment assay of *C. lagenarium*, 750 μ l of conidial suspension in water (5 $\times$ 10⁵ conidia per ml) was placed in a 3.8-cm-diameter glass petri dishes (Iuchi, Osaka, Japan). After 1 h of incubation at 24°C, conidia in marked areas were counted under light microscopy, and then petri dishes were shaken with a reciprocal shaker at 200 rpm for 5 min (Tokyo Rikakikai, Tokyo). After shaking, water in dishes was removed and gently replaced by 750 μ l of water. Conidia remaining in marked areas were counted. As for germination and appressorium formation of *M. grisea*, a conidial suspension (100 μ l) was placed on hydrophobic side of GelBond film (FMC, Rockland, ME), and incubated in a moistened plastic box at 24°C. For *C. lagenarium*, a conidial suspension (5 $\times$ 10⁵ conidia per ml) was placed in 3.8-cm-diameter glass petri dishes, or 3.6-cm-diameter plastic petri dishes (Becton Dickinson, Lincoln Park, NJ), and incubated at 24°C.

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