A Yeast *STE11* Homologue *CoMEKK1* Is Essential for Pathogenesis-Related Morphogenesis in *Colletotrichum orbiculare*

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Several signal transduction pathways, including mitogen-activated protein kinase (MAPK) pathways, are involved in appressorium development in *Colletotrichum orbiculare*, the causal agent of cucumber anthracnose disease. In this study, *CoMEKK1*, a yeast MAPK kinases (MAPKK) kinase *STE11* homolog, was identified as a disrupted gene in an *Agrobacterium tumefaciens*-mediated transformation mutant. The phenotype of *comekk1* disruptant was similar to that of *cmk1*, a *Saccharomyces cerevisiae* *Fus3/Kss1* MAPK homolog mutant. Moreover, *comekk1* and *cmk1* mutants were sensitive to high osmotic and salinity stresses, indicating that *Comekk1p/Cmk1p* signal transduction is involved in stress tolerance. The transformants of the wild type and the *comekk1* mutant expressing a constitutively active form of the *CoMEKK1* showed slower hyphal growth and abnormal appressorium formation, whereas those of the *cmk1* disruptant did not. A *Cmk1p*-green fluorescent protein (GFP) intracellular localization experiment indicated that nuclear localization of the *Cmk1p*-GFP fusion protein induced by salt stress was diminished in *comekk1* mutants. These results indicate that *Comekk1p* functions upstream of *Cmk1p*.

Eukaryotic cells are constitutively stimulated by environmental signals such as growth factors, physical and chemical components, and nutrient starvation. The cells respond to these signals to adapt to specific environmental conditions. Plant-pathogenic fungi possess signal transduction systems to respond to environmental cues. On recognition of the appropriate signals, the plant-pathogenic fungi develop pathogenesis-related structures via signal transduction pathways. Mitogen-activated protein kinase (MAPK) pathways are involved in transducing environmental cues and regulate cell differentiation in the plant-pathogenic fungi (Zhao et al. 2007). MAPK are usually activated by MAPK kinases (MAPKK or MEK), which are activated, in turn, by MEK kinases (MAPKKK or MEKK). These MAPK cascades (MEKK-MEK-MAPK) are conserved in eukaryotic cells and have been studied in several organisms. In the budding yeast *Saccharomyces cerevisiae*, MAPKKK *STE11* initiates activation of the remainder of the MAPK cascade, MAPKK *STE7*, and MAPK *FUS3*, in response to pheromones (Chen and Thorner 2007). In plant-pathogenic

fungi, this pheromone response-related MAPK cascade homolog is essential for pathogenicity (Zhao et al. 2007). In the rice blast fungus *Magnaporthe oryzae*, for instance, the *MST11-MSt7-PMK1* cascade is involved in appressorium formation and infective hyphae development in host leaves (Zhao et al. 2007). In *Ustilago maydis*, a causal agent of corn smut disease, the *Ubc4/Kpp4-Fuz7/Ubc5-Ubc3/Kpp2* cascade is involved in formation of conjugation tubes, filamentous growth, and pathogenicity (Andrews et al. 2000; Banuett and Herskowitz 1994; Mayorga and Gold 1998, 1999; Müller et al. 1999, 2003).

Colletotrichum orbiculare is the causal agent of cucumber anthracnose disease. The infection process is initiated on recognition of an appropriate surface. This fungus undergoes a series of changes in morphology upon recognizing appropriate signals, including formation of a specialized infection structure, the appressorium. Metabolisms essential for appressorium function include melanin synthesis (Kubo and Furusawa 1991; Kubo et al. 1996; Perpetua et al. 1996; Takano et al. 1995; Tsuji et al. 2000), peroxisome metabolism (Asakura et al. 2009; Kimura et al. 2001; Fujihara et al. 2010), cellular polarity (Sakaguchi et al. 2008, 2010), and cell wall integrity (Tanaka et al. 2007, 2009).

Several signal-transduction-related genes associated with these morphological changes have been characterized in *C. orbiculare* and it has been shown that the MAPK and cyclic AMP (cAMP) signaling pathways are linked to infection-related morphological changes in this fungus. The budding yeast *SLT2* homolog *MAF1* is essential for appressorium development but not for pathogenicity (Kojima et al. 2002). A pheromone response MAPK gene *FUS3/KSS1* homolog, *CMK1*, was also identified, and the *cmk1* mutant displayed conidial germination, appressorium formation, and invasive growth (Takano et al. 2000). *CST1*, a homolog of transcriptional factor *STE12*, the downstream target of *FUS3/KSS1*, is essential for formation of infection pegs from the appressoria (Tsuji et al. 2003b). However, upstream MAPK factors have not been identified for *C. orbiculare*.

The cAMP signaling pathway also plays a pivotal role in transducing environmental cues for cell development. Moreover, crosstalk between protein kinase A (PKA) and MAPK signaling is a broad phenomenon in eukaryotes (Stork and Schmitt 2002). The cAMP signaling pathway plays a critical role in regulating conidial germination and pathogenicity in *C. orbiculare* (Takano et al. 2001; Yamauchi et al. 2004). Although mutants of the *RPK1* gene encoding the PKA regulatory subunit form normal melanized appressoria, they are defective in generating penetration hyphae (Takano et al. 2001). Mutants of adenylate cyclase and PKA genes have also been

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shown to be defective in conidial germination and pathogenicity (Yamauchi et al. 2004).

In this study, we identified a putative *C. orbiculare* MAPKK kinase encoding gene *CoMEKK1*, which shows high homology with *S. cerevisiae* *STE11* based on mutant screening. By analyzing *comekk1* disrupted mutants, we elucidated that *CoMEKK1* is essential for pathogenesis-related morphogenesis and, thus, pathogenesis.

RESULTS

Identification of a *STE11* homolog *CoMEKK1* in *C. orbiculare*.

CoMEKK1 was identified by screening *Agrobacterium tumefaciens*-mediated transformation (AtMT) T-DNA mutants of *C. orbiculare* for defective appressorium development on cucumber leaves. We obtained a mutant responsible for defective appressorium development named KENH-2 (Fig. 1A). To isolate genomic DNA segments adjacent to a T-DNA of the KENH-2 strain, T-DNA insertion regions in mutant genomic DNA were amplified by thermal asymmetrical interlaced polymerase chain reaction (TAIL-PCR). Sequence analysis showed that the sequenced TAIL-PCR products had significant homology with *STE11* in *S. cerevisiae*; they were designated as *CoMEKK1*. A primer pair was designed to isolate a *CoMEKK1* genomic clone from a genomic cosmid library of *C. orbiculare*. The full-length sequence of *CoMEKK1* was de-

termined. Based on the preferred translational initiation codon, *CoMEKK1* was predicted to encode a protein of 901 amino acids (aa) (Fig. 1B). Like *STE11* in *S. cerevisiae*, *CoMEKK1* encodes a sterile α motif (SAM) domain at the N terminal (66 to 129 aa) and a C-terminal protein kinase domain (627 to 897 aa). Downstream of the SAM domain, *CoMEKK1* also encodes a Ras-association (RA) domain (251 to 341 aa), which is conserved in *STE11* homologs from other filamentous fungi (Fig. 1C) (Zhao et al. 2007).

To investigate distribution of *CoMEKK1* homologs in other fungal genomes, we searched the National Center for Biotechnology Information nonredundant protein database using BLASTP. The deduced amino acid sequence of *CoMEKK1* showed high similarity with amino acids of the *STE11* homolog in *S. cerevisiae* and filamentous fungi such as *M. oryzae*, *U. maydis*, *Aspergillus nidulans*, and *Neurospora crassa*. A phylogenetic tree, based on the amino acid sequences of *CoMEKK1* homologs and other MAPK kinases, indicated that *Comekk1p* was classified with the *Ste11* MAPKK kinase family (Fig. 2).

CoMEKK1 is essential for infection-related morphogenesis and plant infection.

To determine whether *CoMEKK1* functioned in the *Cmk1* MAPK pathway of *C. orbiculare*, we generated *CoMEKK1* gene-disrupted mutants. An *Agrobacterium tumefaciens* binary gene replacement vector (revS-MEKK1AH3) was introduced into the wild-type *C. orbiculare* 104-T by AtMT (Fig. 3A). Gene replacement and reintroduction of *CoMEKK1* was confirmed by DNA gel blot analysis (Fig. 3B).

Phenotypes of the *comekk1* mutant were quite similar to those of the *cmk1* mutant. The mycelial growth rate of the *comekk1* mutant was also similar to that of the wild-type strain 104-T on potato dextrose agar (PDA) medium (Table 1). On the other hand, conidiation was reduced in the *comekk1* mutant compared with the wild-type strain (Table 1), and conidial morphology was quite similar to *cmk1*, showing round form

Fig. 1. Identification of the *CoMEKK1* gene in *Colletotrichum orbiculare*. **A**, Morphological development of the KENH-2 strain on cucumber cotyledons. The wild-type *C. orbiculare* 104-T and the *comekk1*-disrupted mutant KENH-2 strains were assayed. Conidial suspensions were inoculated on the abaxial surfaces of cucumber cotyledons at 24°C for 24 h. **B**, Schematic representation of the *CoMEKK1* locus in the KENH-2 strain. Exons and an intron are indicated as closed boxes and a hatched box, respectively. The open reading frame of *CoMEKK1*, starting with the predicted initiator ATG (designated as +1) and ending with a TGA termination codon at +1,668, is indicated. The T-DNA insertion position is indicated at +791. **C**, Schematic representation of *Comekk1p*. The lower values indicate the position of the sterile α motif (SAM) domain, Ras-association (RA) domain, and catalytic protein kinase domain. These domains were identified by searching the PROSITE database.

Fig. 2. Phylogenetic analysis of mitogen-activated protein kinase kinase (MEK) kinases in yeast and filamentous fungi. The phylogenetic tree compiled from the amino acid sequences of the putative MAPKKK homologs among fungi was drawn using TreeView software. The phylogenetic analysis was performed using ClustalW based on sequences from *Saccharomyces cerevisiae* (*Ste11p*, *Bck1p*, *Ssk2p*, and *Ssk22p*), *Magnaporthe oryzae* (*Mst11*, *Mck1*, and *MGG_00183*), *Neurospora crassa* (*NRC-1*, *MIK-1*, and *Os-4*), *Aspergillus nidulans* (*SteC*, *SskB*, and *AN4887.2*) and *Ustilago maydis* (*Ubc4/Kpp4*, *UM01662.1*, and *UM01544.1*). The scale bar indicates the evolutionary distance.

with shorter length of the major axis of conidial ellipse. To investigate infection-related morphogenesis of the *comekk1* mutant, we observed conidial germination and appressorium development on glass slides. In the wild type, ectopic transformants, and *CoMEKK1* reintroduced transformants, approximately 90% of conidia germinated and formed darkly

melanized appressoria within 24 h (Fig. 4A). In contrast, the germination frequency of the conidia in the *comekk1* mutant was approximately 1% after 24 h of incubation. This result was similar to that of the *cmk1* mutant (Takano et al. 2000). Conidia of the *cmk1* mutant germinate in yeast extract solution without forming appressoria, indicating that the Cmk1 MAPK pathway was not crucial for nutrient signal response (Takano et al. 2000). Therefore, to assess whether *Comekk1p* is involved in nutrient signal responses, conidia of the *comekk1* mutant were incubated in yeast extract solution. The conidia of

Fig. 3. Gene disruption of *CoMEKK1* in *Colletotrichum orbiculare*. **A**, *CoMEKK1* gene disruption by homologous recombination with the revS-MEKKAH3 vector in which the AH3 fragment (AMP::HPH cassette) was inserted into the *CoMEKK1* gene. Through double crossover, an approximately 10-kb *KpnI* fragment containing wild-type *CoMEKK1* was predicted to be replaced by an approximately 13-kb fragment containing the AH3 fragment. **B**, Southern blot analysis of *comekk1* disruption. Genomic DNA was digested with *KpnI* and probed with an internal fragment of *CoMEKK1*. WT, wild-type 104-T strain; R1-2, the *comekk1* mutant; E1, the *CoMEKK1* ectopic transformant; C1-2, the *CoMEKK1* reintroduced transformant into the *comekk1* mutant.

Fig. 4. Infection-related morphogenesis and pathogenicity assay of the *comekk1* mutants. **A**, Microscopic observation of germlings of the *comekk1* mutant. Conidial suspensions of each strain in distilled water or 0.1% yeast extract solution were incubated on multiwell glass slides at 24°C for 24 h. 104-T, the wild-type strain; RMKK-1, the *comekk1* mutant; Ap, appressoria; Co, conidia; Hy, hyphae. Scale bars = 10 μm. **B**, Percentage of conidial germination and appressorium formation by the *comekk1* mutants. Conidial suspensions of each strain in distilled water or 0.1% yeast extract solution were incubated on multiwell glass slides at 24°C for 24 h. Approximately 200 conidia were observed per well; three independent experiments were performed and mean and standard deviations were noted. 104-T, the wild-type strain; RMKK-1, 2, the *comekk1* mutants; EMKK-1, the *CoMEKK1* ectopic transformant; CMKK-1, 2, the *CoMEKK1* reintroduced transformants; *cmk1Δ*, the *cmk1* mutant. **C**, Pathogenicity assay of *comekk1* mutants on wounded leaves. Mycelial blocks were placed on wound sites prepared by scratching the leaves with a sterile toothpick and inoculated at 24°C for 6 days. 104-T, the wild-type strain; RMKK-1, 2, the *comekk1* mutants; EMKK-1, the *CoMEKK1* ectopic transformant; CMKK-1, 2, the *CoMEKK1* reintroduced transformants; *cmk1Δ*, the *cmk1* mutant.

the *comekk1* mutants germinated with comparable frequency to the wild-type strain in yeast extract solution without forming appressoria like the *cmk1* mutant (Fig. 4A and B).

CMK1 is required for invasive growth in host cucumber leaves (Takano et al. 2000). To assess whether *CoMEKK1* is required for invasive growth in host leaves, *comekk1* mutants were inoculated onto wounded cucumber leaves. Mycelial blocks were incubated at the wound site, which was induced by scratching with a toothpick. The wild-type ectopic transformants and *CoMEKK1*-reintroduced transformants formed lesions at 6 days, whereas the *comekk1* and *cmk1* mutants did not (Fig. 4C).

The *Ste11p* function in the high osmolarity glycerol (HOG) pathway in *S. cerevisiae* (Posas and Saito 1997) and the *Pmk1* MAPK pathway in *M. oryzae* regulates high osmolarity responses independent of the *Osm1* MAPK pathway during vegetative growth (Zhao et al 2005). However, it is unclear whether the *Cmk1* MAPK pathway is involved in the osmotic stress response of *C. orbiculare*. In this study, the growth rate of the *comekk1* and *cmk1* mutants was lower than that of the wild-type strain on PDA medium containing 1 M sorbitol as a high osmotic stressor (Fig. 5). Furthermore, the *comekk1* and *cmk1* mutants showed lower tolerance to salt stress. On PDA medium containing 0.3 M NaCl, the growth rate of the *comekk1* and *cmk1* mutants was lower than that of the wild type (Fig. 5).

A constitutively active *CoMEKK1* allele showed reduction of vegetative growth and abnormal appressorium formation that is dependent on *CMK1*.

To investigate whether *Comekk1p* activates *Cmk1p* in *C. orbiculare*, a putative constitutively active allele of *CoMEKK1* was constructed. We anticipated that constitutively active *Comekk1p* would affect some cellular responses in the wild-type or *comekk1* mutants if *Comekk1p* acts upstream of *Cmk1p*, and that these responses would not be expressed by the *cmk1* mutant. Therefore, *Comekk1p* hyperactive strains were generated from the wild-type strain and the *comekk1* and *cmk1* mutants. In the *STE11-4* allele of *S. cerevisiae*, a constitutively active allele of *STE11*, threonine 596, was replaced by isoleucine adjacent to the invariant Asp-Phe-Gly (DFG) triplet in the subdomain, VII kinase domain (Stevenson et al. 1992). Based on the *STE11-4* data, we replaced serine 775 with isoleucine adjacent to the DFG triplet in the kinase domain of *Comekk1p* and named this encoding gene *CoMEKK1-4*.

The *CoMEKK1-4*-introduced transformants, WME4 and mME4, obtained from the wild-type and *comekk1* mutants,

respectively, showed reduced hyphal growth on PDA. The hyphal growth of WME4 and mME4 was slower than that of the parent strains. Moreover, WME4 and mME4 showed increased tolerance to salt and osmotic stresses compared with the wild-type strain (Fig. 6A). On the other hand, hyphal growth and stress responses in the *CoMEKK1-4* introduced *cmk1* mutant (cME4) showed no distinct difference in these characteristics compared with the *cmk1* mutants (Fig. 6A). Additionally, we investigated whether constitutively active *Comekk1p* affects infection-related morphogenesis (Fig. 6B and C). The conidia of WME4 and mME4 germinated and formed appressoria, and 20 to 40% of the appressoria of these strains displayed a morphologically aberrant phenotype with lateral germination from the appressoria. In contrast, cME4 was not affected by hyperactive *Comekk1p* and conidial germination frequency was very low. These results were similar to those for the *cmk1* mutant (Fig. 6B and C).

Fig. 5. Sensitivity of the *Cmk1* mitogen-activated protein kinase (MAPK) signaling mutant to salt or osmotic stresses. **A**, Growth of the *Cmk1* MAPK signaling mutants on the stress medium. Each strain was grown on potato dextrose agar (PDA) containing 1 M sorbitol or 0.3 M NaCl at 24°C for 5 days. 104-T, the wild-type strain; RMKK-1, the *comekk1* mutants; *cmk1Δ*, the *cmk1* mutant. **B**, Growth rate of each strain on stress medium. Colony extension from 2 to 7 days after inoculation was measured. Five colonies from each strain were measured and the mean and standard deviations were calculated. 104-T, the wild-type strain; RMKK-1, 2, the *comekk1* mutants; EMKK-1, the *CoMEKK1* ectopic transformant; CMKK-1, 2; the *CoMEKK1* reintroduced transformants; *cmk1Δ*, the *cmk1* mutants. Asterisks represent significant differences between the ectopic control and each strain. Student's *t* test: * indicates $P < 0.001$.

Table 1. Growth rate and conidiation of *Cmk1* MAPK kinase signaling mutants in *Colletotrichum orbiculare*

Strain ^a	Vegetative growth (mm/3 days) ^b	Conidia (×10 ⁶ /colony) ^c
104-T	7.73 ± 0.27	18.9 ± 6.04
RMKK-1	7.73 ± 0.10	0.18 ± 0.06
RMKK-2	7.73 ± 0.03	0.16 ± 0.07
EMKK-1	7.93 ± 0.07	23.8 ± 4.52
CMKK-1	7.83 ± 0.19	18.4 ± 4.59
CMKK-2	7.63 ± 0.18	16.7 ± 5.43
<i>cmk1Δ</i>	7.78 ± 0.16	0.19 ± 0.13

^a Strains: 104-T, the wild-type strain; RMKK-1 and 2, *comekk1* mutants; EMKK-1, *CoMEKK1* ectopic transformant; CMKK-1 and 2; *CoMEKK1* reintroduced transformants; *cmk1Δ*, *cmk1* mutant.

^b Each strain was grown on potato dextrose agar at 24°C. Strains were measured from colony edge incubated for 3 days to colony edge incubated for 6 days. Five colonies in each strain were measured and the mean and standard deviation were calculated.

^c Production of conidia was measured in 7-day-old culture. Conidia of five colonies in each strain were counted and the mean and standard deviation were calculated.

Nuclear localization of Cmk1-GFP is affected by Comekk1p.

Once activated by environmental cues, MAPK localize to nuclei and activate downstream targets. In *C. orbiculare*, Osl1 MAPK is transferred to the nuclei following stimulation by osmotic stress. The Fus3 MAPK localizes in the nuclei and cytoplasm. However, Fus3p is more abundant in the nuclei than in the cytoplasm when stimulated by mating pheromones (Blackwell et al. 2003). A *CMK1*-green fluorescent protein (*GFP*) fusion gene was constructed to investigate whether signal transduction via Comekk1p controls localization of Cmk1p. The *CMK1-GFP* fusion gene, expressed under its native *CMK1* promoter, was introduced into the *cmk1* mutant. Using DNA blot analysis, we confirmed that the resulting transformant (CG1) contained one copy of the fusion gene (data not shown). The *cmk1* mutant restored conidial germination, appressorium development, pathogenicity, and NaCl tolerance following the introduction of *CMK1-GFP*, indicating that *CMK1-GFP* was functional (Fig. 7). When induction did not occur, *CMK1-GFP* was not localized at cellular specific sites in the hyphae and conidia of CG1, whereas it was effectively localized in the nuclei under conditions of 5 M NaCl salt

stress (Fig. 8A). Next, we investigated whether localization of *CMK1-GFP* was affected in the *comekk1* mutant or in the CG1 strain expressing *CoMEKK1-4*. The *CMK1-GFP* fusion gene was introduced into the *comekk1* mutant RMKK1-1 strain (designated as the mCG1 strain) and *CoMEKK1-4* was introduced into the CG1 strain (designated as the ACG1 strain). In the conidia of CG1 or ACG1 in 5 M NaCl, GFP signals were conspicuous in the nuclei (Fig. 8B). The percentage of GFP signals observed in the nuclei was approximately 75 and 90% in the conidia of CG1 and ACG1, respectively (Fig. 8C). In contrast, localization of Cmk1-GFP in the nuclei in mCG1 was much lower than that of CG1 and ACG1; the percentage of GFP signals observed in the nuclei was approximately 35% (Fig. 8).

DISCUSSION

There has been an accumulation of data pertaining to MAPK signaling and pathogenicity in plant-pathogenic fungi (Xu 2000; Xu et al. 2007). MAPK signaling pathways are conserved in the plant-pathogenic fungi and yeasts. However, upstream components of the MAPK cascade in some plant

Fig. 6. Expression of the *CoMEKK1-4* allele in mutants. **A**, Growth of the *CoMEKK1-4* transformants on the stress medium. Each strain was grown on potato dextrose agar (PDA) containing 1 M sorbitol at 24°C for 5 days. WT, the wild-type strain; *comekk1*, the *comekk1* mutants; *cmk1Δ*, the *cmk1* mutants; WME4, *CoMEKK1-4* introduced the wild type; mME4, *CoMEKK1-4* introduced the *comekk1* mutant; cME4, *CoMEKK1-4* introduced the *cmk1* mutant. **B**, Appressorium formation by the *CoMEKK1-4* introduced strain. Ap, appressoria; Co, conidia. Scale bars = 10 μm. WT, the wild-type strain; ME4, *CoMEKK1-4* introduced wild-type; mME4, *CoMEKK1-4* introduced *comekk1* mutant; cME4, *CoMEKK1-4* introduced *cmk1* mutant. Conidial suspensions of each strain prepared in distilled water were incubated on multiwell glass slides at 24°C for 24 h. Ap, Appressoria; Co, Conidia. Scale bars = 10 μm. **C**, Percent conidial germination and abnormal appressorium formation. WT, the wild-type strain; ME4-1,2,3, *CoMEKK1-4* introduced into the wild-type strain; cME4-1,2,3, *CoMEKK1-4* introduced into the *cmk1* mutant. Approximately 200 conidia were observed per well and three replicates were examined. Mean and standard deviations were calculated from three independent experiments.

pathogens do not appear to be consistent with those of *S. cerevisiae*. In *S. cerevisiae*, PAK kinase Ste20p is required for Ste11p activation in the mating and filamentous growth pathways (Chen and Thorner 2007). On the other hand, *CHM1* and *MST20*, the PAK kinase genes in *M. oryzae*, have been shown to be dispensable for appressorium formation and may not be involved in activating the Mst11-Mst7-Pmk1 MAPK pathway (Li et al. 2004). Thus, parts of the signaling pathways of the plant-pathogenic fungi, from signal recognition up to cellular response, are not consistent with *S. cerevisiae*.

In *C. orbiculare*, although two MAPK are involved in pathogenicity (Kojima et al. 2002; Takano et al. 2000), the upstream factors influencing these MAPK have not been identified. *CoMEKK1* encodes the SAM domain at the N terminal, followed by the RA domain and protein kinase domain at the C terminal, similar to that for *M. oryzae* *MST11*, *U. maydis* *Ubc4/Kpp4*, and other *STE11* homologs (Andrews et al. 2000; Grimshaw et al. 2004; Muller et al. 2003; Zhao et al. 2005) and it has significant sequence similarity to those genes, suggesting that *CoMEKK1* encodes MAPKK kinase. The phenotype of the *comekk1* disruption mutants showed reduced conidiation, inability to germinate and form appressoria, and

defective invasive growth on host cucumber leaves. In the budding yeast, Ste11 MAPKK kinase, Ste7 MAPK kinase, and Fus3/Kss1 MAPK constitute a cascade and regulate pheromone response and the filamentous growth pathway (Chen and Thorner 2007). Phenotypes of the disruptant of *CMK1*, the *FUS3/KSS1* homolog of *C. orbiculare*, were similar to those of the *comekk1* mutant. Moreover, we showed that both the *comekk1* and *cmk1* mutants, were sensitive to high osmotic and salinity stresses. These data indicate that *CoMEKK1* and *CMK1* regulate the same pathway involving infection-related morphogenesis, pathogenicity, and stress tolerance, and that there is a possibility that their encoded proteins are part of the same cascade.

We induced mutagenesis of *CoMEKK1* in vitro in the region adjacent to the invariant DFG triplet in the subdomain VII kinase domain, as detailed in the study by Stevenson and associates (1992). This kinase domain mutation close to the ATP-binding cleft was likely to escape inhibition by the autoinhibitory domain (van Drogen et al. 2000). Expression of the hyperactive *CoMEKK1-4* allele affected several phenotypes of the wild-type strain and the *comekk1* mutant. Thus, it is suggested that repression of kinase activity by the autoinhibitory domain is conserved between Ste11p and Comekk1p.

Expression of the *CoMEKK1-4* allele rescued the defect in conidial germination, appressorium formation, and osmotic stress tolerance in *comekk1* mutants but not in *cmk1* mutants. This result indicates that MEKK encoded by *CoMEKK1* likely functions upstream of *CMK1*. *S. cerevisiae* Ste11p is involved in regulation of the Fus3/Kss MAPK pathway and the Hog1 MAPK pathway (Chen and Thorner 2007; Posas and Saito 1997). In *C. orbiculare*, the Osc1 MAPK pathway (a Hog1-related pathway) regulates osmotic stress response (Kojima et al. 2004). Moreover, it has been shown in this study that the Cmk1 MAPK pathway also regulates osmotic tolerance. Introduction of *CoMEKK1-4* into the wild-type *C. orbiculare* strain or the *comekk1* mutant conferred tolerance against osmotic stress while it did not have that effect on the *cmk1* mutant. These results support the hypothesis that Comekk1p is a direct upstream component of the Cmk1 MAPK pathway. The strains in which *CoMEKK1-4* had been introduced also showed significant reduction in vegetative growth on PDA, indicating that proper regulation of the Cmk1 MAPK pathway is required for normal vegetative growth in this fungus. On the other hand, expression of *MST7^{S212D T216E}*, a dominant active MEK allele, did not affect vegetative growth in *M. oryzae* (Zhao et al. 2005). The differences in phenotype of the dominant active introduced strains suggest that *MST7* and *CoMEKK1* regulate different signaling pathways.

In this experiment, Comekk1p hyperactivation influenced appressorium morphogenesis. Previous reports have shown that phosphorylation of Mps1 MAPK is repressed by expression of the dominant active *MST7* allele and by activation of Pmk1 during appressorium formation, suggesting that Pmk1 activation may have a negative effect on Mps1 phosphorylation in *M. oryzae*. Maf1, the Mps1 MAPK of *C. orbiculare*, regulates appressorium formation, and disruption of *MAF1* has been shown to cause defective appressorium development (Kojima et al. 2002). Therefore, there is the possibility that hyperactivation of Comekk1p negatively regulates phosphorylation of Maf1p and affects appressorium morphogenesis.

MAPK responses are dependent on activation by MEK and their subsequent translocation to the nuclei where they phosphorylate transcription factors. Signal-induced nuclear translocation has been observed in some MAPK (Chen and Thorner 2007). The budding yeast Hog1 MAPK exhibits a dramatic translocation from the cytoplasm to the nuclei when the cells are subjected to hyperosmotic shock (Reiser et al. 1999). Ac-

Fig. 7. Complementation test for pathogenicity and stress tolerance of *cmk1* mutant by introduction of the *CMK1*-green fluorescent protein (GFP) fusion gene. **A**, Appressorium formation by the *CMK1*-GFP gene introduced transformant into the CG1 strain and the wild-type strain 104-T. Conidial suspensions of each strain, prepared in distilled water, were incubated on multiwell glass slides at 24°C for 24 h. Ap, Appressoria; Co, Conidia. Scale bars = 10 μm. **B**, Pathogenicity assay of *CMK1*-GFP-introduced transformants on intact cucumber cotyledons. Conidial suspensions were droplet inoculated onto detached cucumber cotyledons and incubated at 24°C for 6 days. WT, wild-type 104-T; CG1-3, *CMK1*-GFP-introduced transformant into the *cmk1* mutant. Mock, distilled water control. **C**, Growth of *CMK1*-GFP-introduced transformants on the stress medium. Wild-type (WT), *CMK1*-GFP-introduced transformants (CG1), and *cmk1* mutants were grown on potato dextrose agar (PDA) containing 0.3 M NaCl at 24°C for 5 days.

cordingly, OSC1 MAPK, the Hog1-related MAPK in *C. orbiculare*, has been shown to translocate into the nuclei after stimulation by the addition of fludioxonil or KCl (Kojima et al. 2004). On the other hand, several MAPK constitutively shuttle between the cytoplasm and nuclei and do not show an obvious nuclear concentration when activated. Fus3p constitutively shuttles between the cytoplasm and nuclei and is easily detectable in both compartments. A quantitative assay has shown increased nuclear localization of Fus3p in response to pheromone stimulation (Blackwell et al. 2003). In *M. oryzae*, although a slight GFP-Pmk1 signal was detected in the conidia, germ tubes, and vegetative hyphae, the signal increased

in the nuclei of the appressoria where nuclear localization was observed (Bruno et al. 2004). On the other hand, strong Cmk1-GFP signals were detected in the cytoplasm of vegetative hyphae and the conidia of *C. orbiculare*. The GFP signals were also detected in the nuclei of the conidia under conditions of high-salt stress. To test the possibility that localization of Cmk1-GFP was affected by Comekk1p activation, we examined Cmk1-GFP nuclear translocation in the *comekk1* mutant. Nuclear localization of Cmk1-GFP in the *comekk1* mutant was reduced compared with the wild-type or hyperactive Comekk1p strains, indicating that nuclear translocation of Cmk1 is dependent on Comekk1p activity.

Fig. 8. Involvement of *CoMEKK1* in the localization of Cmk1-green fluorescent protein (GFP) in stress condition. **A**, Cmk1-GFP localized to nuclei in the CG1 strain. The conidia of the CG1 strain were observed in 5 M NaCl solution and stained with Hoechst 33342 (5 mg/ml) for 5 min. Scale bars = 10 μm. **B**, Cmk1-GFP localized to nuclei in the ACG1 strain but not in the mCG1 strain. The conidia of each strain were observed in 5 M NaCl solution. Scale bars = 10 μm. **C**, Percent nuclear localization of Cmk1-GFP in conidia in distilled water (DW, upper graph) or 5 M NaCl solution (lower graph). More than 100 conidia harvested as suspension in DW or NaCl solution were observed per experiment. Four independent experiments were conducted and mean and standard deviations were noted. Cmk1 localization was categorized as follows: nuclei < cytoplasm, nuclei = cytoplasm, and nuclei > cytoplasm. GFP, strain expressing GFP; CG1, the *CMK1-GFP*-introduced strain into the wild-type. mCG1, CG1, the *CMK1-GFP*-introduced strain into the *comekk1* mutant; ACG1, the *CoMEKK1-4* introduced strain to CG1.

In *S. cerevisiae*, the Ste11p is also involved in the HOG pathway (Posas and Saito 1997). *CoMEKK1* gene mutation caused higher sensitivity against high osmotic stress, indicating that Comekk1p also regulates osmotic regulatory pathways in *C. orbiculare*. In our experiment, hyperactivation of Comekk1p conferred high osmotic tolerance to the wild type and the *comekk1* mutant but not to the *cmk1* mutant. Furthermore, the *cmk1* mutant also showed increased sensitivity against high osmotic stress at level similar to that of *comekk1*. These results suggested that Comekk1p is required for Cmk1p function to respond to osmotic stress, and this regulation would be independent of the Osl1 MAPK pathway in *C. orbiculare*. However, to verify this, a phosphorylation assay of the MAPK kinase upstream of the Osl1 MAPK-like Ste11p would be essential.

In conclusion, our results suggest that Comekk1p MAPKK kinase acts upstream of Cmk1 MAPK and regulates stress tolerance, appressorium development, and pathogenesis in *C. orbiculare*.

MATERIALS AND METHODS

Fungal and bacterial strains.

Strain 104-T (MAFF240422) of *C. orbiculare* (Berk. & Mont.) Arx (syn. *C. lagenarium* (Pass.) Ellis & Halst.) was used as the wild-type strain. All *C. orbiculare* strains used in this study are listed in Table 2 and were cultured on PDA media (3.9% [wt/vol] PDA; Difco Laboratories, Detroit) at 24°C. *Escherichia coli* DH5- α was used as a host for gene manipulation. *E. coli* was cultured in Luria-Bertani media (Sambrook et al. 1989) at 37°C. The medium was supplemented when necessary with ampicillin at 50 μ g/ml or with kanamycin at 50 μ g/ml. *A. tumefaciens* C58C1 was used to transform *C. orbiculare* by AtMT. *A. tumefaciens* was cultured in AB-minimal medium at 28°C. The medium was supplemented when necessary with kanamycin at 50 μ g/ml and rifampicin at 100 μ g/ml.

Fungal transformation.

The AtMT protocol was applied with slight modifications of the previously described method (Tsuji et al. 2003a). The hygromycin-resistant transformants were selected on PDA medium containing hygromycin B at 100 μ g/ml (Wako Chemicals, Osaka, Japan), cefotaxim at 100 μ g/ml (Wako Chemicals), and spectinomycin at 100 μ g/ml (Wako Chemicals). The bialaphos-resistant transformants were selected on SD medium containing bialaphos at 10 μ g/ml (Meiji Seika Kaisha, Ltd., Tokyo), cefotaxim at 100 μ g/ml, and spectinomycin at 100 μ g/ml. The sulfonyleurea-resistant transformants were selected on SD medium containing chlorimuronethyl at 4 μ g/ml

(Chem Service, West Chester, PA, U.S.A.), cefotaxim at 100 μ g/ml, and spectinomycin at 100 μ g/ml.

Genomic DNA blot analysis.

Total DNA of *C. orbiculare* was isolated from mycelia incubated in potato-sucrose liquid medium for 3 to 4 days, and DNA blot analysis was performed according to the method previously described (Takano et al. 1997). DNA digestion, gel electrophoresis, labeling of probes, and hybridization were performed according to the manufacturer's instructions and standard methods (Sambrook et al. 1989). DNA probes were labeled with digoxigenin (DIG)-dUTP using a BcaBEST DIG labeling kit (Takara Bio, Ohtsu, Japan). Hybridized DNA was detected with anti-DIG antibody Fab fragments conjugated to alkaline phosphatase (Roche Diagnostics, Tokyo) and light emission from the enzymatic dephosphorylation of CDP-Star Detection Reagent (GE Healthcare, Tokyo) was detected using the LAS-1000 Plus Gel Documentation System (Fujifilm, Tokyo).

Cloning and sequencing.

The analysis of fungal genomic DNA flanking the T-DNA inserts was achieved using the TAIL-PCR protocol performed as described previously (Tsuji et al. 2003a). Amplified PCR products were analyzed by agarose gel electrophoresis and sequenced with the Big-Dye terminator cycle sequencing ready reaction kit (Applied Biosystems Japan Ltd., Tokyo) and an ABI PRISM 310 automated DNA sequencer (Applied Biosystems Japan Ltd.). To identify the T-DNA insertion gene in the KENH-2 strain, a TAIL-PCR protocol was applied to the mutant genomic DNA (Liu et al. 1995). Fungal genomic DNA flanking the T-DNA insert was analyzed as previously described (Tsuji et al. 2003a). Amplified PCR products were sequenced with the Big-Dye terminator cycle sequencing ready reaction kit and an ABI PRISM 310 automated DNA sequencer. Genomic clones of *CoMEKK1* were selected from a cosmid library of *C. orbiculare* 104-T by PCR using primer pair KEH2-S1 (5'-CATGCGAAGAGGTCATGAGA-3') and KEH2-AS1 (5'-ATCAAACGGTTACGCTCAGG-3') designed from the obtained DNA sequence. The sequencing of the entire region of the *CoMEKK1* open reading frame was as follows. The EZ::TN <KAN-2> Insertion Kit (Epicenter Technologies, Madison, WI, U.S.A.) was used to insert the transposon containing the selection marker into the target DNA. The clones of the target DNA containing the <KAN-2> transposon were then sequenced with the Big-Dye terminator cycle sequencing ready reaction kit using primers KAN2-RP2 and KAN2-FP3. The *CoMEKK1* sequence is available in the DDBJ database under accession no. AB546841.

Table 2. Fungal strains used in this study

Strain	Description	Reference
104-T	Wild-type strain of <i>Colletotrichum orbiculare</i>	NIAS, Japan Genebank MAFF240422
RMKK-1	<i>comekk1</i> disruptant of 104-T	This study
RMKK-2	<i>comekk1</i> disruptant of 104-T	This study
EMKK-1	<i>CoMEKK1</i> ectopic transformant of 104-T	This study
CMKK-1	RMKK-1 complemented with <i>CoMEKK1</i>	This study
CMKK-2	RMKK-1 complemented with <i>CoMEKK1</i>	This study
<i>cmk1</i> Δ	<i>CMK1</i> deletion mutant of 104-T	Takano et al. 2000
WME4	104-T transformed with hyperactive <i>CoMEKK1-4</i>	This study
mME4	RMKK-1 transformed with hyperactive <i>CoMEKK1-4</i>	This study
cME4	<i>cmk1</i> Δ transformed with hyperactive <i>CoMEKK1-4</i>	This study
CG1	<i>cmk1</i> Δ transformed with <i>CMK1</i> -green fluorescent protein (<i>GFP</i>) fusion gene	This study
CG2	<i>cmk1</i> Δ transformed with <i>CMK1-GFP</i> fusion gene	This study
CG4	<i>cmk1</i> Δ transformed with <i>CMK1-GFP</i> fusion gene	This study
mCG1	RMKK-1 transformed with <i>CMK1-GFP</i> fusion gene	This study
ACG1	CG1 transformed with hyperactive <i>CoMEKK1-4</i>	This study
KENH-2	<i>comekk1</i> mutant of 104-T obtained by <i>Agrobacterium tumefaciens</i> -mediated transformation mutant screening	This study

Plasmid constructs.

To create the *CoMEKK1* gene-replacement revs-MEKK1AH3 vector, the pCB-CoMEKK1 plasmid was constructed by introducing an approximately 4.3-kb *HindIII* genomic fragment containing *CoMEKK1* from a cosmid clone (MEKKcosA10, which contained *CoMEKK1*) into the *HindIII* site of pCB1004 (Carroll et al. 1994). An approximately 2.7-kb EZ::TN <KAN-2> fragment (the AH3 fragment, in which the kanamycin resistance gene was replaced by the ampicillin resistance gene and the hygromycin resistance gene) was then inserted into pCB-CoMEKK1H. A pCB-MEKK1AH3 plasmid containing the AH3 fragment, 483 bp from the start codon, was selected. Finally, *Bam*HI and *Apa*I fragments containing the AH3 fragment with *CoMEKK1* from the pCB-MEKK1AH3 plasmid were cloned into the *A. tumefaciens* binary vector pBIG4MRSrev, which contained a sulfonyleurea resistance gene and was used as the gene replacement plasmid.

To perform a complementation assay of the *comekk1* mutant, we first constructed the *CoMEKK1* complement vector revs-CoMEKK1. The full region of *CoMEKK1* was amplified by PCR with primers CoMEKK1P-S (5'-CGGGATCCGCGG GACAAGGACAACAC-3') and CoMEKK1T-AS (5'-CGTCT AGACGCTTGGTGCAGAGAAAAC-3') from the cosmid clone MEKKcosA10. The CoMEKK1P-S primer contained a terminal *Bam*HI site, whereas the CoMEKK1T-AS primer contained a terminal *Xba*I site. The amplified product was digested with *Bam*HI and *Xba*I, introduced into the *Bam*HI and *Xba*I sites of pBluescriptII SK+, and named pBS-CoMEKK1. Finally, the *Bam*HI and *Xba*I fragments containing *CoMEKK1* from the pBS-CoMEKK1 plasmid were cloned into pBIG4MRSrev. To construct the constitutively active *Comekk1p* expression vector, we constructed pBS-CoMEKK1-4 from pBS-CoMEKK1 using the primer pair Mekk1-4S1 (5'-TCGATTTTGGTATTTCCAA GAAGCT-3') and Mekk1-4AS1 (5'-AATCTTGATGGTGCC CTTG-3') and a KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Finally, the *Bam*HI and *Xba*I fragments containing CoMEKK1-4 from the pBS-CoMEKK1-4 plasmid were cloned into pBIG4MRSrev.

To construct the CMK1-GFP fusion plasmid, we first constructed the pBI-BglyGFP plasmid in which the hygromycin resistance gene was replaced with the bialaphos resistance gene from the pBI-HglyGFP plasmid (Sakaguchi et al. 2008). The *CMK1* gene containing the full open reading frame of *CMK1* with 1 kb of the 5' upstream region was amplified by PCR using the primers CM1BMS (5'-CGGGATCCTCGAAG TCTATGGCGTCA-3') and CM1HAS (5'-GACCAAGCTTCC GCATAATCTCCTGGTAGA-3') (Takano et al. 2000). The amplified product was digested with *Bam*HI and *Hind*III and introduced into pBI-BglyGFP. The resultant clone was designated as pBI-CMGFPB.

Pathogenicity tests.

The inoculation assay on cucumber cotyledons (*Cucumis sativus* L. "Suyo") was performed as described by Tsuji and associates (1997). The conidia of *Colletotrichum orbiculare* were obtained from 7-day-old cultures and drops (10 μ l) of conidial suspension (5×10^5 conidia/ml) were spotted on the surfaces of cucumber cotyledons. To assess invasive growth ability, mycelial blocks incubated on PDA were placed on wound sites prepared by scratching the surfaces of the cotyledons with a sterile toothpick. After inoculation, the cotyledons were incubated at 24°C with 16 h of light per day for 6 days.

Microscopy.

For appressorium formation assays in vitro, the conidia were harvested from PDA cultures aged 7 days or older and suspended in distilled water or 0.1% yeast extract solution. The

suspension, adjusted to 1×10^5 conidia/ml, was poured onto a multitest glass slide (Glass 8-well multitest slide; ICN Bio-medicals, Aurora, OH, U.S.A.) and incubated at 24°C in the dark. Germlings were observed with a Nikon ECLIPSE E600 microscope with differential-interference contrast optics. GFP fluorescence was observed on a fluorescent microscope with the GFP(R)-BP filter (460- to 500-nm excitation wavelength, 505-nm wavelength dichroic mirror, and 510- to 560-nm wavelength barrier filter). For staining the nuclei, the conidia were incubated in Hoechst 33342 (5 mg/ml) for 5 min. These conidia were washed with distilled water and observed under a fluorescent microscope with a UV-1A filter (excitation wavelength of 365/10 nm; dichroic mirror wavelength of 400 nm, and barrier filter wavelength of 400 nm).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

TreeView software: taxonomy.zoology.gla.ac.uk/rod/treeview.html