The Mitogen-Activated Protein Kinase Gene *MAF1* Is Essential for the Early Differentiation Phase of Appressorium Formation in *Colletotrichum lagenarium*

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Colletotrichum lagenarium, the causal agent of cucumber anthracnose, invades host plants by forming a specialized infection structure called an appressorium. In this fungus, the mitogen-activated protein kinase (MAPK) gene *CMK1* is involved in several steps of the infection process, including appressorium formation. In this study, the goal was to investigate roles of other MAPKs in *C. lagenarium*. The MAPK gene *MAF1*, related to *Saccharomyces cerevisiae* *MPK1* and *Magnaporthe grisea* *MPS1*, was isolated and functionally characterized. The *maf1* gene replacement mutants grew normally, but there was a significant reduction in conidiation and fungal pathogenicity. The *M. grisea* *mps1* mutant forms appressoria, but conidia of the *C. lagenarium* *maf1* mutants produced elongated germ tubes without appressoria on both host plant and glass, on which the wild type forms appressoria, suggesting that *MAF1* has an essential role in appressorium formation on inductive surfaces. On a nutrient agar, wild-type conidia produced elongated germ tubes without appressoria. The morphological phenotype of the wild type on the nutrient agar was similar to that of the *maf1* mutants on inductive surfaces, suggesting repression of the *MAF1*-mediated appressorium differentiation on the nutrient agar. The *cmk1* mutants failed to form normal appressoria but produced swollen, appressorium-like structures on inductive surfaces, which is morphologically different from the *maf1* mutants. These findings suggest that *MAF1* is required for the early differentiation phase of appressorium formation, whereas *CMK1* is involved in the maturation of appressoria.

Anthracnose fungi belonging to the genus *Colletotrichum* cause severe diseases on a wide range of crops and ornamental plants (Agrios, 1988). *C. lagenarium* (Pass.) Ellis & Halst. (syn. *C. orbiculare* (Berk. & Mont.) Arx) is the causal agent of cucumber anthracnose. This fungus forms a swollen infection structure called an appressorium. In several cases, it is known that fungal pathogens form appressoria in response to distinct physical and chemical cues in their environment. The bean rust fungus, *Uromyces appendiculatus*, forms appressoria upon en-

countering ridges formed by the guard cells surrounding a stomatal opening. Hoch and colleagues (1987) demonstrated that artificial ridges mimicking those formed by guard cells are sufficient to induce appressorium formation in this fungus. On the other hand, the rice blast fungus, *Magnaporthe grisea*, forms appressoria efficiently on inert hydrophobic surfaces in a droplet of water, whereas appressoria are much less efficiently formed on hydrophilic surfaces, suggesting that surface hydrophobicity induces appressorium formation (Lee and Dean, 1994). In *M. grisea*, the cAMP signaling pathway has been shown to be involved in appressorium differentiation through sensing of surface hydrophobicity (Adachi and Hamer, 1998; Choi and Dean, 1997; Lee and Dean, 1993). In *C. lagenarium*, knockout analysis of *RPK1*, encoding a regulatory subunit of cAMP-dependent protein kinase (PKA), indicated that proper regulation of PKA is required for appressorium function (Takano et al., 2001a).

Mitogen-activated protein kinase (MAPK) cascades have also been shown to play pivotal roles in fungal infection processes (Xu, 2000). MAPK cascades generally consist of three conserved kinases, MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAPK, which function sequentially and mediate appropriate cellular responses to distinct environmental inputs (Nishida and Gotoh, 1993; Schaeffer and Webber, 1999). The budding yeast *Saccharomyces cerevisiae* has five MAPKs, encoded by *FUS3*, *KSS1*, *HOG1*, *MPK1* (*SLT2*), and *SMK1*, respectively (Herskowitz, 1995; Hunter and Plowman, 1997). These MAPK cascades regulate fungal growth and differentiation processes such as pheromone response, pseudohyphal growth, osmoregulation, cell wall integrity, and ascospore formation. Fus3/Kss1-related MAPK genes of several phytopathogenic fungi, including *PMK1* in *M. grisea*, *CHK1* in *Cochliobolus heterostrophus*, *UBC3* (*KPP2*) in *Ustilago maydis*, *CMK1* in *C. lagenarium*, *BMP1* in *Botrytis cinerea*, *FMK1* in *Fusarium oxysporum*, and *PTK1* in *Pyrenophora teres*, play important roles in fungal pathogenicity (Di Pietro et al., 2001; Lev et al., 1999; Mayorga and Gold, 1999; Muller et al., 1999; Ruiz-Roldan et al., 2001; Takano et al., 2000; Xu and Hamer, 1996; Zheng et al., 2000). Furthermore, *PMK1*, *CHK1*, *CMK1*, *FMK1*, and *PTK1* are required for appressorium formation, suggesting conserved roles of Fus3/Kss1-related MAPKs in the formation of infection structures by plant pathogens. However, knockout mutants of the MAPKs in each of the fungi show different phenotypes in growth, conidiation, and germination, which suggests diverse roles of Fus3/Kss1-related MAPKs in phytopathogenic fungi.

In contrast with the extensive investigation of Fus3/Kss1-related MAPKs, the roles of Mpk1-related MAPK pathways are less well studied. At present, functional analysis of the *MPK1*-

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type MAPK gene is limited to *MPS1* in *M. grisea* (Xu et al., 1998). It is known that the Mpk1 pathway in *S. cerevisiae* is a part of the signaling pathway controlling cell wall integrity (Lee et al., 1993). *M. grisea mps1* mutants are hypersensitive to cell-wall-digesting enzymes, suggesting that *MPS1* is also involved in cell wall integrity (Xu et al., 1998). Phenotypic analysis of *mps1* mutants during infection indicated that *MPS1* is dispensable for appressorium formation but is required for penetration by appressoria (Xu et al., 1998).

In this report, we identify the *C. lagenarium* MAPK gene, *MAF1* (MAPK essential for appressorium formation), related to *MPK1* of *S. cerevisiae* and *MPS1* of *M. grisea*. We have investigated the roles of the *MAF1*-encoded MAPK cascade in *C. lagenarium* by assessing the phenotype of knockout mutants. The *maf1* mutants grew normally but exhibited significant reductions in conidiation and pathogenicity. In contrast with *M. grisea mps1* mutants, which are able to form appressoria, *C. lagenarium maf1* mutants failed to form appressoria on both host plant and glass, on which the wild type forms appressoria, demonstrating that the Maf1 MAPK is essential for appressorium formation in *C. lagenarium*. Further analyses, including comparison of *maf1* mutants with *cmk1* mutants having a defect in appressorium formation, indicated that two MAPKs (Maf1 and Cmk1) regulate distinct steps in appressorium development in *C. lagenarium*.

RESULTS

Identification of the *MAF1* gene encoding a MAPK related to yeast Mpk1.

We isolated *MPK1*-related MAPK genes in *C. lagenarium* by polymerase chain reaction (PCR) using degenerate oligonucleotide primers designed from amino acid sequences con-

served in Mpk1-type MAPKs in other organisms. A cDNA pool synthesized from mRNA isolated from conidia during appressorium formation (after 2 h of incubation) was used as a template for PCR screening (Inagaki et al., 2000). Amplified products were cloned into pBluescript and sequenced. The DNA sequence of an insert fragment in one PCR clone, pMP1, showed a high degree of similarity to Mpk1-type MAPKs in other organisms. The insert DNA was used as a probe to isolate genomic clones containing the *MPK1*-related MAPK gene of *C. lagenarium*, designated *MAF1*. The entire nucleotide sequence of the *MAF1* gene was determined. *MAF1* contains an open reading frame composed of 418 codons and four introns (Fig. 1 and data not shown). Maf1 contains the sequence TEY, a site for threonine and tyrosine phosphorylation required for kinase activation (Fig. 1). The deduced amino acid sequence of Maf1 was found to have 91% identity with *M. grisea* Mps1 and 60% with *S. cerevisiae* Mpk1.

Gene disruption of *MAF1*.

To investigate the roles of *MAF1* in *C. lagenarium*, a gene disruption strategy was employed. A gene replacement vector, pGDMAF1, containing a hygromycin B phosphotransferase (*hph*) gene cassette and 5' and 3' flanking regions of *MAF1*, was constructed and introduced into the wild-type strain 104-T (Fig. 2A). Eight of 20 hygromycin-resistant transformants formed colonies different from those of the wild type on nutrient agar plates (Fig. 2C). We tested whether *MAF1* was disrupted in the transformants that exhibited the different colony phenotype. Gene replacement of *MAF1* was investigated by DNA gel blot analysis (Fig. 2B). Genomic DNAs were isolated from the wild-type 104-T; the transformant DMA8, which forms wild-type colonies; and the transformants DMA5, DMA13, DMA15, and DMA16, which form colonies distinct

Fig. 1. The *Colletotrichum lagenarium* MAPK gene *MAF1* related to *Saccharomyces cerevisiae* *MPK1*. Amino acid sequence alignment of Maf1 of *C. lagenarium* with Mpk1 of *S. cerevisiae* and Mps1 of *Magnaporthe grisea*. Sequences were aligned with the CLUSTAL W program (Thompson et al., 1994). Identical amino acids are indicated by white letters on a black background. Similar residues are indicated by a gray background. Gaps introduced for alignment are indicated by hyphens. The protein kinase domains are indicated by Roman numerals above sequences. Sequence TEY, which is required for kinase activation, is marked by asterisks.

from those of the wild type. Isolated genomic DNAs were digested with *Pst*I and probed with a 6.7-kb *Pst*I fragment containing *MAF1*. Strains 104-T and DMA 8 contained the common 6.7-kb *Pst*I fragment (Fig. 2B, lanes 1 and 6). DMA8 also contained additional bands, indicating ectopic integration of pGDMAF1. On the other hand, DMA5, DMA13, DMA15, and DMA16 did not contain the 6.7-kb *Pst*I fragment but contained common 2.8-kb and 3.8-kb *Pst*I fragments (Fig. 2B, lanes 2 to 5), which is consistent with the lengths expected in gene replacement. We concluded that the *MAF1* gene was disrupted in transformants DMA5, DMA13, DMA15, and DMA16. These *maf1*Δ strains grew normally, at growth rates slightly higher than that of the wild type on potato–dextrose agar (PDA) (Table 1 and Fig. 2C). Also, on minimal medium agar plates, the *maf1* mutants grew like the wild type (data not shown). These results indicate that *MAF1* is not essential for mycelial growth on nutrient agar plates. On the other hand, conidiation of the *maf1* mutants was significantly reduced, in comparison with that of the wild-type strain (Table 1 and Fig. 2C). The number of co-

nidia produced by the *maf1* mutants was approximately 10% of that produced by the wild type. To determine whether conidiation in the *maf1* mutants could be complemented by the introduction of *MAF1*, the *maf1* mutant DMA5 was transformed with plasmid pCBMAF1, containing *MAF1* with the bialaphos resistance gene (Fig. 3A). Bialaphos-resistant transformants were obtained and analyzed by genomic DNA gel blot analysis (Fig. 3B). The analysis detected the 2.4-kb *Hind*III-*Pst*I fragment containing *MAF1* in the transformants RMA1 and RMA2 but not in the *maf1* mutant DMA5, indicating that the *MAF1* gene had been introduced into RMA1 and RMA2. In RMA2, one additional band was detected, suggesting integration of the truncated *MAF1* fragment. In both RMA1 and RMA2, conidiation was similar to that of the wild-type strain (Table 1). We concluded that the *MAF1*-encoded MAPK pathway is involved in the conidiation of *C. lagenarium*.

As was mentioned earlier, it has been reported that *MPK1*-type MAPKs are involved in cell wall integrity in several fungi, including the plant pathogen *M. grisea*. To evaluate whether *MAF1* is involved in cell wall integrity, we investigated sensitivity to cell-wall-digesting enzymes in the *maf1* mutants. Mycelia of tested strains were treated with the cell-wall-digesting enzymes Driselase and β-D-glucanase, protoplasts released from the mycelia were examined 3 and 6 h after the treatment (Fig. 4). The number of protoplasts released by the *maf1* mutant was similar to that of the wild-type strain, which suggests that *MAF1* is not essential for cell wall integrity in *C. lagenarium*.

MAF1 is essential for appressorium formation.

Infection-related morphogenesis of the *maf1*Δ strains on a glass surface was investigated by microscopic observation. Germination of the *maf1* mutants on glass was the same as that of the wild type (Table 1). Conidia of the wild-type strain formed melanized appressoria on glass after 12 h of incubation, while germ tubes of the *maf1* mutants elongated continuously and failed to differentiate into appressoria (Fig. 5A and B and Table 1). The *maf1* mutants did not form appressoria, even after 48 h of incubation. The *maf1* mutants also failed to form appressoria on the surface of host cucumber cotyledons (Fig. 5D and E). In contrast, RMA1 and RMA2, the *MAF1*-introduced transformants of the *maf1*Δ strain DMA5, formed darkly melanized appressoria on both glass and host surfaces (Fig. 5C

Fig. 2. Gene disruption of *MAF1*. **A**, *MAF1* locus and gene replacement vector pGDMAF1, containing a hygromycin-resistant gene (*hph*) cassette flanked by border sequences of *MAF1*. By homologous recombination through double crossing, the entire region of *MAF1* was replaced by the *hph* cassette. **B**, Genomic DNA gel blot analysis of *MAF1* gene replacement transformants. Genomic DNAs were extracted from the wild-type strain 104-T (lane 1); *MAF1* gene replacement transformants DMA5, DMA13, DMA15, and DMA16 (lanes 2–5); and ectopic transformant DMA8 (lane 6). All genomic DNA samples were digested by *Pst*I. The blot was probed with the 6.7-kb *Pst*I fragment shown in **A**. **C**, Colony morphology of the wild-type strain 104-T and *maf1*Δ strain DMA5 incubated on potato–dextrose agar plates at 24°C for 14 days.

Table 1. Characteristics of *maf1* mutants of *Colletotrichum lagenarium*

Strain ^a	Vegetative growth ^b (mm)	Conidia ^c (× 10 ⁶)	Germination ^d (%)	Appressorium formation ^e (%)
104-T	34.0 ± 2.0	54.3 ± 22.5	95.9 ± 2.0	95.8 ± 2.1
DMA5	39.3 ± 1.1	5.4 ± 3.1	98.3 ± 1.2	0.0 ± 0.0
DMA13	36.7 ± 2.1	5.4 ± 1.8	96.8 ± 2.0	0.0 ± 0.0
DMA15	38.3 ± 2.1	4.0 ± 1.3	92.8 ± 6.1	0.0 ± 0.0
DMA16	36.7 ± 3.1	3.7 ± 0.5	94.6 ± 2.3	0.0 ± 0.0
DMA8	33.3 ± 2.3	43.7 ± 5.7	94.8 ± 0.9	93.4 ± 1.4
RMA1	33.3 ± 1.5	53.3 ± 9.1	96.2 ± 0.1	94.4 ± 1.6
RMA2	34.0 ± 1.0	35.7 ± 4.1	93.1 ± 0.2	92.3 ± 1.0

^a Strains tested were the wild-type strain 104-T; *MAF1*-disrupted transformants DMA5, DMA13, DMA15, and DMA16; ectopic integration transformant DMA8; and two *MAF1*-reintroduced transformants of DMA5, designated RMA1 and RMA2.

^b Diameter of colonies grown on potato–dextrose agar at 24°C for 7 days.

^c Conidia collected from 7-day-old cultures.

^d Percentage of conidia that germinated after incubation on glass slides at 24°C for 12 h. At least 200 conidia were examined to calculate percentage germination. The mean and standard deviation were calculated from three independent experiments.

^e Percentage of germinating conidia that formed appressoria after incubation for 12 h. The mean and standard deviation were calculated from three independent experiments.

and F and Table 1). From these results, we conclude that *MAF1* is essential for appressorium formation in *C. lagenarium*.

We compared the behavior of an *maf1* mutant with that of the wild type during conidial incubation on a glass surface with nuclei stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). After 3 h of incubation, conidia of both the *maf1* mutant DMA5 and the wild-type strain 104-T had germinated (more than 90% germination; $n = 300$), but nuclear division was not observed. Germinating conidia of the wild type formed appressoria accompanied by a single round of nuclear division, and one of the two daughter nuclei had moved to the appressorium by 6 h (Takano et al., 2001b, and this study). At 12 h, the wild-type strain had formed highly melanized appressoria (more than 95% of germinating conidia formed appressoria; $n = 300$) and had not undergone further nuclear division. Multiple nuclear divisions after the first nuclear division occurred in DMA5, in contrast to the wild-type strain. After 12 h of incubation, 60% of germinating conidia of DMA5 ($n = 200$) contained four nuclei (Fig. 5G and H). At further long incubation, DMA5 continued nuclear division. These results suggest that the disruption of *MAF1* affected the nuclear duplication process during infection-related morphogenesis.

***MAF1* is required for fungal pathogenicity.**

To investigate the pathogenicity of the *maf1* Δ strains, an infection assay was performed. Suspensions of conidia of strains 104-T, DMA5, and RMA1 were sprayed on detached cucumber leaves. Many yellowish lesions formed on cucumber leaves treated with the wild-type 104-T or with RMA1, while only a few lesions formed on leaves treated with the *maf1* Δ strain DMA5 (Fig. 6A). Other *maf1* mutants were examined, with identical results (data not shown). These results show that disruption of *MAF1* significantly reduces the pathogenicity of *C. lagenarium*. However, when 20- μ l drops of a conidial suspension of DMA5 were spotted on cucumber leaves, lesions formed more frequently than on spray-inoculated leaves, although the efficiency of lesion formation was still lower than that of the wild type (data not shown). Strains obtained from these lesions exhibited the original *maf1* phenotype in mycelial culture, appressorium formation, and pathogenicity, suggesting that the formation of lesions is not due to suppressor mutations (data not shown). These results indicate that *MAF1* is important in the pathogenicity of *C. lagenarium*, but gene disruption of *MAF1* did not cause complete loss of pathogenicity. To assess invasive growth ability, leaves were inoculated with *maf1* mutants via wounds (Fig. 6B). The melanin-deficient mutant 79215, carrying a mutation in *PKS1*, was used as a positive control (Takano et al., 1995). This strain is not pathogenic on intact leaves, because of a lack of appressorial melanization, but it is invasive at wound sites (Takano et al., 2000). Strain SGC1, isolated as a suppressor mutant from the PKA regulatory subunit (Rpk1) mutant, was used as a negative control. This strain does not cause lesions, even on leaves inoculated at wound sites (Takano et al., 2001a). Yellowish lesions formed on cucumber leaves at all wound sites spotted with a conidial suspension of DMA5 or the *pkS1* mutant 79215, but not on leaves spotted with SGC1. This result indicates that the *maf1* Δ strains retain the ability to grow invasively in host plant tissues. Thus, we conclude that the reduced pathogenicity of the *maf1* mutants is due to attenuated penetration of the host plant, probably caused by a failure of appressorium formation.

***MAF1* is required in the early phase of appressorium formation, whereas *CMK1* is involved in appressorium maturation.**

The wild-type strain 104-T of *C. lagenarium* forms melanized appressoria on a glass surface at 24°C (Fig. 7A). How-

ever, conidia of the wild-type strain incubated under certain conditions exhibited a phenotype morphologically similar to that of the *maf1* mutants. Conidia of the wild type incubated on PDAS nutrient agar (3.9% PDA and 0.6 M sucrose) instead of glass germinated and produced elongated germ tubes without appressoria (Fig. 7D); 66% of germinating conidia of the wild type ($n = 200$) on PDAS had two rounds of nuclear division after 12 h of incubation, resulting in four nuclei, which seemed similar to the phenotype of the *maf1* mutants on an inductive glass surface. These similarities suggest a possibility that the nondifferentiation of appressoria of wild-type *C. lagenarium* on the nutrient agar is associated with the Maf1 MAPK cascade.

It has been shown by Takano et al. (2000) that the *CMK1* MAPK gene is required for appressorium formation. We compared the morphological phenotypes of *cmk1* and *maf1* mutants during conidial incubation on an inductive glass surface. In previous studies, *cmk1* mutants suspended in water failed to germinate on a glass surface, but the addition of yeast extract restored their germination (Takano et al., 2000). However, even when germination of the *cmk1* mutants was restored by yeast extract, the germinating conidia failed to form normal appressoria; rather, they formed swollen, appressorium-like structures that exhibited curling (Takano et al., 2000) (Fig. 7C). In the presence of yeast extract, the *maf1* mutants produced elongated germ tubes without forming any curled, swollen structures, the same as the mutants in water (Fig. 7B). This indicates that the morphological phenotype of the *maf1* mutants differs from that of the *cmk1* mutants when they are incubated on an inductive glass surface, and it suggests that *MAF1* is required for the ear-

Fig. 3. Reintroduction of *MAF1* into the *maf1* Δ strain. **A**, Plasmid pCBMAF1, carrying a bialaphos resistance gene and the 4.5-kb *PstI* fragment containing *MAF1*, was introduced into *maf1* Δ strain DMA5, and bialaphos-resistant transformants were obtained. **B**, Genomic DNA gel blot analysis of *MAF1*-reintroduced transformants of DMA5. Genomic DNAs were extracted from the wild-type strain 104-T (lane 1), DMA5 (lane 2), and two *MAF1*-reintroduced transformants, RMA1 and RMA2 (lanes 3 and 4). All genomic DNA samples were digested by *Hind*III and *PstI*. The blot was probed with the 1.7-kb *MAF1* fragment shown in A. As a control, the blot was also probed with a fragment containing the *CMK1* gene (Takano et al., 2000).

lier differentiation step of appressorium formation, whereas *CMK1* is involved in later maturation steps. The phenotype of *maf1* mutants on PDAS was similar to that of the mutants on an inductive glass surface (Fig. 7E). The *cmk1* mutants incubated on PDAS, like the wild type and the *maf1* mutants, also produced elongated germ tubes without forming curled swollen structures (Fig. 7F).

DISCUSSION

The *maf1* mutants, generated by targeting gene disruption, had growth rates similar to that of the wild type on nutrient media. This suggests that, like the *Cmk1* MAPK, the *Maf1* MAPK is dispensable for vegetative growth. However, the two MAPKs have different roles in fungal invasive growth in cucumber leaves. The *maf1* mutants formed lesions after inoculation at wound sites, indicating that *MAF1* is dispensable for invasive growth in the host plant, whereas *CMK1* is necessary for invasive growth (Takano et al., 2000). Similarly, the *MPS1* MAPK gene of *M. grisea*, homologous to *MAF1*, is not essential for invasive growth in rice (Xu et al., 1998). Also, conidiation was reduced in the *maf1* mutants, as it is in *M. grisea mps1* mutants, suggesting a conserved role of *MPK1*-type MAPK genes for conidiation in both *C. lagenarium* and *M. grisea*. We have shown that disruption of *CMK1* caused a reduction in conidiation (Takano et al., 2000), suggesting the involvement of two different MAPKs (*Maf1* and *Cmk1*) in the conidiation of *C. lagenarium*.

The *Mpk1*-type MAPKs have been shown to be involved in the maintenance of cell wall integrity in several fungi, such as *S. cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, and *M. grisea* (Lee et al., 1993; Navarro-García et al., 1995; Toda et al., 1996; Xu et al., 1998). In contrast, we did not find any differences in sensitivity to cell-wall-digesting enzymes in the wild-type strain and *maf1* mutants of *C. lagenarium*, sug-

gesting that the *MAF1*-encoded MAPK is dispensable for cell wall integrity. There is a possibility that (1) *Maf1* redundantly regulates the maintenance of cell wall integrity with one or more other unidentified signaling pathways or (2) *Maf1* is not involved in the maintenance of cell wall integrity.

The *FUS3/KSS1*-type MAPK genes are commonly required for appressorium formation in *M. grisea*, *C. heterotrophus*, *C. lagenarium*, and *P. teres* (Lev et al., 1999; Ruiz-Roldan et al., 2001; Takano et al., 2000; Xu and Hamer, 1996). In contrast with conserved roles of *Fus3/Kss1* MAPKs for appressorium formation, the different phenotypes in appressorium formation by *C. lagenarium maf1* and *M. grisea mps1* mutants imply that *MPK1*-type MAPK genes have diverse roles in appressorium formation in phytopathogenic fungi. In *S. cerevisiae*, the *Mpk1* MAPK is involved in other aspects, including actin organization, in addition to the maintenance of cell wall integrity (Costigan and Snyder, 1994; Heinisch et al., 1999; Helliwell et al., 1998; Madden et al., 1997; Philips and Herskowitz, 1997). Our experiments using actin-depolymerizing drugs suggest that rearrangement of the actin cytoskeleton plays an important role in appressorium formation by *C. lagenarium* (E. Oshiro, Y. Takano, and T. Okuno, unpublished results). The inability of the *maf1* mutants to form appressoria might be due to a failure of the actin organization required for appressorium formation.

Few lesions formed on host plants spray-inoculated with the *maf1* mutants, but lesions formed more frequently on drop-inoculated leaves. These results indicate that the *maf1* mutants retained some pathogenicity, although they never formed appressoria. In *Colletotrichum* species, mechanical penetration using appressoria is thought to be a major pathogenic factor (Bechinger et al., 1999). The finding that the *maf1* mutants did not completely lose pathogenicity suggests that other pathogenicity factors are also involved in infection by *C. lagenarium*. In the drop inoculation tests, a big group of the *maf1* mutant conidia in conidial suspension drops may produce degrading enzymes or toxic compounds sufficient for penetration and lesion formation without appressoria.

M. grisea forms appressoria efficiently on hydrophobic surfaces but not on hydrophilic surfaces (Lee and Dean, 1994). In contrast, *C. lagenarium* forms appressoria on both hydrophobic and hydrophilic surfaces, although these surface properties do seem to affect germination efficiency (Takano et al., 2000). Also, *M. grisea* on a nutrient agar has been reported to produce elongated germ tubes without forming appressoria. This is similar to the phenotype of *C. lagenarium* on the nutrient agar (Adachi and Hamer, 1998; DeZwaan et al., 1999). These results imply that fungal pathogens commonly do not develop appressoria on nutrient agar via sensing mechanisms of environmental conditions. We showed that the *maf1* mutants on inductive surfaces exhibited a phenotype similar to that of the wild-type *C. lagenarium* on the nutrient agar, which suggests that incubation on the nutrient agar represses the appressorium differentiation pathway mediated by *MAF1*. We consider that the *Maf1* MAPK cascade is under putative environmental sensing mechanisms that control appressorium differentiation.

Comparison of the *maf1* mutants with the *cmk1* mutants on inductive glass surfaces suggests that *MAF1* is required for the early phase of appressorium formation, whereas *CMK1* is involved in appressorium maturation. The *cmk1* mutants formed swollen, appressorium-like structures on a glass surface but not on the nutrient agar, indicating that they can respond to the glass surface signal for appressorium formation. These results suggest that *CMK1* is not involved in early appressorium differentiation mediated by contact on the inductive glass surface and that *CMK1* may function downstream of the contact-mediated pathway that triggers appressorium differentiation.

Fig. 4. Sensitivity of *maf1* mutants to cell-wall-digesting enzymes. **A**, Quantitative analysis of protoplasts released by mycelia of the wild-type strain 104-T (open bars) and *maf1*Δ strain DMA5 (shaded bars) 3 and 6 h after treatment with the cell-wall-digesting enzymes β -D-glucanase and Driselase. **B**, Protoplasts released from mycelia of 104-T and DMA5 6 h after treatment with cell-wall-digesting enzymes. Bars = 50 μ m.

In this study, we present evidence that two MAPKs, Maf1 and Cmk1, independently control different steps in appressorium development in *C. lagenarium*. Further studies to identify upstream regulators and downstream targets of these MAPK cascades will elucidate MAPK-mediated infection mechanisms of fungal pathogens and will offer novel strategies for protecting plants from fungal infection.

MATERIALS AND METHODS

Fungal strains and culture conditions.

C. lagenarium 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 medium (3.9% PDA, wt/vol) (Difco, Detroit, MI, U.S.A.) at 24°C. Conidia were obtained by gently scraping cultures incubated for 7 days.

MAF1 cloning.

A nested PCR was performed to isolate MAPK genes using cDNA synthesized from polyadenylated RNA prepared from conidia 2 h after the start of incubation for appressorium formation. Three degenerate primers—primer LVN, 5'-YTNGTNAAYGC-NGAYTG-3' (LVNADC); primer CDF, 5'-GGACGGATCCTG-

YGAYTTYGGNYTNGC-3' (CDFGLA); and primer WYR, 5'-CCCAGAAATCTCNGGNGCNCRTACCA-3' (WYRAPE)—were designed, based on the conserved amino acids in Mpk1-type MAPKs in other organisms. Primer CDF contains a terminal *Bam*HI site. Primer WYR contains a terminal *Eco*RI site. The first PCR was performed with primers LVN and WYR with Ex Taq polymerase (Takara, Ohtsu, Japan). Nested PCR was performed with primers CDF and WYR. PCR conditions were 30 cycles at 94°C for 1 min, 52°C for 2 min, and 72°C for 2 min, with a final extension at 72°C for 5 min. Nested PCR products were digested with *Bam*HI and *Eco*RI, cloned into pBS (KS⁻) (Stratagene, La Jolla, CA, U.S.A.), and sequenced. Clone pMP1, having homology with *S. cerevisiae* MPK1 and *M. grisea* MPS1, was used as a probe to screen genomic clones containing the *MAF1* gene from the *C. lagenarium* cosmid library. The DNA sequence was determined with a Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Warrington, U.K.) and an automated DNA sequencer (Applied Biosystems Model 310).

Fungal transformation.

Preparation of protoplasts and transformation of *C. lagenarium* were performed according to a method described previously (Takano et al., 2001b). Hygromycin- and bialaphos-resistant

Fig. 5. *MAF1* is essential for appressorium formation. **A–F**, Conidia of the wild-type strain 104-T (**A** and **D**), the *maf1* mutant DMA5 (**B** and **E**), and the *MAF1*-reintroduced transformant RMA1 (**C** and **F**) incubated for 12 h on glass slides (**A–C**) or on the lower epidermis of cucumber cotyledons (**D–F**): a, appressorium; c, conidium; g, germ tube. **G** and **H**, Conidia of DMA5 incubated on glass for 12 h and then fixed and stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) to visualize nuclei (arrows): differential interference contrast image (**G**) and DAPI image (**H**). Bars = 10 μm.

Fig. 6. *MAF1* is required for fungal pathogenicity. **A**, Detached cucumber leaves sprayed with suspensions of conidia of the wild-type strain 104-T, *maf1*Δ mutant DMA5, and the *MAF1*-reintroduced transformant RMA1 and incubated at 25°C for 7 days. **B**, Inoculation assay of wounded cucumber leaves. Detached cucumber leaves wounded by scratching with a needle and inoculated by spotting wound sites with suspensions of conidia of DMA5 and SGC1, a growth suppressor mutant isolated from the PKA regulatory subunit mutant (Takano et al., 2001a), on the right half of the leaf, and with the melanin-deficient mutant 79215 (*pks1*) on the left half of the leaf as a positive control. Inoculated leaves were incubated at 25°C for 7 days.

transformants were selected on regeneration medium containing hygromycin B (100 µg/ml) (Wako Pure Chemicals, Osaka, Japan) and bialaphos (250 µg/ml) (kindly provided by Nobuyuki Fuchigami, Meiji Seika Kaisha), respectively.

Genomic DNA blot analysis.

Total DNA of *C. lagenarium* was isolated from mycelia by a method described previously (Takano et al., 1997). DNA digestion, gel electrophoresis, labeling of probes, and hybridization were performed according to the manufacturers' instructions and standard methods (Sambrook et al., 1989). DNA probes were labeled digoxigenin-dUTP (Roche Molecular Biochemicals) with the BcaBEST digoxigenin labeling kit (Takara). Hybridization was carried out as described previously (Takano et al., 1997).

Plasmid construction.

To construct the gene disruption vector pGDMAF1, two DNA fragments (a 7.5-kb *ApaI* fragment and a 6.7-kb *PstI* fragment) containing the *MAF1* gene were subcloned into pBSII(KS⁻) (Stratagene) from the cosmid clone, to produce pMAFA and pMAFP, respectively. The 1.1-kb fragment containing the 3' flanking region of *MAF1* was amplified by PCR using primer MAFD1A (5'-CGCAAGGGGCCAGGTTATGACGAGAGCG-3') and M13 reverse primer from pMAFA. Primer MAFD1A contains a terminal *ApaI* site. PCR was performed with LA-taq polymerase (Takara) for high fidelity of DNA synthesis. The amplified fragment was digested with *ApaI* and introduced into *ApaI* sites of pCB1636 (Sweigard et al., 1997), containing the *hph* gene, to produce plasmid pCBMAF3A. The 2.8-kb fragment containing the 5' flanking region of *MAF1* was amplified by PCR using primer MAFD2P (5'-ACAAAACCTGCAGTGCTTCTTGTGAATCT-3') and

Fig. 7. Morphological phenotype of the wild-type strain 104-T (**A** and **D**), the *maf1* mutant DMA5 (**B** and **E**), and the *cmk1* mutant DCM1 (**C** and **F**) on glass slides (**A–C**) and on nutrient agar (**D–F**): a, appressorium; c, conidium; g, germ tube; s, swollen appressorium-like structure. Conidia of the wild-type strain and the *maf1* mutant suspended in 0.1% yeast extract were incubated for 12 h at 24°C. Conidia of the *cmk1* mutant in 0.1% yeast extract were incubated for 18 h at 24°C, because of delayed germination of this mutant (Takano et al., 2000). Bars = 10 µm.

M13 forward primer from pMAFP. Primer MAFD2P contains a terminal *Pst*I site. The amplified fragment was digested with *Pst*I and introduced into *Pst*I sites of pCBMAF3A, to produce plasmid pGDMAF1. To construct plasmid pCBMAF1 for use in a complementation test of the *maf1* mutant, a genomic fragment was amplified from pMAFP by PCR using primer MAF1CP (5'-AAACTGCAGTGTCTGTGTTTCATTCG-3') and M13 forward primer. Primer MAF1CP contains a terminal *Pst*I site. The amplified fragment was digested with *Pst*I and introduced into *Pst*I sites of pCB1531, containing a bialaphos-resistant gene (Sweigard et al., 1997) to produce pCBMAF1.

Pathogenicity tests.

Conidia were collected from 7-day-old PDA cultures and resuspended in water (approximately 5×10^5 conidia per ml). The conidial suspension was sprayed on the surface of detached cucumber leaves (*Cucumis sativus* L. 'Suyo'). Inoculated leaves were placed in humid petri dishes and incubated at 25°C for 7 days. For microscopic observation, 20 µl of conidial suspension was spotted on the lower epidermis of cucumber cotyledons. The epidermal layers were peeled off and observed under light microscopy 12 h after inoculation (Takano et al., 1997). For leaf wound inoculation, wounds (1 to 2 mm²) were produced by scratching with 26G_{1/2} needles, and leaves were inoculated with a conidial suspension spotted on the wound sites.

Microscopy.

For synchronous infection-related morphogenesis on glass slides, 20 µl of a conidial suspension (1×10^5 conidia per ml in 0.1% yeast extract solution) was placed on an eight-well multitest glass slide (INC Biochemicals, Aurora, OH, U.S.A.) and incubated at 24°C (Takano et al., 2001b). After 1 h of incubation, 0.1% yeast extract solution was changed to distilled water, and the samples were subjected to further incubation. For incubation on PDAS medium, containing PDA (3.9%, wt/vol) and 0.6M sucrose (Difco, Detroit, MI, U.S.A.), the glass slide was coated with PDAS prior to placement of the conidial suspension. In morphological comparisons of the *maf1* mutant with the *cmk1* mutant, conidia of the tested strains were incubated continuously in the presence of yeast extract to restore germination of the *cmk1* mutant. For staining of nuclei, samples on glass slides were fixed with 3.75% formaldehyde, 50 mM phosphate buffer (pH 7.0), and 0.2% Triton X-100 at room temperature for 30 min (Takano et al., 2001b). Fixed samples were rinsed with distilled water, stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 µg/ml) (Sigma, St. Louis, MO, U.S.A.) for 30 min, and then rinsed again with distilled water. Stained cells were observed with a microscope with a Zeiss filter set (excitation 365 nm, dichroic 395 nm, emission 420 nm). Images were captured with a chilled charge-coupled device camera (Argus 50, Hamamatsu Photonics, Hamamatsu, Japan).

For cell wall integrity assays, mycelia of tested strains grown in PSY (potato-sucrose broth supplemented with 0.2% yeast extract) were transferred to a solution of 1.2 M MgSO₄ and 10 mM Na₂HPO₄ with β-D-glucanase (5 mg/ml) (Inter-Spex Products, Inc., San Mateo, CA, U.S.A.) and Driselase (10 mg/ml) (Sigma) and incubated at 30°C. Protoplasts released from mycelia 3 and 6 h after the enzyme treatment were examined under a microscope, and the concentration of protoplasts in a sample solution was calculated.

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