Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses

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ABSTRACT The rice blast fungus, *Magnaporthe grisea*, generates enormous turgor pressure within a specialized cell called the appressorium to breach the surface of host plant cells. Here, we show that a mitogen-activated protein kinase, Mps1, is essential for appressorium penetration. Mps1 is 85% similar to yeast Slt2 mitogen-activated protein kinase and can rescue the thermosensitive growth of *slt2* null mutants. The *mps1–1Δ* mutants of *M. grisea* have some phenotypes in common with *slt2* mutants of yeast, including sensitivity to cell-wall-digesting enzymes, but display additional phenotypes, including reduced sporulation and fertility. Interestingly, *mps1–1Δ* mutants are completely nonpathogenic because of the inability of appressoria to penetrate plant cell surfaces, suggesting that penetration requires remodeling of the appressorium wall through an Mps1-dependent signaling pathway. Although *mps1–1Δ* mutants are unable to cause disease, they are able to trigger early plant-cell defense responses, including the accumulation of autofluorescent compounds and the rearrangement of the actin cytoskeleton. We conclude that *MPS1* is essential for pathogen penetration; however, penetration is not required for induction of some plant defense responses.

The plant cell wall provides such an effective barrier that many microorganisms can infect plants only through wounds or natural openings in the wall (1). A variety of fungal pathogens have evolved a complex morphogenetic program to sense specific components and properties of the plant cell surface and to differentiate specialized infectious cells, called appressoria, for penetrating the plant cell wall (2). Appressoria are produced by a wide range of taxonomically diverse fungal and nonfungal species (e.g., *Oomycetes*), suggesting that appressorium differentiation is a widespread microbial adaptation that assists in plant cell-wall penetration.

The rice blast fungus, *Magnaporthe grisea*, infects most of the economically important cereal crops, particularly rice (3). Dispersed fungal spores (conidia) attach tightly to the plant surface, and under high moisture conditions, they germinate and differentiate a dome-shaped appressorium (4–6). The appressorium attaches tightly to the plant surface, and over several hours, turgor pressures within the appressorium reach 3–8 MPa, the highest measured turgor pressures for any cell (7, 8). The fact that appressoria are capable of denting and penetrating inert hydrophobic surfaces (4) suggests that turgor pressure is the major force used to drive a thin penetration hypha through the plant cell wall.

Turgor within the *M. grisea* appressorium is generated through a dramatic rise in intracellular glycerol levels (9). The efflux of glycerol from within the appressorium is prevented partially by a wall layer rich in polyketide-derived melanin.

Mutations or chemicals that block the formation of the melanin wall layer allow the rapid efflux of glycerol, impair the maintenance of appressorium turgor, and prevent penetration (7, 9). Other cell-wall or membrane components may also be important for penetration. Ultrastructural studies reveal that several additional wall layers are laid down during appressorium formation, and these may be necessary to maintain cell-wall integrity under conditions of membrane stress and high turgor pressure (5). How turgor is directed to the highly localized site of penetration-hypha formation is not currently known. Actin is localized to the site of penetration-hypha formation, suggesting that polarization of the cytoskeleton may be involved in localized wall modification (10).

In *Saccharomyces cerevisiae*, the control of cell-wall growth in response to membrane stress is mediated by a protein kinase C pathway that activates the mitogen-activated protein kinase (MAPK), Slt2/Mpk1 (11–13). The Δ*slt2* mutants display defects in cell-wall integrity, including sensitivity to cell-wall-degrading enzymes and an inability to grow at elevated temperatures in the absence of osmotic stabilizers. The homologous MAPK in *Schizosaccharomyces pombe* (14, 15) also plays a role in the maintenance of cell-wall integrity; however, the function of related kinases in filamentous fungi are currently unknown. Here, we show that *M. grisea* has a MAPK designated Mps1 that is related functionally to the yeast Slt2 kinase. We show that *MPS1* is essential for the maintenance of cell-wall integrity during specific phases of the life cycle and, most importantly, for turgor-driven penetration of plant cells. Unexpectedly, although the *mps1–1Δ* mutant strains are nonpathogenic and fail to penetrate the cell wall, they still activate plant defense responses.

MATERIALS AND METHODS

Strains, Growth Conditions, and Pathogenicity Assays. Growth, mating assays, transformation, and nucleic-acid extraction from *M. grisea* were performed as described (16–18). Infection assays on rice, epidermal-cell penetration assays on onions, germination assays, and appressorium-formation assays were performed as described (18, 19). *S. cerevisiae* strains were cultured on 1% yeast extract/1% peptone, supplemented with either 1% galactose/1% raffinose or 1% dextrose.

Cloning of *MPS1*. Procedures for amplifying and cloning MAPK-related genes from *M. grisea* are described in ref. 18. Amplified DNA fragments were used to screen cDNA and genomic libraries. Automated fluorescent DNA sequencing was performed on an ALF-Express DNA sequencer (Pharmacia). DNA-protein sequence database searches were performed with the BLAST programs (20). Amino-acid-sequence comparisons and alignments were made with the BESTFIT,

PILEUP, and BOXSHADE programs from the Genetics Computer Group's software package (Madison, WI).

Yeast Complementation. The *MPS1* ORF was amplified with primers M3E5 (GGAATTCTAATGTCGGATCTC-CAGGGCC) and M3S3 (GCCGAGCTCTCACCTCCTCT-GATCCAAA), and subcloned between *Eco*RI and *Sac*I sites in the yeast expression vector pYES2 (Invitrogen). This construct was then transformed into yeast strain DL456 (*MAT***a** Δ*slt2 ura3-52*). Ura⁺ transformants were tested for the ability to grow at 37°C.

MPS1 Gene Replacement. A 1.4-kb *Hin*dIII fragment extending upstream of *MPS1* was end-filled with DNA polymerase I and cloned into the *Sma*I site on pBluescript II KS(+) as pM3L7. The 2.4-kb *Xho*I–*Hin*dIII fragment (downstream of *MPS1*) from pM3R24 was cloned into the *Xho*I–*Hin*dIII sites on pM3L7. The hygromycin phosphotransferase gene was then cloned into the *Eco*RI site to create the *mps1–1*Δ gene-replacement vector pM3H22.

Microscopy. Onion epidermal peels were isolated, and conidia were allowed to germinate on the adaxial surface (19). After 48 h in the dark, epidermal peels were fixed for 1 h with 500 μM 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Sigma) prepared in PBS, 0.1% Triton X-100, and 0.5% dimethyl sulfoxide. Peels were rinsed of fixative by three 10-min washes with PBS, and then filamentous-actin stained for 45 min with 0.33 μM rhodamine-phalloidin (Molecular Probes) in PBS plus 5% methanol. After two 10-min PBS washes, the peels were mounted in SlowFade reagent (Molecular Probes) and visualized by fluorescence microscopy.
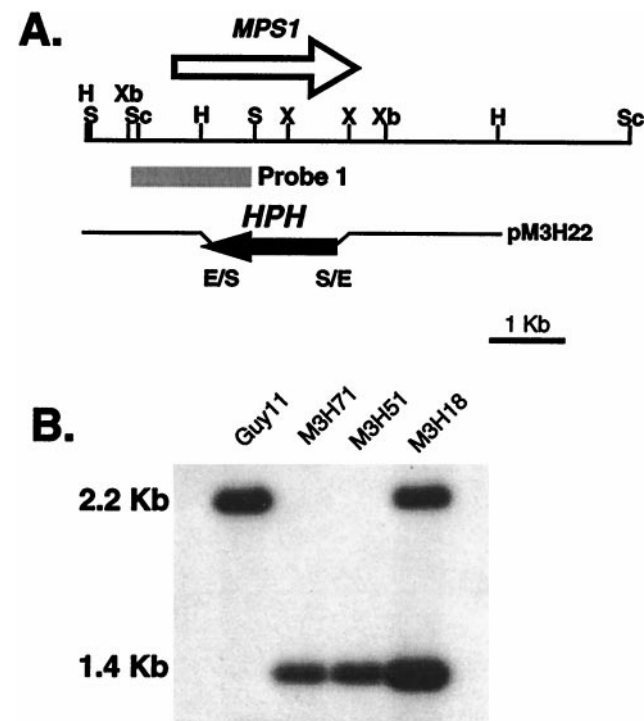
Cell-Wall Integrity Assays. *M. grisea* cells at various stages of germination and appressorium formation were incubated in 1 M sorbitol with 5 mg/ml Novozyme 234 (InterSpex Products, Foster City, CA). Spheroplast release and cell-wall degradation were examined with differential-interference-contrast microscopy after incubation for various periods at 30°C.

RESULTS

Mps1 Is Functionally Related to the Yeast Slt2 MAPK. We identified sequences from the rice blast fungus that are related to yeast MAPKs (ref. 18; see *Materials and Methods*). The cDNA and genomic sequence of *MPS1* identified an ORF encoding a 415-aa peptide (Fig. 1*A*) that is 80% similar and 64% identical to *S. cerevisiae* Slt2/Mpk1 and 77% similar and 65% identical to *S. pombe* MAPK Spm1/Pmk1 (14, 15). Phylogenetic analysis indicated that Mps1 clustered with kinases related to Slt2/Mpk1 and was less related to *S. cerevisiae* Hog1 or Fus3/Kss1 MAPKs (Fig. 1*B*). To test whether *MPS1* could function in place of *SLT2*, the *MPS1* ORF was cloned into a galactose-inducible yeast expression vector and transformed into yeast strain DL456 (*MAT***a** Δ*slt2 ura3-52*). Ura⁺ transformants were tested for the ability to grow at 37°C with either glucose or galactose as a carbon source (Fig. 1*C*). *MPS1* complemented the Δ*slt2* autolysis defect at 37°C (Fig. 1*C*). This complementation was specific for *MPS1*, as another MAPK gene from *M. grisea*, *PMK1*, failed to complement the Δ*slt2* defect (data not shown).

MPS1 Is Required for Efficient Sporulation, Cell-Wall Integrity, and Female Fertility. The *MPS1* gene-replacement vector pM3H22 (Fig. 2*A*) was constructed by replacing the

FIG. 1. Structure, function, and relatedness of *MPS1*. (*A*) Sequence alignment of Mps1 with *S. cerevisiae* Slt2 and *S. pombe* Spm1. Identical residues are shown in black boxes and similar residues are shaded. The 11 protein-kinase subdomains are labeled with roman numerals. (*B*) Relatedness of Mpk1 to yeast MAPKs. GenBank accession nos. used for yeast MAPKs are StyI (X89262), Hog1 (L06279), Spm1 (U65405), Slt2 (X59262), Spk1 (X57334), Kss1 (M26398), Fus3 (M31132), and Smk1 (L35047). Accession nos. for *M. grisea* MAPKs are Mps1 (AF020316) and Pmk1 (U70134). A third MAPK from *M. grisea*, Pmk2, is most closely related to yeast Hog1 and StyI kinases (J.-R.X. and N. Talbot, unpublished work). The phylogram was prepared with the GROWTREE program from the Genetics Computer Group's software package (Madison, WI). (*C*) Complementation of a yeast *slt2* mutant. *S. cerevisiae* strains numbered 2–6 are DL456 (*slt2*Δ, *ura3–52*); strain 1 is CG219 (*ura3–52 SLT2*); strains numbered 2, 3, and 4 contain the plasmid pNX9 (*MPS1* controlled by the *GAL1* promoter in pYES2); strains 5 and 6 contain the vector pYES2. The upper plate (1% yeast extract/1% peptone/1% dextrose) was incubated at 30°C and replicated to 1% yeast extract/1% peptone/1% galactose/1% raffinose (lower right) or 1% yeast extract/1% peptone/1% dextrose (lower left) plates that were incubated at 37°C before being photographed.

FIG. 2. *MPS1* gene-replacement vector and transformants. (*A*) Physical map of the *MPS1* genomic region and the gene-replacement vector pM3H22. The restriction enzymes are E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sc, *Sac*I; X, *Xho*I; Xb, *Xba*I. (*B*) Southern blot of wild-type strain Guy11, ectopic-integration transformant M3H18, and *MPS1* gene-replacement transformants M3H51 and M3H71. All DNA samples were digested with *Sal*I. The blot was probed with the 1.6-kb *Sac*I–*Sal*I fragment. Guy11 and transformant M3H18 contained the *MPS1* 2.2-kb *Sal*I fragment. M3H18 also contained a 1.4-kb *Sal*I fragment, indicative of an ectopic insertion event. Transformants M3H51 and M3H71 do not contain the 2.2-kb *Sal*I fragment but have the 1.4-kb *Sal*I fragment, indicative of a gene replacement.
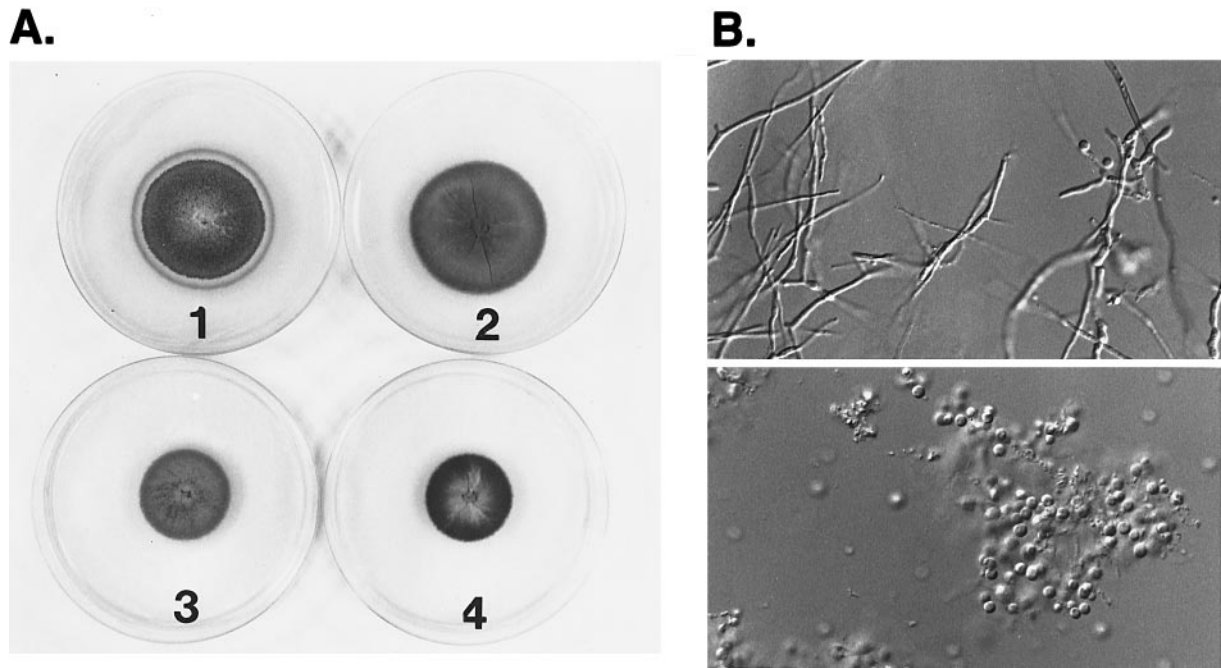
A.

B.

Fig. 3.   *mps1-1Δ* mutants have defects in cell-wall integrity. (*A*) Growth of *mps1–1Δ* strains on complete media without 1 M sorbitol (plate 1); growth of *MPS1* strains on complete media without 1 M sorbitol (plate 2); growth of *mps1–1Δ* strains on complete media with 1 M sorbitol (plate 3); and growth of *MPS1* strains on complete media with 1 M sorbitol (plate 4). *mps1–1Δ* mutants sporulate poorly and undergo progressive autolysis in the absence of osmotic stabilization. Radial growth rates are identical to those of wild-type strains. (*B*) Sensitivity of *mps1–1Δ* strains to cell-wall-digesting enzymes. Wild-type (*Upper*) or *mps1–1Δ* (*Lower*) strains were photographed 15 min after treatment with Novozyme 234. The *mps1–1Δ* mutants are reduced to spheroplasts with very short enzyme treatment. (×230.)

*Hin*dIII–*Xho*I fragment, containing the majority of the *MPS1* coding region, with the hygromycin phosphotransferase gene, *HPH*. This construct was used to transform wild-type strain Guy11. Strains containing the *MPS1* deletion (*mps1–1Δ*) were characterized by Southern blot analysis (Fig. 2*B*). Guy11 and transformant M3H18 contained the *MPS1* 2.2-kb *Sal*I fragment. M3H18 also contained a 1.4-kb *Sal*I fragment from an ectopic insertion event. Transformants M3H51 and M3H71 do not contain the 2.2-kb *Sal*I fragment, but have the 1.4-kb fragment indicative of the gene-replacement event (Fig. 2*B*). The *mps1–1Δ* mutants were crossed with wild-type strain 2359 (*MAT1-1*; ref. 21). Progeny that segregated for the *mps1–1Δ* allele (*n* = 34: 15 *mps1–1Δ*, hygromycin-resistant; 19 *MPS1*, hygromycin-sensitive) were isolated and used to confirm cosegregation of all *mps1–1Δ* phenotypes (described below).

The *mps1–1Δ* mutants have radial growth rates on plate media similar to those of wild-type strains (Fig. 3*A*). Mycelial dry-weight determinations of *mps1–1Δ* mutant and wild-type strains grown in liquid complete media also show no difference (data not shown). However, *mps1–1Δ* mutants were reduced dramatically in aerial hyphal growth and conidiation (Fig. 3*A*). Wild-type strains produced an average of 10^7 conidia per plate, whereas *mps1–1Δ* mutants produced <4,000 conidia per plate. The reduction in conidiation was caused by the limited ability of *mps1–1Δ* strains to produce aerial hyphae. Conidia produced by *mps1–1Δ* strains have normal morphology and germination rates (data not shown).

mps1–1Δ colonies underwent progressive autolysis beginning at the central part of the colony and radiating outwards (Fig. 3*A*). This novel growth-related phenotype was suppressed by the addition of 1 M sorbitol. *mps1–1Δ* mutants are also hypersensitive to the fungal cell-wall-digesting enzyme Novozyme 234 (Fig. 3*B*). Enzyme digestion of mycelia from *mps1–1Δ* strains released spheroplasts within 5 min and resulted in the dissolution of all intact hyphae after 30 min at 30°C (Fig. 3*B*). These findings suggest that *MPS1* has a role similar to that of the *SLT2* gene in *S. cerevisiae* and is required

to maintain cell-wall integrity in *M. grisea*. However, unlike Δ*slt2* mutants, *mps1–1Δ* mutants are not temperature-sensitive for growth, and unlike *S. pombe spm1⁻/pmk1⁻* mutants (15), they are not sensitive to growth in medium containing either 1 M sorbitol, 1 M NaCl, 1 M $MgCl_2$, or 1.4 M KCl.

A novel phenotype was observed during genetic analysis of strains carrying the *mps1–1Δ* allele. The *mps1–1Δ* strains and segregants mated only as males. As female sterility is encountered commonly in cultures of *M. grisea*, the 1.7-kb *Sac*I fragment containing the *MPS1* gene was reintroduced at an ectopic site in the *mps1–1Δ* strain M3H51. All cotransformed strains (*n* = 20) were rescued for the *mps1–1Δ* phenotypes and were restored for female fertility.

MPS1 Is Required for Pathogenicity. Infection assays performed on rice seedlings showed that *mps1–1Δ* mutants were completely nonpathogenic (Fig. 4). However, when *mps1–1Δ* conidia are sprayed onto an abraded leaf surface (not shown) or injected into the leaf sheath, they produce small lesions (Fig. 4). The *mps1–1Δ* mutants could be recovered from these lesions (data not shown). These results show that *MPS1* is essential for plant penetration but not necessarily for invasive growth in rice plants.

The kinetics of conidial germination, germ tube differentiation, and appressorium formation in *mps1–1Δ* mutants were indistinguishable from those of isogenic wild-type strains (data not shown). In addition, appressorium formation could be stimulated by 10 mM cAMP (data not shown; ref. 22). Calcofluor (cell wall) and Hoechst 33258 (nuclei) staining showed that septation, cell-wall deposition, nuclear division, and migration were also indistinguishable from those of wild type (data not shown). Finally, *mps1–1Δ* mutant appressoria underwent melanization and were resistant to plasmolysis by 1 M KCl (23) suggesting that they were not osmotically sensitive (data not shown).

***mps1* Mutants Elicit Plant Defense Responses.** To define more precisely the pathogenicity defects of *mps1–1Δ*, we examined infection-related morphogenesis with a sensitive

penetration assay of onion epidermal cells (19). In this assay, wild-type strains form appressoria, penetrate into epidermal cells, and produce invasive hyphae (Fig. 5 *Top*). *mps1–1Δ* mutant strains also formed appressoria efficiently in this assay, but the mutant strains were unable to produce intracellular infectious hyphae and were not observed to be able to penetrate plant cells (*n* = 200). Because *mps1–1Δ* mutants can infect plant cells through wounds, we conclude that the primary pathogenicity defect in *mps1–1Δ* mutants is their failure to penetrate plant cells.

Interestingly, although there was no penetration of onion epidermal cells by *mps1–1Δ* mutants, we noticed that the plant cells that underlie appressoria seemed to undergo cytoplasmic alterations. Fluorescence microscopy showed the accumulation of intense plant-cell-derived autofluorescence directly around *mps1–1Δ* mutant appressoria, consistent with the activation of early plant defense responses (Fig. 5 *Lower*). Similar levels of plant-cell autofluorescence and cytoplasmic alterations were visible at early stages of infection by wild-type appressoria (data not shown).

Because previous studies suggested that plant cells respond to pathogen attack with rearrangement of cytoskeletal elements (24, 25), onion epidermal peels were inoculated with either wild-type or *mps1–1Δ* mutant strains and stained with rhodamine-phalloidin to visualize the plant actin cytoskeleton. Uninfected onion epidermal cells contain prominent longitudinal bundles and a fine cortical meshwork of actin microfilaments (Fig. 6*A*). After the formation of an appressorium by a wild-type strain on the epidermal cell surface, the longitudinal actin cables accumulated and seemed to radiate from the penetration site (Fig. 6 *B* and *C*). Moreover, the fine cortical network and longitudinal actin cables were reduced substan-

tially elsewhere in the plant-cell cytoplasm. Although they fail to penetrate, the *mps1–1Δ* mutant appressoria elicited an identical cytoplasmic-actin response in the onion epidermal cells (Fig. 6 *D* and *E*).

DISCUSSION

Several of the most economically devastating fungal crop pathogens use appressoria to penetrate the plant cell wall. Elegant experiments with the rice blast fungus on synthetic surfaces showed that penetration is driven by the generation of enormous turgor pressure (5, 6). Here, we provide evidence that a signaling pathway related to the yeast protein kinase C/Slt2/Mpk1 cell-integrity pathway is required to allow turgor-driven penetration of plant cells by fungal appressoria. In the rice blast fungus, this pathway is also required for sporulation and the maintenance of hyphal cell-wall integrity. Components of this signaling pathway could provide targets for the development of antifungals that would block both penetration by appressoria and sporulation from developing lesions.

The compromised cell integrity of *mps1–1Δ* mutants of *M. grisea* is reminiscent of *Δslt2/Δmpk1* mutants of yeast but with several important differences. *S. cerevisiae* mutants undergo lysis at high temperature, and analogous mutants in *S. pombe* are salt-sensitive as well as temperature-sensitive. These phenotypes were not observed with *M. grisea* mutants. Rather,
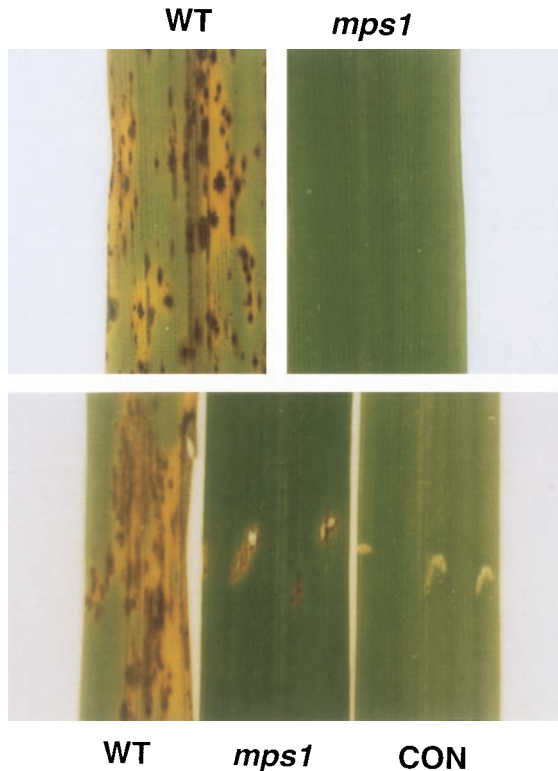

FIG. 4.   Infection assays. (*Upper*) Typical infected leaf areas from 14-day-old rice seedlings spray-inoculated with either wild-type (WT) or *mps1–1Δ* (*mps1*) strains. (*Lower*) Typical infected areas from rice leaves inoculated by leaf sheath injection of either wild-type (WT) or *mps1–1Δ* (*mps1*) strains. A wounded control (CON) inoculated with water is also shown. Small areas of fungal growth occur near the injection site in *mps1–1Δ* strains.
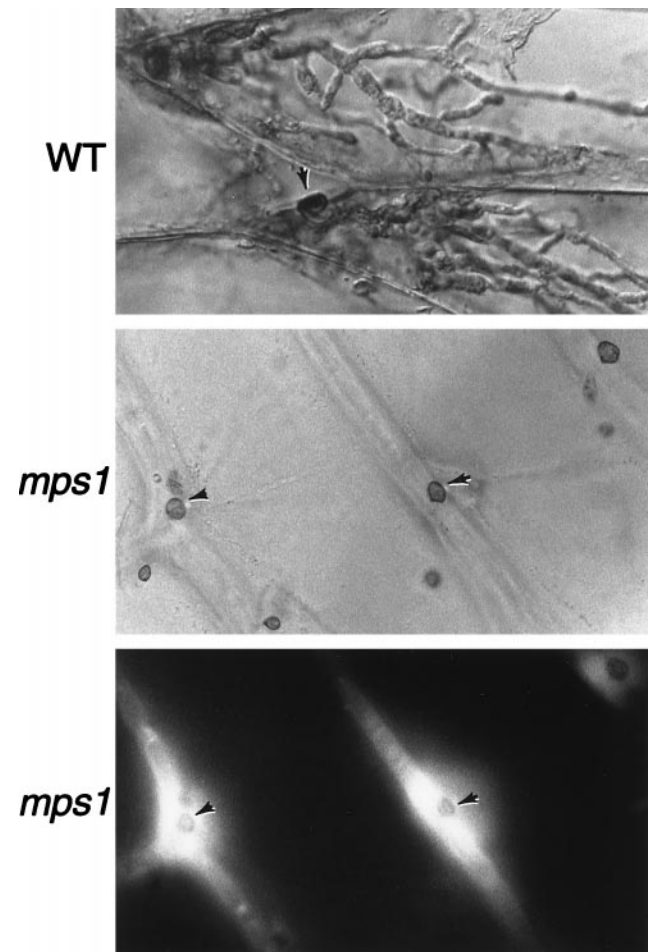

FIG. 5.   Fungal penetration assays on plant epidermal cells. (*Top*) Wild-type strains (WT) form appressoria (arrow) that penetrate and produce abundant infectious hyphae. (*Middle* and *Bottom*) Strains containing the *mps1–1Δ* allele (*mps1*) produce numerous appressoria (arrows) but do not penetrate or form infectious hyphae. *Lower* shows fluorescence (465 nm) illumination of the same area depicted in *Middle*. Plant-cell-derived autofluorescence surrounds nonpenetrating appressoria. (×465.)
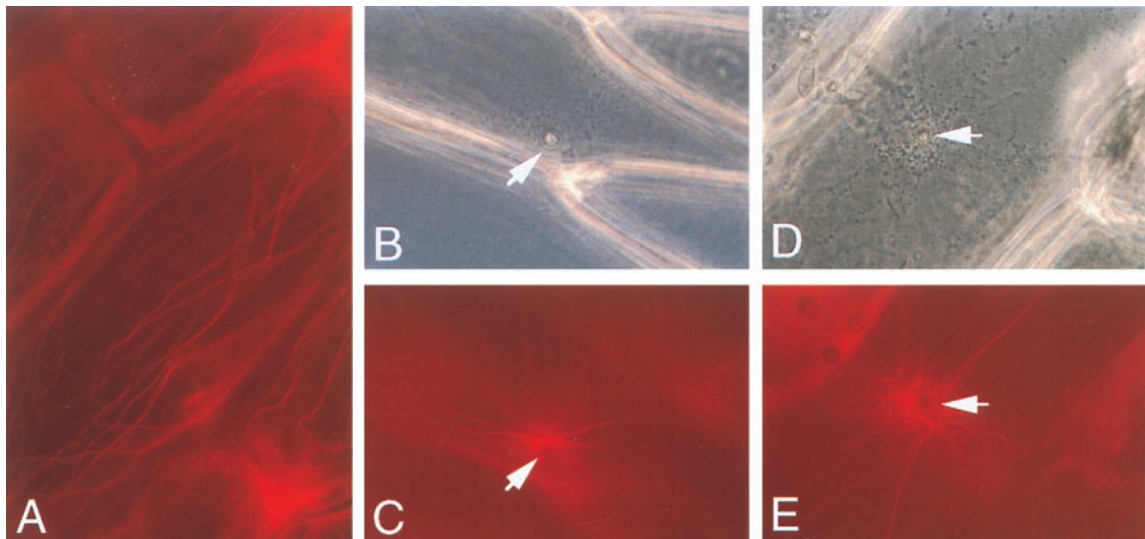
FIG. 6.   Actin rearrangements in response to *mps1–1Δ* mutants. Onion epidermal cells were left untreated (*A*), inoculated with wild-type conidia (*B* and *C*), or inoculated with *mps1–1Δ* mutants (*D* and *E*). Plant cells were visualized with phase-contrast microscopy (*B* and *D*) and with epifluorescence (*C* and *E*) after staining with rhodamine-phalloidin. Arrows indicate appressoria (*B* and *D*) and underlying actin rearrangements (*C* and *E*). (×280.)

cells became osmotically sensitive as a result of cell aging. In addition, specific defects were observed in certain developmental processes, such as aerial-hypha formation, conidiation, female fertility, and appressorium-driven penetration of plant cells. These findings suggest that the Mps1 signaling pathway in *M. grisea* is required for cell-wall remodeling in response to specific developmental cues. It has been shown that yeast Mpk1 activates a MADS-box transcription factor, Rlm1, that may be required to activate cell-wall biosynthetic genes (26). Although we do not know which gene products could be activated by this pathway in *M. grisea*, it is known that, for emergent growth and sporulation, filamentous fungi secrete a group of fungal cell-wall proteins, called hydrophobins, that are arrayed on the outer cell-wall surface (27). Interestingly, the hydrophobin Mpg1p from *M. grisea* has been shown to be necessary for sporulation and pathogenicity (16, 17). It will be interesting to see whether developmental expression of Mpg1p depends on Mps1 signaling.

Mps1 signaling is required for appressorium penetration of plant cells. Mps1 may be required to bring about modifications to the appressorium cell wall in response to increasing turgor. These modifications are not immediately obvious; *mps1–1Δ* mutant appressoria undergo melanization normally, and Calcofluor staining did not reveal anomalies in appressorium formation. In addition, *mps1–1Δ* appressoria did not undergo autolysis during prolonged incubations on artificial surfaces, a condition that leads to dramatic increases in intracellular turgor (7). The Pkc1-Mpk1 signaling pathway of *S. cerevisiae* is also required for bud emergence and polarization of the actin cytoskeleton (28). Thus we conclude, by analogy to yeast, that Mps1 signaling may also be necessary for polarization of the actin cytoskeleton to facilitate the formation of a penetration hypha. This role must be rather stage-specific, because Mps1 seems to be dispensable for actin-dependent polarized growth of fungal hyphae.

Mps1 is one of two MAPKs found to be essential for the infection process in *M. grisea*. Pmk1, functionally homologous to the yeast MAPKs Fus3/Kss1, is required specifically for the differentiation of appressoria and plays no role in mating processes (18). Studies of *S. cerevisiae* have shown that outputs from the pheromone signaling pathway are required for activation of the Mpk1-cell-integrity pathway (29). An analysis of the phenotypes of Δ*pmk1* strains (failure to form appressoria) and *mps1–1Δ* strains (appressoria that fail to penetrate plant

cells) also would suggest that Mps1 function is subsequent to Pmk1 function during the infection process. It is tempting to speculate that although the output of MAPK-signaling pathways between yeast and the rice blast fungus are very different, remarkably, their integration into a common cell-morphogenesis pathway may be conserved (30).

A surprising finding is that although *mps1–1Δ* mutants are nonpathogenic and fail to penetrate plant cell walls, *mps1–1Δ* mutants still elicit certain physiological behaviors characteristic of the plant defense response. These include accumulation of autofluorescent compounds, changes in the pattern of cytoplasmic streaming, and dramatic rearrangement of the actin cytoskeleton. Reorganization of the plant cytoskeleton has been observed usually as a consequence of pathogen penetration (24), particularly in the case of barley–*Erysiphe graminis* interactions (25). Experiments with actin-microfilament inhibitors suggest that this response is part of a general defense mechanism to prevent fungal penetration (31). Here, the surprising finding is that these dramatic cytological events can be triggered in the complete absence of fungal penetration. Although we have not investigated whether these effects also occur in rice plants, careful examination of rice leaves inoculated with *mps1–1Δ* mutants did not reveal any hypersensitive plant cell death and indicates that *mps1–1Δ* mutants most likely failed to penetrate rice cells. Together, these findings suggest that certain aspects of the rice-plant defense response may be activated before fungal penetration and thus may be elicited at the plant cell surface. It remains to be determined whether this plant response is triggered by mechanical pressure exerted by appressoria attempting (but failing) to penetrate or by soluble factors released from the invading fungus. The array of *M. grisea* mutants defective in various aspects of appressorium formation and penetration should allow a more detailed investigation of this process.

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