Independent Signaling Pathways Regulate Cellular Turgor during Hyperosmotic Stress and Appressorium-Mediated Plant Infection by *Magnaporthe grisea*

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The phytopathogenic fungus *Magnaporthe grisea* elaborates a specialized infection cell called an appressorium with which it mechanically ruptures the plant cuticle. To generate mechanical force, appressoria produce enormous hydrostatic turgor by accumulating molar concentrations of glycerol. To investigate the genetic control of cellular turgor, we analyzed the response of *M. grisea* to hyperosmotic stress. During acute and chronic hyperosmotic stress adaptation, *M. grisea* accumulates arabitol as its major compatible solute in addition to smaller quantities of glycerol. A mitogen-activated protein kinase–encoding gene *OSM1* was isolated from *M. grisea* and shown to encode a functional homolog of *HIGH-OSMOLARITY GLYCEROL1* (*HOG1*), which encodes a mitogen-activated protein kinase that regulates cellular turgor in yeast. A null mutation of *OSM1* was generated in *M. grisea* by targeted gene replacement, and the resulting mutants were sensitive to osmotic stress and showed morphological defects when grown under hyperosmotic conditions. *M. grisea* Δ*osm1* mutants showed a dramatically reduced ability to accumulate arabitol in the mycelium. Surprisingly, glycerol accumulation and turgor generation in appressoria were unaltered by the Δ*osm1* null mutation, and the mutants were fully pathogenic. This result indicates that independent signal transduction pathways regulate cellular turgor during hyperosmotic stress and appressorium-mediated plant infection. Consistent with this, exposure of *M. grisea* appressoria to external hyperosmotic stress induced *OSM1*-dependent production of arabitol.

INTRODUCTION

Cellular turgor in eukaryotic cells is regulated in response to the environment or as a component of developmental programs. The most apparent stimulus affecting cellular turgor is hyperosmotic stress, which cells contend with by generating high concentrations of compatible solutes to maintain cellular turgor and thus prevent water loss. Adjustment of cellular turgor is, however, also vital for the generation of the mechanical force that plants and microorganisms need for growth and proliferation, particularly in terrestrial environments. The importance of cellular turgor to microorganisms is exemplified by the plant pathogenic fungus *Magnaporthe grisea*, which causes a serious disease of rice (Talbot, 1995; Howard and Valent, 1996). *M. grisea* elaborates specialized cells called appressoria to breach the plant cuticle. *M. grisea* appressoria are dome-shaped cells that penetrate rice cuticles by generating me-

chanical force. This is achieved by producing enormous turgor pressure within the appressorium estimated to be as high as 8 mPa (Howard et al., 1991; Money and Howard, 1996).

Appressorium turgor is focused on a small area at the base of the appressorium, allowing a penetration peg to rupture the underlying cuticle. Precisely how turgor is translated into mechanical force remains unclear, but it probably involves localized cell wall dissolution at the base of the appressorium and reorientation of the cytoskeleton to allow polarization of the penetration peg (Bourett and Howard, 1990, 1992). The infection process may be accelerated by the action of extracellular enzymes, but the fact that *M. grisea* appressoria can penetrate inert plastic surfaces shows that mechanical force is the primary means of infection (Howard et al., 1991). Recently, cellular turgor in appressoria of *M. grisea* was shown to be generated by accumulation of high concentrations of intracellular glycerol (de Jong et al., 1997). Glycerol concentrations in developing appressoria rise to as high as 3.2 M during turgor generation. Glycerol is maintained in the appressorium by means of the heavily melanin-pigmented cell wall, which provides

a glycerol-impermeable layer (de Jong et al., 1997; Money, 1997). Nonmelanized mutants of *M. grisea* lack this wall layer and are nonpathogenic and unable to generate turgor (Howard and Ferrari, 1989; Chumley and Valent, 1990; de Jong et al., 1997). In this study, we set out to investigate the control of cellular turgor in *M. grisea* by characterizing the response of the fungus to external osmotic stress and comparing the mechanism of this response to that of pressure generation in appressoria.

Cellular turgor in eukaryotes appears to be controlled by a conserved mitogen-activated protein kinase (MAPK) signaling system called the HOG (for high-osmolarity glycerol) pathway, which was first discovered in the budding yeast *Saccharomyces cerevisiae* (Brewster et al., 1993). In *S. cerevisiae*, the HOG pathway is composed of the *SSK2-*, *SSK22-*, and *STE11*-encoded MAP kinase kinase kinases (MAPKKKs), the *PBS2*-encoded MAP kinase kinase (MAPKK), and the *HOG1* MAPK (Gustin et al. 1998). The HOG pathway can be activated in two ways. First, a membrane-bound osmosensor encoded by *SHO1* can act on the MAPKKK Ste11p and the MAPKK Pbs2p, triggering phosphorylation of Hog1p (Maeda et al., 1995). In the second pathway, the *SSK2-* and *SSK22*-encoded MAPKKKs are activated by a three-component histidine–aspartate kinase complex, related to the two- and three-component sensors common in prokaryotes, encoded by *SLN1*, *YPD1*, and *SSK1* (Maeda et al., 1994; Posas et al., 1996; Posas and Saito, 1998). Stimulation of the HOG pathway in budding yeast leads to production of glycerol by transcriptional activation of *GPD1*, which encodes NADH-dependent glycerol-3-phosphate dehydrogenase, and *HOR1*, which encodes glycerol-3-phosphatase (Albertyn et al., 1994; Hirayama et al., 1995; Norbeck et al., 1996). The resulting accumulation of glycerol in yeast cells maintains cellular turgor even after exposure to severe hyperosmotic stress. Elements of the HOG pathway have also been found in mammals, *Drosophila*, and plants (Han et al., 1994, 1998; Popping et al., 1996) and shown either to be required for hyperosmotic adaptation (Sheikh Hamad et al., 1998) or to be capable of complementing corresponding yeast mutants (Kumar et al., 1995; Han et al., 1998).

In this study, we have characterized the cellular response of *M. grisea* to hyperosmotic stress. We show that *M. grisea* accumulates mannitol and trehalose as storage carbohydrates during normal growth and development. Arabitol, however, accumulates as the principal compatible solute during the response to hyperosmotic stress. We also present evidence that arabitol accumulation and resistance to hyperosmotic stress are controlled by *OSM1*, an osmosensory MAPK-encoding gene that is functionally homologous to *S. cerevisiae HOG1*. Remarkably, although cellular turgor is controlled by OSM1 during the response to external osmotic shock, appressorium turgor is unaltered in Δ*osm1* mutants. This suggests that plant pathogenic fungi have evolved specific signaling pathways for appressorium-mediated plant infection that operate independently of the

conserved eukaryotic regulatory mechanism for modulation of cellular turgor.

RESULTS

Cellular Turgor Generation in the Rice Blast Fungus *M. grisea*

Hyperosmotic stress results in the production of compatible solutes, which cells use to prevent water loss and regulate their cellular turgor. In fungi, compatible solutes are predominantly polyols, including glycerol, which is generated in *S. cerevisiae*, and erythritol, which is used by *Aspergillus nidulans* (Blomberg and Adler, 1992). In contrast, plants tend to accumulate proline or glycine betaine, whereas mammalian cells accumulate sorbitol, betaine, or ionic solutes (Lewis and Smith, 1967; Blomberg and Adler, 1992). We investigated compatible solute generation by *M. grisea* during both chronic and acute hyperosmotic stress. First, the nature of compatible solutes accumulating during hyperosmotic stress was determined by using gas–liquid chromatography. During normal growth and development, *M. grisea* accumulates trehalose and mannitol as its major storage carbohydrates in mycelium, as shown in Figure 1A. During hyperosmotic stress, which was imposed by incubating fungal mycelium in 0.4 M NaCl, levels of mannitol remained broadly constant, whereas trehalose levels fell. Arabitol, which is present only in trace amounts during growth of *M. grisea* under isoosmotic conditions, accumulated rapidly in response to hyperosmotic stress, as shown in Figure 1B. Smaller amounts of glycerol were also present in mycelial extracts and increased during osmotic stress. Arabitol was found to accumulate in direct proportion to external osmolarity (data not shown) and was the major solute accumulating under severe hyperosmotic conditions. Gas–liquid chromatography and ^{13}C nuclear magnetic resonance analyses failed to identify any amino acids or other noncarbohydrate solutes accumulating in osmotically stressed mycelium (data not shown). Based on these observations, we conclude that *M. grisea* responds to hyperosmotic stress by accumulating arabitol as its principal osmolyte, with lower concentrations of glycerol also present in stressed mycelium.

Appressoria of *M. grisea* accumulate large amounts of glycerol during turgor generation and plant infection. Glycerol has previously been measured in bulk appressorial extracts by use of an enzymatic assay (de Jong et al., 1997). The internal concentration of glycerol in these small cells (*M. grisea* appressoria are 7 to 8 μm in diameter) can be estimated more readily, however, using a cytorrhysis (cell collapse) assay (Howard et al., 1991; de Jong et al., 1997). Appressoria were allowed to form in water drops on the hydrophobic side of Gelbond membranes. Glycerol was then carefully added to the drop to give a final concentration of

A

B

C
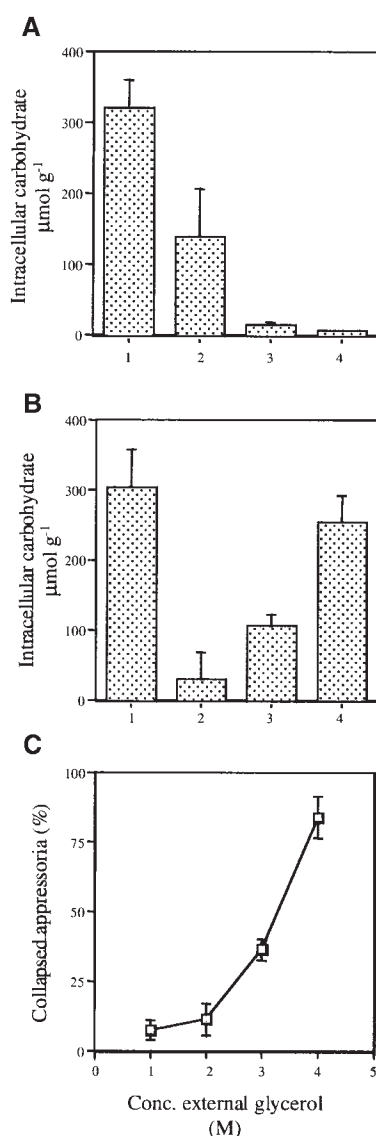
Figure 1. Compatible Solute Production in *M. grisea* during Hyperosmotic Stress and Appressorium Turgor Generation.

Compatible solute accumulation was assayed in *M. grisea* mycelium from the wild-type strain Guy11 after hyperosmotic stress. The mycelium was grown for 48 hr in complete medium (CM) before being transferred to CM or CM plus 0.4 M NaCl for an additional 24 hr. Carbohydrates were extracted and quantified by gas–liquid chromatography. Error bars indicate standard deviation from three repeats of the experiment.

(A) Major storage carbohydrates accumulating in *M. grisea* mycelium during growth in isoosmotic conditions (CM). 1, mannitol; 2, trehalose; 3, glycerol; 4, arabitol.

(B) Major storage carbohydrates accumulating in *M. grisea* mycelium after acute hyperosmotic stress (CM + 0.4 M NaCl). *M. grisea* accumulates mannitol and trehalose as its main storage carbohydrates during normal growth and development. Arabitol accumulates as the principal compatible solute during hyperosmotic stress.

between 1 and 5 M glycerol. When the external glycerol concentration exceeds the intracellular glycerol concentration, appressoria collapse (de Jong et al., 1997), allowing an estimate of appressorial turgor to be made (Figure 1C). A concentration of 3.2 M glycerol was sufficient to collapse 50% of the mature appressoria, indicating that the mean glycerol concentration within appressoria (allowing for the presence of other cellular constituents) was within the 2 to 4 M range.

Identification of the *OSM1* Gene Encoding a MAPK from *M. grisea*

A candidate gene encoding the stress-activated *M. grisea* Hog1/p38 MAPK was isolated by polymerase chain reaction (PCR) amplification with primers designed from conserved regions of the *S. cerevisiae* amino acid sequence (see Methods). The resulting PCR product was then used to probe a *M. grisea* genomic library and a cDNA library derived from *M. grisea* conidia. A genomic clone was isolated, and two genomic subclones were sequenced. Sequencing of 4 kb of DNA identified an open reading frame of 1071 nucleotides putatively encoding a 357–amino acid protein, as shown in Figure 2. The open reading frame was divided by nine potential introns, confirmed by sequencing a full-length cDNA clone (data not shown). The predicted amino acid sequence showed 80.3% identity and 89.3% similarity to *S. cerevisiae* Hog1 and 83.9% identity and 93.4% similarity to the Sty1 MAPK from *Schizosaccharomyces pombe* (Figure 2). The gene was named *OSM1*.

To test whether *OSM1* could function in the yeast HOG pathway, we subcloned a full-length cDNA corresponding to *OSM1* as a 1.17-kb XbaI fragment into the yeast expression vector pYES1 for expression under the *S. cerevisiae GAL1* promoter in the *hog1Δ* mutant JBY10 (Brewster et al., 1993). Osmotic sensitivity of the resulting transformants was determined by streaking yeast cells onto galactose-containing agar medium supplemented with 0.4 M NaCl. Transformants carrying the pYES1:OSM1 construct complemented the osmotic sensitivity of *hog1Δ* (Figure 3A), although we found that the osmotolerance of these transformants under more severe stress was not as high as the (nonisogenic) wild-type *S. cerevisiae* strain tested (Figure 3B). Deletion of *HOG1* in *S. cerevisiae* is known to result in morphological aberrations

1, mannitol; 2, trehalose; 3, glycerol; 4, arabitol.

(C) Cytorrhysis (cell collapse) assay to determine the concentration of glycerol present in appressoria during maturation. Appressoria were allowed to form for 48 hr on the hydrophobic side of Gelbond membranes. Glycerol was added to a final concentration of 1 to 5 M. The number of appressoria is proportional to the mean concentration (conc.) of glycerol within the cells.
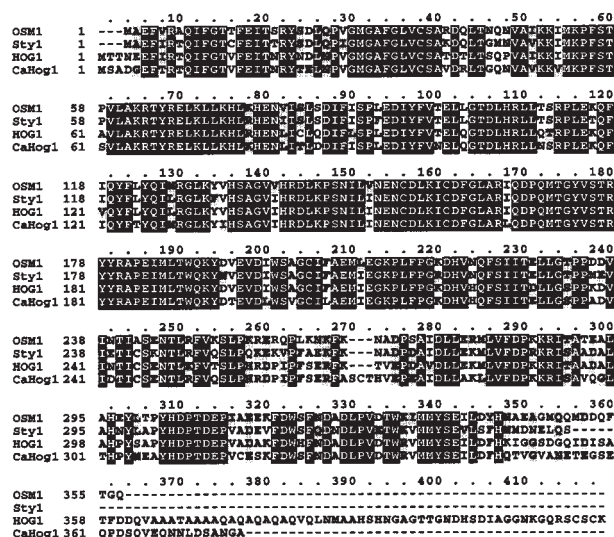
Figure 2. Predicted Amino Acid Sequence of the *M. grisea OSM1* Gene.

Amino acid sequence alignment of the predicted *M. grisea* OSM1 MAPK gene product with *S. pombe* Sty1, *S. cerevisiae* Hog1, and *Candida albicans* CaHog1. Sequences were aligned with the CLUSTALW program (Thompson et al., 1994). Identical amino acids are highlighted on a black background, conserved amino acids are shown on a dark gray background, and similar amino acids are shown on a light gray background. Dashes indicate gaps in the alignments. OSM1 contains the conserved TGY activation loop at positions 174 to 176 found in the stress-activated protein kinase subgroup of MAPKs (Robinson and Cobb, 1997). OSM1 has Gen-Bank accession number AF184980.

when cells are grown under osmotic stress (Brewster and Gustin, 1994). Introduction of *OSM1* complemented this phenotype, and cells shaped like the wild type were observed after exposure to 0.4 M NaCl (Figures 3C). We conclude that *OSM1* is a homolog of *HOG1* and encodes a functional MAPK.

Inactivation of *OSM1* Leads to Osmotic Sensitivity

A *Δosm1* deletion mutant was generated using a one-step gene replacement, as shown in Figures 4A to 4C. A gene replacement vector (pΔOSM1) was constructed to delete 1.1 kb of the *OSM1* coding region and to replace it with the hygromycin B phosphotransferase (*HPH*) gene cassette (Carroll et al., 1994). The region deleted includes the threonine and tyrosine residues (Thr-174 and Tyr-176) within the activation loop of the conserved kinase domain that is known to mediate MAPK activity (Johnson et al., 1996; Robinson and Cobb, 1997). Due to the likelihood that the gene replacement would lead to osmotic sensitivity, the *M.*

grisea transformation protocol, which depends on regeneration of protoplasts on osmotically stabilized media, was altered. Protoplasts were regenerated in osmotically stabilized liquid medium for 6 hr before being plated directly to nonstabilized growth medium (see Methods). Transformants were selected on hygromycin B–containing medium. The
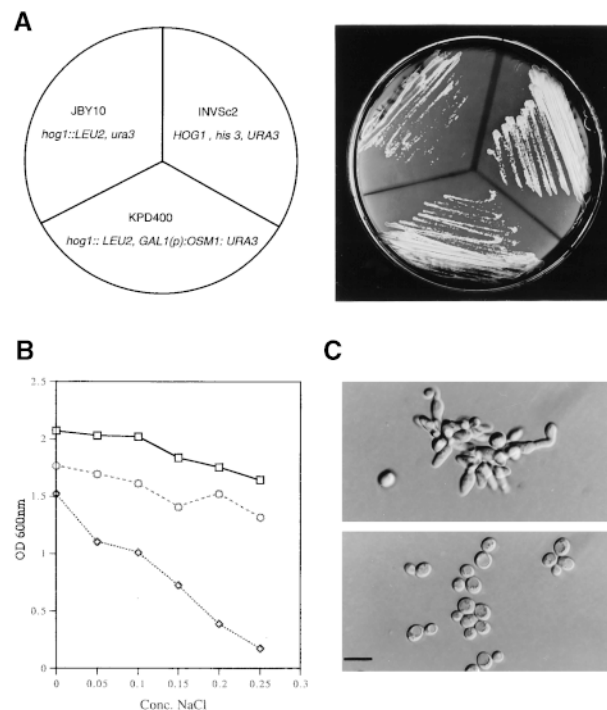

Figure 3. The *M. grisea OSM1* Gene Is a Functional Homolog of the *S. cerevisiae* Osmosensory MAPK-Encoding Gene *HOG1*.

(A) Growth of *S. cerevisiae* strains on a galactose-supplemented minimal medium agar plate in the presence of 0.4 M NaCl. JBY10 is an osmotically sensitive *hog1Δ* strain. KPD400 is a transformant of JBY10 expressing the *M. grisea OSM1* gene under the control of the *S. cerevisiae GAL1* promoter. INVSc2 is a nonisogenic wild-type *S. cerevisiae* strain.
(B) Growth of the same *S. cerevisiae* strains in galactose-supplemented minimal media in the presence of a range of NaCl concentrations. Growth was determined spectrophotometrically by recording the OD_{600nm}. Square data points are INVSc2, circles are KPD400, and diamonds are JBY10. Expression of *M. grisea OSM1* restored the ability of *S. cerevisiae hog1Δ* mutants to grow in conditions of hyperosmotic stress, although not to levels observed by a wild-type (nonisogenic) strain. Each data point represents the mean of three replicate samples. Error bars representing the standard deviation did not exceed the size of the symbols used to plot the data.
(C) Restoration of wild-type morphology of the *S. cerevisiae hog1Δ* mutant JBY10 by expression of *M. grisea OSM1*. JBY10 cells (top) after exposure to 0.2 M NaCl were typically polarized with misshapen budding. Below, the wild-type morphology of KPD400 cells is shown under the same conditions. Bar = 10 μm.
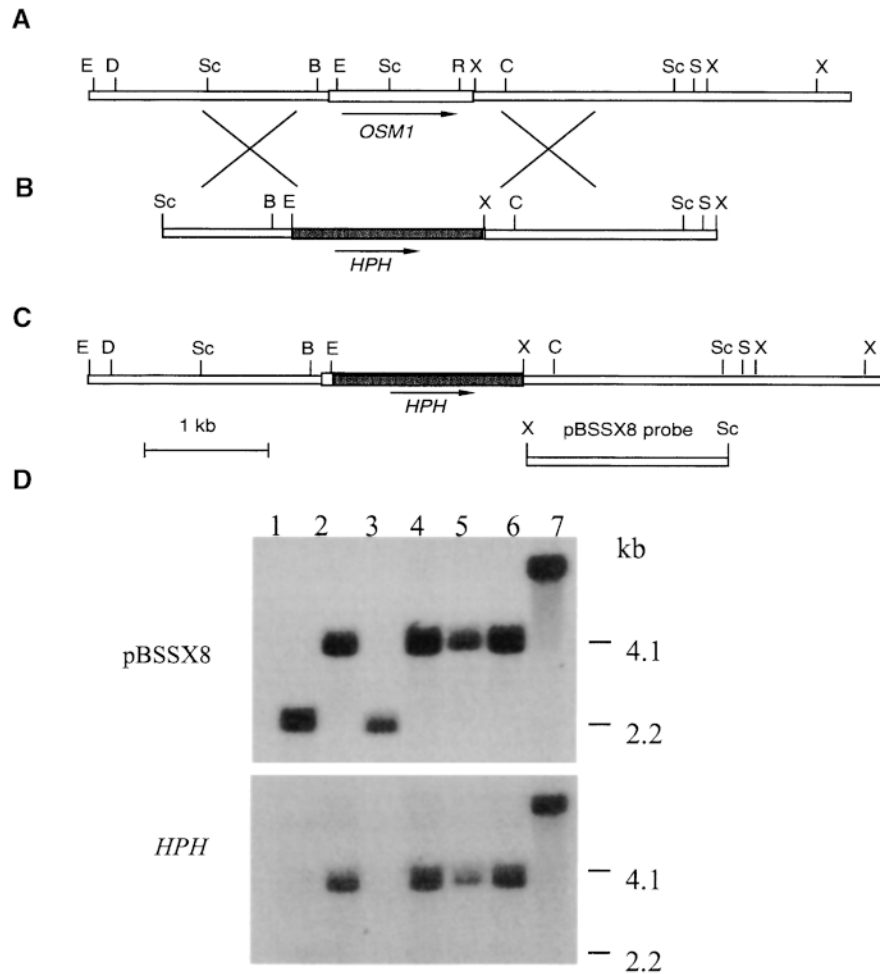
Figure 4. Targeted Replacement of the *M. grisea OSM1* MAPK Gene.

(A) Restriction map of the *OSM1* locus. The arrow shows the orientation of the *OSM1* open reading frame.

(B) Gene replacement vector pΔOSM1.

(C) Restriction map of the *Δosm1* deletion allele. The arrow indicates the orientation of the *HPH* open reading frame.

(D) DNA gel blot analysis of pΔOSM1 transformants. Genomic DNA was prepared from the wild-type strain Guy11 (lane 1), *Δosm1* deletion mutants JH73, JH33, JH28, and JH22 (lanes 2 and 4 to 6), an ectopic integration transformant JH8 (lane 7), and nontransformed strain JH69 (lane 3). Genomic DNA was digested with SacI and separated in a 1% agarose gel. The blot was probed first with pBSSX8, which contains the 3′ end of OSM1, and then with the *HPH* gene cassette (Carroll et al., 1994).
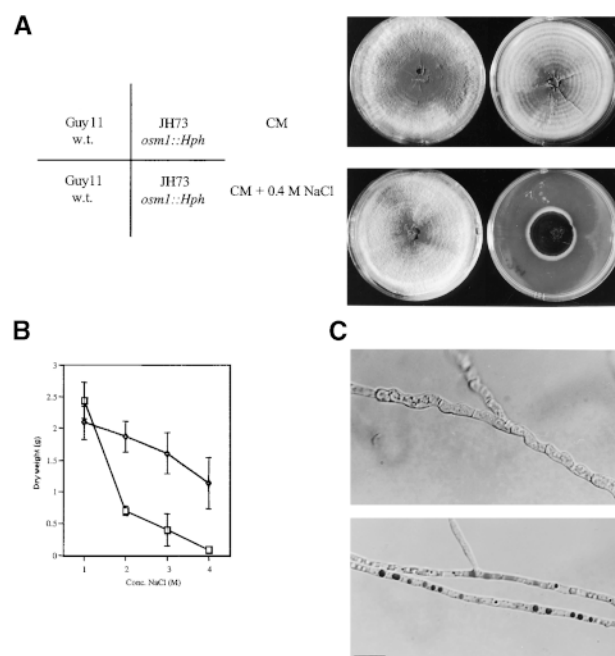
The Xs between **(A)** and **(B)** indicate a crossover event. B, BamHI; C, ClaI; D, HindIII; E, EcoRI; R, EcoRV; S, SalI; Sc, SacI; X, XhoI.

presence of the *Δosm1* deletion allele was verified by DNA gel blotting. Hybridization analysis with probe pBSSX8 showed that the isogenic wild-type strain Guy11 and one of the putative transformants, JH69, contained a 2.2-kb SacI fragment containing the 3′ end of *OSM1*. Four of the transformants, JH73, JH33, JH28, and JH22, contained a larger hybridizing 4.1-kb Sac I fragment, which is indicative of a gene replacement (Figure 4D). Consistent with this, the same fragment hybridized with an *HPH* probe. One of the transformants selected showed an ~10-kb hybridizing fragment, indicating ectopic insertion of the gene replacement vector.

To ensure that any phenotypes attributed to the *osm1⁻* mutation were due to the gene replacement event, we crossed JH73 to an *M. grisea* strain of opposite mating type, TH3. The resulting random ascospore progeny were subjected to DNA gel blot analysis to determine whether *OSM1* was present. All hygromycin-resistant progeny were found also to carry the *Δosm1* null mutation (data not shown).

Inactivation of *OSM1* did not cause any measurable defect in the growth and development of *Δosm1* mutants in standard growth medium, although a reduction in conidiogenesis was consistently observed in *Δosm1* mutants.

Conidial numbers were ~10-fold lower in plate cultures of $\Delta osm1$ mutants (mean = 4.1×10^5 mL^{-1}) than those of the isogenic strain Guy11 (3.8×10^6 mL^{-1}). The $\Delta osm1$ deletion mutant showed dramatically reduced growth compared with the Guy11 strain after exposure to hyperosmotic stress, as shown in Figure 5A. The response to chronic hyperosmotic stress was determined based on growth in the presence of 0.4 M NaCl. The reduction in growth was found to be pro-

portional to the external concentration of solute when analyzed by determination of dry weight (Figure 5B). Similar reductions in growth were observed when the $\Delta osm1$ mutant was subject to chronic hyperosmotic stress by exposure to concentrations of sorbitol or KCl generating identical osmotic potentials (data not shown). A pronounced morphological difference was also observed when the $\Delta osm1$ mutants were exposed to hyperosmotic stress. Hyphae became wider and took on a pseudohyphal, budding appearance (Figure 5C). Hyphae were also conspicuously less vacuolated than were those of the Guy11 strain under these conditions (Figure 5C).
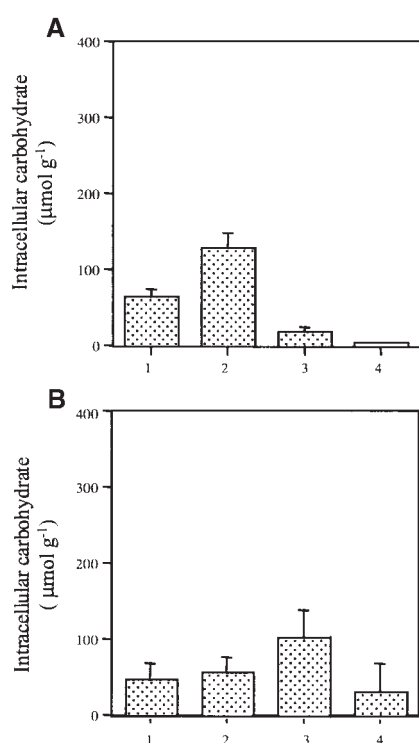
Accumulation of the Compatible Solute Arabitol Is Controlled by *OSM1*

To determine the effect of the $\Delta osm1$ mutation on the cellular response to hyperosmotic stress, we made mycelial extracts from JH73 cultures under isoosmotic and hyperosmotic conditions. Under normal conditions, the $\Delta osm1$ mutant accumulated trehalose normally, although amounts of the other major storage carbohydrate mannitol were depleted (Figure 6A). During hyperosmotic stress, arabitol did not accumulate to the high levels observed in the Guy11 strain (Figures 1, 6A, and 6B). In contrast, smaller amounts of glycerol accumulated in both $\Delta osm1$ and Guy11 mycelium in response to hyperosmotic stress. These results indicate that accumulation of the compatible solute arabitol is regulated by *OSM1* and that the $\Delta osm1$ mutation may have further pleiotropic effects on polyol metabolism in *M. grisea*.

M. grisea $\Delta osm1$ Mutants Produce Functional Appressoria

The role of *OSM1* in appressorium turgor generation was determined by estimation of internal turgor using cytorrhysis. The wild-type *M. grisea* strain Guy11 and the isogenic $\Delta osm1$ mutant JH73 both required >3 M glycerol to collapse 50% of the developed appressoria, suggesting a mean internal glycerol concentration of between 2 and 4 M, as shown in Figures 1C and 7A. To confirm this result, we made appressorium extracts and assayed glycerol quantitatively by using an enzymatic assay. Glycerol was found in nearly identical quantities in both Guy11 and JH73 (mean = 3.13 ± 1.1 M after 48 hr). Appressorium-mediated penetration was determined by using an onion epidermis penetration assay (Chida and Sisler, 1987) and by checking the pathogenicity of *M. grisea* strains on a susceptible rice cultivar, CO39. Appressorium-mediated penetration and the ability to cause rice blast disease symptoms were unaffected in the $\Delta osm1$ gene replacement mutant, as shown in Figure 7B. We conclude that *OSM1* is not required for appressorium turgor generation by *M. grisea*.

The fact that deletion of *OSM1* did not affect appresso-

Figure 5. Osmotic Sensitivity and Aberrant Hyphal Morphology of *M. grisea* $\Delta osm1$ Deletion Mutants.

(A) *M. grisea* strain Guy11 and isogenic $\Delta osm1$ deletion mutant JH73 on CM agar and CM agar supplemented with 0.4 M NaCl. Plates were inoculated with a 5-mm^2 plug of mycelium and allowed to grow for 12 days at 24°C. Strains on each plate are given in the panel on the left.
(B) Growth of the wild-type *M. grisea* strain Guy11 and isogenic $\Delta osm1$ deletion mutant JH73 in CM broth supplemented with varying concentrations of NaCl. Liquid cultures were prepared in 20 mL of CM with a uniform inoculum of blended mycelia and allowed to grow for 4 days with aeration. The mycelia were removed by filtration, and dry weights were determined. The data points represent the means of three replicates. Square data points are measurements of the growth of JH73, and diamonds are that of Guy11. Error values represent the standard deviation. The experiment was repeated with very similar results.
(C) Hyphal morphology of $\Delta osm1$ deletion mutant JH73 (top panel) and the iosgenic wild-type strain Guy11 after exposure to 0.4 M NaCl. Hyphae appeared bulbous and budded. The hyphae were stained with neutral red to reveal vacuoles and viewed under Hoffman modulation contrast. Guy11 showed increased vacuolation under hyperosmotic stress. This response was not observed in the $\Delta osm1$ deletion mutant JH73. Bar = 10 μm.

Figure 6. Compatible Solute Production by the *Δosm1* Mutant JH73 during Hyperosmotic Stress.

Mycelium was grown for 48 hr in CM broth before being transferred to CM or CM plus 0.4 M NaCl broth for a further 24 hr. Carbohydrates were extracted and quantified by gas–liquid chromatography. Error bars indicate standard deviation from three repeats of the experiment.
(A) Major storage carbohydrates accumulating in *M. grisea Δosm1* mycelium during growth in isoosmotic conditions (CM). 1, mannitol; 2, trehalose; 3, glycerol; 4, arabitol.
(B) Major storage carbohydrates accumulating in *M. grisea Δosm1* mycelium after acute hyperosmotic stress (CM broth plus 0.4 M NaCl). The *Δosm1* mutation affects the ability to accumulate arabitol in response to hyperosmotic stress. Levels of mannitol stored during normal growth and development are also affected. 1, mannitol; 2, trehalose; 3, glycerol; 4, arabitol.

rium function suggested that the *M. grisea* HOG pathway does not regulate turgor generation within infection cells. One possibility, however, is that the pathway does not operate within differentiated appressoria but only in hyphae exposed to hyperosmotic stress. Therefore, we decided to investigate the response of appressoria to hyperosmotic stress to determine whether the *OSM1*-mediated hyperosmotic signal transduction pathway is capable of functioning at the same time as appressorium turgor generation. Appressoria were allowed to form in water drops on the hydrophobic side of Gelbond for 24 hr and then subjected to acute osmotic stress by addition of 0.4 M NaCl. The appressorial contents were then extracted, and internal polyol con-

centrations were analyzed by gas–liquid chromatography. In unstressed wild-type appressoria, glycerol accumulated as expected (de Jong et al., 1997), but in appressoria exposed to acute stress with 0.4 M NaCl, trace amounts of arabitol were also present (Figure 8B). In contrast, the mutant did not accumulate arabitol in its appressoria after acute osmotic stress (Figure 8A). We conclude that the *OSM1*-mediated MAPK signaling pathway acts independently of the signal transduction pathway leading to appressorium turgor.

Inactivation of *OSM1* Has Pleiotropic Effects on Appressorium Morphogenesis

Although *OSM1* is not required for appressorium function, deletion of *OSM1* does have pronounced effects on appressorium development under conditions of chronic osmotic stress (Figures 9A to 9C). Conidia were germinated in the presence of 0.2 or 0.4 M NaCl and allowed to develop appressoria on the hydrophobic surface of Gelbond. Appressoria formed normally in the Guy11 strain. However, in *Δosm1* mutants, multiple appressoria were produced from the ends of germ tubes. This response was proportional to the external osmotic potential, and as many as 24 ± 11% of the conidia produced compound appressoria after developing in the presence of 0.4 M NaCl. The presence of enhanced appressorium development during hyperosmotic stress in the absence of *OSM1* may indicate that one function of the MAPK encoded by *OSM1* is to negatively regulate appressorium development during severe osmotic stress. This suggests that the *OSM1*-mediated hyperosmotic signaling pathway may communicate with the known signal transduction cascade(s) required for appressorium morphogenesis in *M. grisea*.

DISCUSSION

This study sought to characterize the control of cellular turgor in *M. grisea*. We found that two distinct polyols form the major compatible solutes during turgor generation either in response to hyperosmotic stress or during plant infection. The enormous turgor reported in appressoria of *M. grisea* apparently requires the highly soluble polyol glycerol, which can accumulate to the very high concentrations required without precipitation or toxicity (de Jong et al., 1997). In contrast, the five-carbon polyol arabitol accumulates in hyphae of *M. grisea* in addition to glycerol when they are exposed to chronic or acute hyperosmotic stress. Arabitol biosynthesis has not been studied extensively, although an NAD-dependent arabitol dehydrogenase–encoding gene has been isolated from the yeast *Candida albicans* (Wong et al., 1993), suggesting one possible route for its accumulation in *M. grisea*. Hyperosmotic stress tolerance is likely to be an important process during the life cycle of *M. grisea*,

A

B

Guy11   JH73
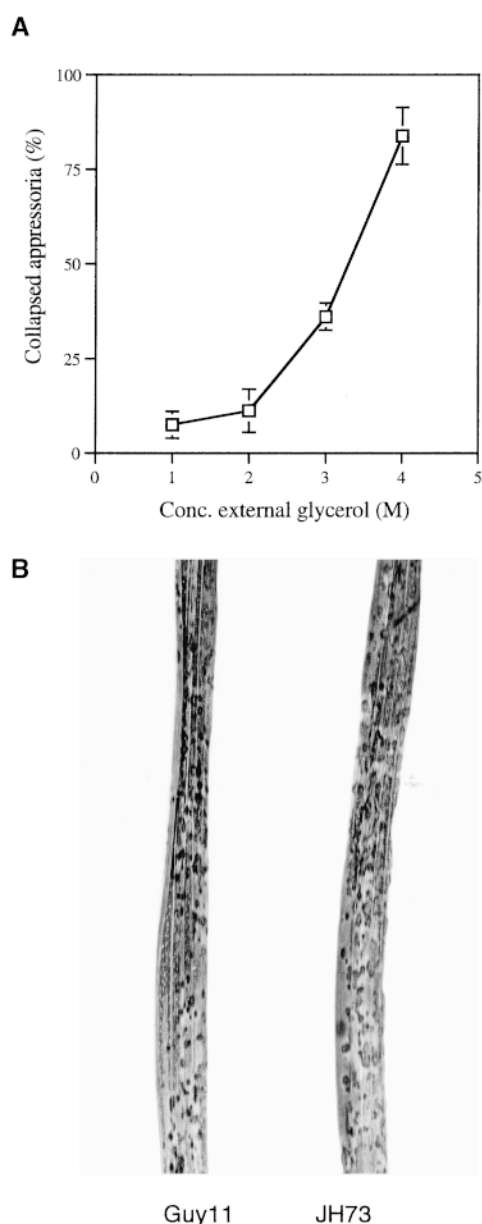
Figure 7. *M. grisea* Δ*osm1* Deletion Mutants Produce Appressoria with High Cellular Turgor.

(A) Appressorium turgor was determined using a cytorrhysis assay (Howard et al., 1991; de Jong et al., 1997). Appressoria were allowed to form on hydrophobic plastic cover slips for 50 hr and then incubated in a range of glycerol concentrations (conc.). The number of appressoria from the Δ*osm1* mutant JH73 collapsing after this treatment was recorded. In this experiment, 3 M glycerol caused ~35% of JH73 appressoria to collapse, suggesting a mean internal glycerol concentration of 2 to 4 M. Turgor was not significantly different from the wild-type *M. grisea* strain Guy11. Error bars indicate standard deviation from three repeats of the experiment.

(B) Rice blast symptoms caused by inoculation of the susceptible rice cultivar CO39 with Guy11 and the Δ*osm1* mutant JH73. Lesion density values were not significantly different.

and high levels of both glycerol and arabitol have previously been reported in blast-infected rice leaves (Hwang et al., 1989).

To determine whether celluar turgor is controlled in *M. grisea* via the conserved eukaryotic signaling pathway, we isolated and characterized the *OSM1* gene, which is functionally related to *S. cerevisiae HOG1*. Recent evidence has shown that fungal pathogens have adapted several conserved signaling pathways for regulating pathogenicity-related functions such as appressorium development or plant tissue colonization, including MAPK elements of the pheromone response pathway of *S. cerevisiae*, components of the Ras-adenylate cyclase pathway, and heterotrimeric G proteins (Gold et al., 1994; Mitchell and Dean, 1995; Banuett and Herskowitz, 1996; Gao and Nuss, 1996; Xu and Hamer, 1996; Alspaugh et al., 1997; Beckerman et al., 1997; Choi and Dean, 1997; Liu and Dean, 1997; Xu et al., 1997). Identification of *OSM1*, and its subsequent targeted deletion, revealed that *M. grisea* possesses a MAPK-mediated hyperosmotic stress pathway.

The OSM1 MAPK shows very high similarity to the stress-activated MAPKs Hog1 from *S. cerevisiae* and Sty1 from *S. pombe*. The similarity extends throughout the catalytic domains of the proteins, and the conservation of an activation loop within the kinase domain (the TGY motif at residues 174 to 176) indicates that OSM1 is activated via threonine and tyrosine phosphorylation by a MAPKK homologous to Pbs2 from *S. cerevisiae* or Wis1 from *S. pombe* (Herskowitz, 1995; Robinson and Cobb, 1997). Consistent with the high degree of conservation, *OSM1* was able to complement the osmotic sensitivity and morphological aberrations of a *hog1Δ* mutant of *S. cerevisiae*. The morphological defects of *S. cerevisiae hog1Δ* mutants during osmotic stress are due to the role of Hog1 in regulating reorganization of the cytoskeleton during exposure to high solute concentrations (Brewster and Gustin, 1994). The reduced polarization and budding morphology of Δ*osm1* null mutants after exposure to hyperosmotic stress indicate that *OSM1* probably performs this function in *M. grisea* hyphae as well. Increased vacuolation of *M. grisea* hyphae during hyperosmotic stress was also observed and found to be *OSM1* dependent. Formation of vacuoles is likely to be a consequence of compatible solute generation and adjustment of cellular turgor.

The HOG pathway in budding yeast appears to act specifically to regulate the cellular response to hyperosmotic stress, whereas in fission yeast, the same pathway, which is mediated by the Sty1 MAPK, controls the response to a wider selection of environmental stresses, including oxidative stress, ultraviolet light exposure, and heat shock. To determine whether the OSM1 pathway in *M. grisea* resembles either of these distinct situations, we exposed conidia from *M. grisea* Δ*osm1* mutants to UV light, oxidative stress (exposure to 10 mM H_2O_2), or heat shock. Conidial viability of Δ*osm1* mutants was reduced compared with the isogenic wild type after UV exposure but was not affected by the other stresses (data not shown). This indicates that the

A

B
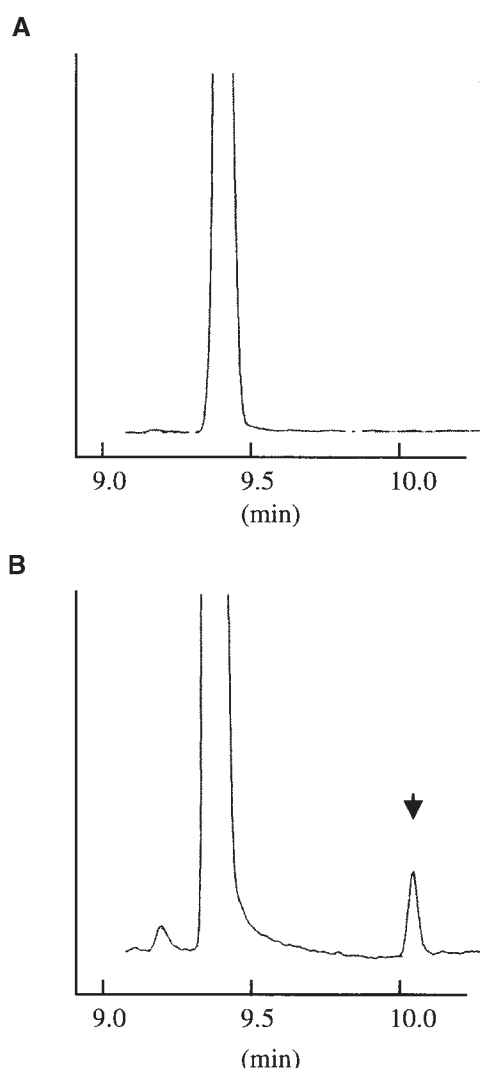
Figure 8. Compatible Solute Production in *M. grisea* Appressoria after Exposure to Acute Hyperosmotic Stress.

Appressoria were allowed to form on the hydrophobic surface of Gelbond for 24 hr and then subjected to acute hyperosmotic stress by the addition of 0.4 M NaCl. Appressorial extracts were prepared and analyzed by gas–liquid chromatography. An enlarged section of the gas–liquid chromatography trace from this experiment is shown with retention times in minutes. Arabitol had a retention time of 10.04 min in this experiment (arrow). The large peak shown at 9.4 min represents the internal standard, ribose.
(A) Gas–liquid chromatography trace from appressorial extract of the *Δosm1* mutant JH73 exposed to hyperosmotic stress.
(B) Gas–liquid chromatography trace from appressorial extract of Guy11 exposed to hyperosmotic stress.

OSM1 signaling cascade may respond to stresses other than osmotic shock, resembling, at least in part, the Sty1/Wis1 pathway in fission yeast (Millar et al., 1995; Gustin et al., 1998).

Regulation of Appressorium-Mediated Infection in *M. grisea*

We tested whether *OSM1* was required for appressorium function and found (to our surprise) that *M. grisea* appressoria can operate effectively in the absence of this MAPK. This indicates that the massive increase in cellular turgor that occurs in *M. grisea* appressoria during plant infection is not controlled using the conserved mechanism for modulation of cellular turgor. We were aware, however, that hyperosmotic stress experiments were all done by analyzing the response of fungal hyphae; therefore, there was a possibility that the OSM1 pathway cannot operate effectively in appressoria. We therefore exposed preformed appressoria to hyperosmotic stress and found that arabitol began to accumulate in an *OSM1*-dependent manner. This shows that the OSM1 pathway is still able to operate within appressoria and must therefore be independent of the pathway mediating turgor generation. Although we cannot preclude the possibility that there are shared components in both signaling cascades, the fact that osmotically stressed appressoria generate arabitol and glycerol points to both pathways being fully active at the same time.

OSM1 is the third MAPK-encoding gene identified in *M. grisea*, and all three MAPKs play a role in infection-related development. *PMK1* is a functional homolog of the *S. cerevisiae* MAPK-encoding gene *FUS3* from the pheromone response pathway (Xu and Hamer, 1996). *M. grisea PMK1* regulates the formation of appressoria, probably acting downstream of a cAMP-dependent pathway involving a heterotrimeric G-protein encoded by *MAGB* (Lee and Dean, 1993; Liu and Dean, 1997) and adenylate cyclase encoded by *MAC1* (Choi and Dean 1997; Adachi and Hamer, 1998). *Δpmk1* mutants are unable to elaborate appressoria and are also blocked in all subsequent stages of pathogenic development because mutants fail to colonize plant tissue even when conidia are injected directly into leaves (Xu and Hamer, 1996). The MPS1 MAPK is required for appressorium function in *M. grisea*. Here its function appears to be in regulating cell wall remodeling and polarity establishment for penetration peg emergence (Xu et al., 1998). *MPS1* is a functional homolog of the MAPK-encoding gene *SLT2*, which is required for cell wall integrity under low osmotic conditions in budding yeast. *Δmps1* mutants fail to penetrate plant cuticles, although they still generate turgor pressure and activate plant defense responses (Xu et al., 1998).

OSM1 does not regulate appressorium turgor, but it does appear to play a role in appressorium morphogenesis. Chronic osmotic stress induced a multiple appressorium phenotype in *Δosm1* mutants, which indicates that one

A

[NaCl] (M) surrounding germinating conidia

B

C
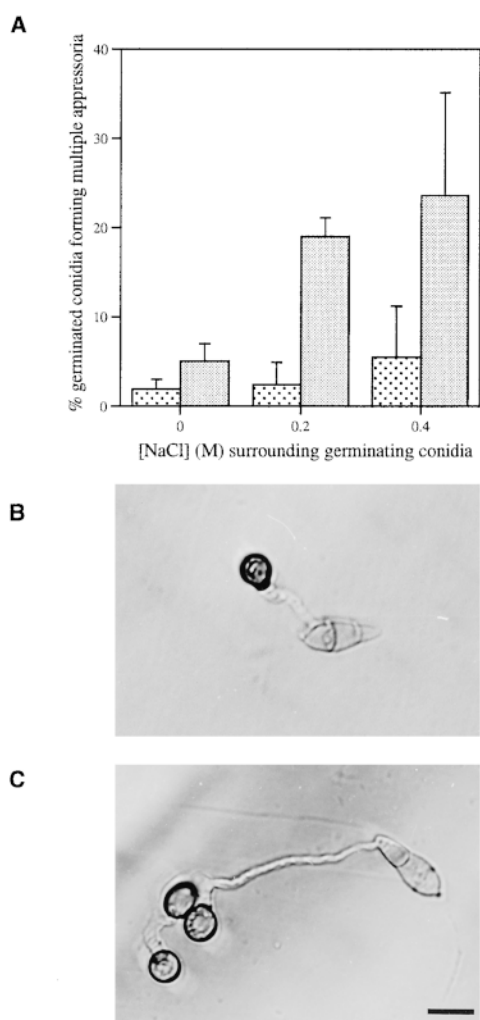
Figure 9. Development of Multiple Appressoria by the *M. grisea* Δ*osm1* Mutant JH73 during Chronic Hyperosmotic Stress.

M. grisea conidia of Guy11 and the Δ*osm1* mutant JH73 were allowed to germinate on hydrophobic Gelbond membranes in the presence of increasing concentrations of NaCl.
(A) The number of conidia elaborating more than one appressorium was recorded. Each data point represents the mean percentage from observing 200 conidia. Error values are the standard deviation.
**(B)** Normal infection structure in *M. grisea*. A conidium of Guy11 has elaborated a single appressorium from a short germ tube.
(C) Multiple appressorium formation by the Δ*osm1* mutant JH73 in the presence of 0.4 M NaCl. Bar = 10 μm.

function for *OSM1* might be to prevent cross-talk between the hyperosmotic stress pathway and the *PMK1* appressorium morphogenesis pathway. In *S. cerevisiae*, for example, Hog1p prevents miscommunication between the HOG pathway and pheromone response pathway to ensure that mat-

ing does not occur during times of hyperosmotic stress (O'Rourke and Herskowitz, 1998). Mutations in the *HOG1* gene therefore allow osmolarity-induced activation of the pheromone response pathway in *S. cerevisiae* (Hall et al., 1996). In *M. grisea*, the PMK1 pathway, which controls appressorium development, is functionally related to the pheromone response pathway from *S. cerevisiae*. It may be worth speculating, therefore, that *OSM1* normally acts to prevent inappropriate activation of this pathway during hyperosmotic stress (appressorium development is severely impeded in the presence of >0.5 M NaCl). In the absence of *OSM1*, the PMK1 pathway may be overstimulated in the presence of osmotic stress, thereby resulting in multiple rounds of appressorium formation. A summary of the MAPK signaling pathways of *M. grisea* is given in Figure 10.

Identification of *OSM1* has shown that the action of *M. grisea* appressoria is likely to be regulated in a novel manner, distinct from the mechanism that underlies turgor generation in most eukaryotic cells. It is clear from many recent studies that fungal phytopathogens have coopted and adapted several regulatory pathways that condition mating, nutrition, or the response to environmental stress in budding yeast and used them to regulate pathogenicity functions, including infection cell development and plant tissue invasion
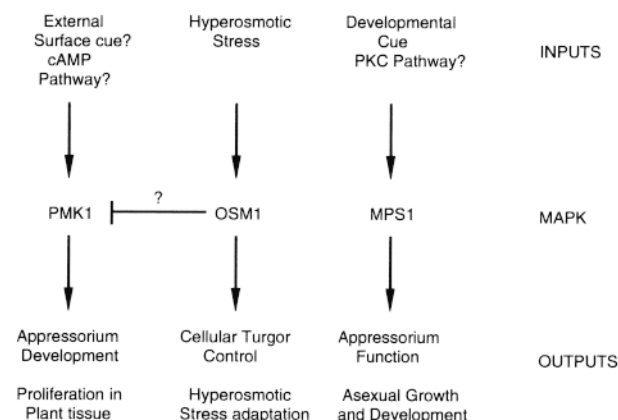
Figure 10. Model Showing the MAPK Signaling Pathways in the Rice Blast Fungus *M. grisea*.

Three genes encoding MAPKs have been identified and characterized in *M. grisea*. *PMK1* regulates appressorium development and subsequent proliferation of the fungus in plant tissue. PMK1 may operate downstream of (or parallel to) the cAMP signaling pathway, which regulates appressorium formation (Xu and Hamer, 1996). *MPS1* regulates appressorium-mediated infection, potentially by controlling formation of the penetration peg. By analogy to *S. cerevisiae*, MPS1 may act downstream of a protein kinase C (PKC) pathway (Xu et al., 1998). *OSM1* regulates hyperosmotic stress adaptation and clearly acts in response to that stress. *OSM1* may also (?) prevent the *PMK1* signaling pathway from operating during stressful environmental conditions.

(Banuett, 1998). This study shows that in addition to these regulatory pathways, novel signaling networks may well have evolved specifically to regulate plant infection.

METHODS

Fungal Isolates

Strains of *Magnaporthe grisea* from this study are stored in the laboratory of N.J. Talbot. Strain TH3 was kindly donated by Dr. J.L. Notteghem (Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Montpellier, France). Standard growth procedures were used for *M. grisea* (Crawford et al., 1986), with oatmeal and complete medium (Talbot et al., 1993). Long-term storage of *M. grisea* was performed by growing the fungus through sterile filter paper discs, desiccating these for 48 hr, and then storing them under desiccation at $-20°C$.

Genetic Crosses

Genetic crosses were performed as described by Valent et al. (1991). Briefly, strains of opposite mating type were paired on oatmeal agar and grown until the mycelia joined. Approximately 14 days after inoculation, flask-shaped perithecia were visible at the mycelial margins. These were transferred to a 4% distilled water agar plate with the use of fine forceps. The perithecia were rubbed gently to remove adhering mycelium and conidia before being broken open to reveal asci. Mature asci were removed with a sterile fine needle, and ascospores were dissected from them. These ascospores were transferred individually to a 24-cell well plate (Sarstedt, Nümbrecht, Germany) containing complete medium and incubated for 4 to 5 days (Talbot et al., 1993). At this time, monoconidial isolations were made from each well and grown in individual plates for storage.

Rice Infections

Fourteen-day-old rice seedlings were infected with suspensions of *M. grisea* conidia prepared in 0.2% gelatin at a concentration of 10^4 conidia mL^{-1}. Two-week-old seedlings of the susceptible rice cultivar CO39 were sprayed using an artist's airbrush (Badger Co., Franklin Park, IL). Plants were incubated in plastic bags for 48 hr to maintain high humidity and then transferred to controlled environment chambers at 24°C, 84% relative humidity, with 900 μE m^{-2} sec^{-1} tungsten illumination and a 14-hr day length. Plants were incubated for 96 to 144 hr for full disease symptoms to become apparent. The first disease symptoms were observed 96 hr after seedling inoculation. Lesion densities were routinely scored from 40 randomly chosen 5-cm-long leaf tips, and means and standard deviations were determined.

Assays for Infection-Related Morphogenesis

Appressorium development by *M. grisea* was observed on plastic cover slips (Fisher Scientific, Loughborough, UK) or the hydrophobic side of Gelbond membranes (FMC, Chicago, IL), as described by Hamer et al. (1988). A 100-μL drop of a conidial suspension at a concentration of 10^5 mL^{-1} was placed on the surface of a cover slip and left in a humid environment at 24°C. The frequency and morphology of appressorium formation were determined by counting the number of appressoria that had developed from 300 conidia after 14 hr (Talbot et al., 1993). Hyphal staining of *M. grisea* to determine the extent of vacuolation was performed by staining with Neutral Red solution containing 0.15% (w/v) neutral red (BDH, Poole, Dorset, UK) and 3.75% (w/v) PVP (Sigma) in 50 mM Tris-maleate buffer, pH 7.5. Hyphae and appressoria were routinely viewed with an Optiphot-2 microscope (Nikon, Kingston, UK) under Hoffman modulation contrast.

DNA Isolation and Analysis

Genomic DNA was extracted from fungal mycelium by using a CTAB (hexadecyltrimethylammonium bromide) procedure described by Talbot et al. (1993). Gel electrophoresis, restriction enzyme digestion, and DNA gel blot hybridizations were all performed using standard procedures (Sambrook et al., 1989). DNA hybridization probes were labeled by the random primer method (Feinberg and Vogelstein, 1983), using the Stratagene Prime-It kit, and high-stringency washes were performed as previously described (Talbot et al., 1993). DNA sequencing was performed using an ABI 377 automated sequencer (Perkin-Elmer) and dye terminator cycle sequencing Ready Reaction kit (Amersham Pharmacia Biotechnology, Amersham, UK) with Ampli Taq DNA Polymerase FS (Amersham Pharmacia Biotechnology), according to the manufacturer's instructions. DNA/protein sequence databases were searched using the BLAST algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (Bethesda, MD), which can be accessed via the World Wide Web.

Identification of the *OSM1* Mitogen-Activated Protein Kinase–Encoding Gene

OSM1 was cloned using polymerase chain reaction (PCR) with degenerate primers designed to conserved regions of mitogen-activated protein kinases (MAPKs). The gene was cloned by two independently working coauthors (K.P.D. and J.-R.X.). *OSM1* was obtained as a 400-bp sequence amplified by PCR with the degenerate primers KPD1 (5′-CTTGGATCCMGNGCNCCNGARATHATGYTN-3′) and KPD2 (5′-CTTGGATCCRTANGGRTGNGCNARNGC-3′), where R is purine; Y is pyrimidine; M is alanine or cytosine; H is thymine, cytosine, or alanine; and N is any nucleotide. Optimal conditions for the PCR reactions were as follows: 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C for 35 cycles with a 10-min extension at 72°C. All PCR reactions were performed in a Perkin-Elmer GeneAmp PCR System 2400 machine using 1 unit of *Tth* DNA polymerase (Promega) per reaction. The resulting PCR amplicons were gel purified with standard methods using a Qia-ex kit (Qiagen, Chatsworth, CA), according to the manufacturer's instructions, and cloned into Promega pGEM-T, and the plasmids were digested with BamHI to liberate the recombinant insert. *OSM1* was also isolated using primers MAK2, MAK4, and MEK3, described by Xu and Hamer (1996) and used previously to identify *PMK1* and *MPS1* from *M. grisea* (Xu and Hamer, 1996; Xu et al., 1998). PCR amplicons showing similarity to *S. cerevisiae HOG1* were used to screen a λZAPII (Stratagene) cDNA library from nitrogen-starved Guy11 mycelium (Xu and Hamer, 1996) and a Guy11 λGEM-11 genomic library (Talbot et al., 1993).

Complementation of *Saccharomyces cerevisiae hog1Δ*

An *S. cerevisiae hog1Δ* strain (JBY10:*MATa ura3 leu2 his3 ade2 lys2 try1 hog1-Δ*::*TRP1*) was kindly donated by M. Gustin (Rice University, Houston, TX). Plasmid pGAL1(p):OSM1, placing *OSM1* under control of the yeast *GAL1* promoter, was constructed by amplifying a 1168-bp fragment from a full-length cDNA clone (pH g110-3) containing the entire *OSM1* open reading frame, using the primers KD5HOG (5′-GCTCTAGATGAAACCTGCTTCCCGACGGT-3′) and KD3HOG (5′-GGTCTAGAGAATGAAATCATGAACGGAAA-3′), which also added XbaI sites to each end of the fragment. The amplicon was digested with XbaI and cloned into pYES2 (Invitrogen, Carlsbad, CA). Yeast cells of strain JBY10 (*hog1Δ*) were transformed using the lithium acetate method described by Gietz and Schiestl (1995). The resulting (Ura⁺) transformants were confirmed by colony PCR and dot blot hybridization with pH g110-3 (data not shown). *S. cerevisiae* Ura⁺ transformants were tested for osmosensitivity by growth on galactose-supplemented minimal media without uracil in the presence of 0.2 M NaCl (Brewster et al., 1993). For analysis of *S. cerevisiae* growth in liquid medium, precultures of the wild-type strain INVSc2, *hog1Δ* strain JBY10, and *GAL1p:OSM1* strain KPD400 were prepared by overnight incubation at 30°C in yeast extract–peptone–dextrose medium (Brewster et al., 1993). The titer of cells was equalized, and 100 μL of preculture was used to inoculate 10-mL shaking cultures in supplemented minimal media in the presence of galactose and a range of NaCl concentrations from 0.1 to 0.4 M. Cultures were incubated overnight at 30°C while being shaken at 220 rpm. The OD_{600nm} of the resulting cell suspensions was determined in a Pharmacia Ultrospec 2000 spectrophotometer.

Construction of the Gene Replacement Vector pΔOSM1

Plasmid pΔOSM1 was constructed by first cloning a 1.9-kb EcoRI fragment (5′ flanking region of *OSM1*) into pBCKS (Stratagene) to create pBHE12. This clone was digested with SacI and religated to create pBHS12, which carries a 1-kb SacI-EcoRI fragment (5′ flank of *OSM1*). A 1.8-kb XhoI fragment (the 3′ end of *OSM1* and downstream flanking sequence) was cloned into pBHS12, and the orientation of the insert was checked by SalI digestion. The correct clone was named pNX2. The hygromycin phosphotransferase gene cassette (*HPH*) (Carroll et al., 1994) was inserted into the unique EcoRI site to create pΔOSM1.

Fungal Transformation

Protoplast preparation and transformation were performed as described previously (Talbot et al., 1993). The transformation protocol for generation of the *Δosm1* gene replacement was adapted by incubating polyethylene glycol–treated transformed Guy11 protoplasts for 28 hr in liquid TB3 medium (3 g L^{-1} yeast extract, 3 g L^{-1} casamino acids, and 10 g L^{-1} glucose) osmotically stabilized with 0.8 M sucrose. The regenerating protoplasts were collected by centrifugation at 1000*g* for 6 min and resuspended in unmodified TB3. The protoplasts were then immediately plated onto nonosmotically stabilized complete medium (CM; Talbot et al., 1993) agar containing 150 μg mL^{-1} hygromycin B (Calbiochem), and an overlay of CM agar containing 250 μg mL^{-1} hygromycin B was added after 18 hr. Transformants were selected at 24°C after 6 to 10 days.

Identification of Compatible Solutes in *M. grisea*

M. grisea liquid cultures were prepared, and resulting mycelia were harvested via filtration through sterile Miracloth (Calbiochem). The mycelium was washed in isoosmotic concentrations of $CaCl_2$, frozen in liquid nitrogen, and freeze dried under vacuum for 3 to 5 days. The dry weight of these samples was then recorded, and carbohydrates were extracted using methylene dichloride. Briefly, lyophilized samples were ground using a pestle with a mortar containing 4 mL of MMW (methanol–methylene dichloride–double-distilled H_2O [v/v] 12:5:3). A further 6 mL of MMW was added gradually to the ground mycelium, and the suspension was decanted into 50-mL sterile Oakridge tubes (Nalgene, Rochester, NY). The suspension was incubated at 40°C for 30 min, with vortexing every 10 min. Samples were then processed by centrifugation for 30 min at 3000*g* at 4°C. The supernatant was decanted into sterile Oakridge tubes and stored on ice. The pellet was reextracted with 5 mL MMW and vortexed for 1 min followed by centrifugation at 3000*g*. The resulting supernatants were pooled, and 7.5 mL of sterile double-distilled H_2O and 7.5 mL of methylene dichloride were added. This solution was vortexed for ~1 min, and the resulting emulsion was centrifuged at 3000*g* for 30 min at 4°C. The aqueous phase was then carefully decanted into a sterile universal and frozen at −80°C overnight. Extracts were then lyophilized for 3 to 5 days. The lyophilized material was resuspended in 0.5 to 1 mL of sterile double-distilled H_2O and stored at −20°C.

For gas–liquid chromatography, 500-μL aliquots of extracted carbohydrate sample were dried in a gentle stream of air in Reacti-vials (Pierce, Rockford, IL). In each case, a 50-μL aliquot of the internal standard (100 mM ribose) was first added. Samples were oximated with 500 μL of 0.3 M *O*-methyl hydroxyl amine (Sigma) in pyridine (HPLC grade; Sigma) by incubation at 70°C for 30 min and then silylated by the addition of 100 μL of *N*-trimethylsilylimidazole (Sigma) and incubation at room temperature for 60 min. The samples were extracted using 500 μL of hexane (Sigma) and analyzed using a capillary gas–liquid chromatograph (model GC14-A; Shimadzu, Columbia, MD) with a BP5 25-m column with an internal diameter of 0.25 mm. Quantitative estimates of carbohydrate concentration were made by integrating peaks, normalizing values to the internal standard, and comparing them with peaks from replicate derivatized (100 mM) standards of fructose, glucose, mannitol, arabitol, glycerol, and trehalose.

Appressorial glycerol was extracted by scraping cells from the surface of plastic cover slips (Fisher Scientific, Loughborough, UK) in 80% ethanol. The suspension was then sonicated on ice for 1 min. This was repeated five times. The appressorial debris was then removed by centrifugation at 3000*g*, and the supernatant was evaporated to dryness in a rotary evaporator (Jencons Scientific, Bridgeville, PA). The extract was resuspended in double-distilled H_2O, and glycerol content was quantified using a spectrophotometric glycerol enzymatic assay (Roche Molecular Biochemicals, Lewes, UK).

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