MgHog1* Regulates Dimorphism and Pathogenicity in the Fungal Wheat Pathogen *Mycosphaerella graminicola

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The dimorphic ascomycete pathogen *Mycosphaerella graminicola* switches from a yeastlike form to an infectious filamentous form that penetrates the host foliage through stomata. We examined the biological function of the mitogen-activated protein kinase-encoding gene *MgHog1* in *M. graminicola*. Interestingly, *MgHog1* mutants were unable to switch to filamentous growth on water agar that mimics the nutritionally poor conditions on the foliar surface and, hence, exclusively developed by a yeastlike budding process. Consequently, due to impaired initiation of infectious germ tubes, as revealed by detailed in planta cytological analyses, the *MgHog1* mutants failed to infect wheat leaves. We, therefore, conclude that *MgHog1* is a new pathogenicity factor involved in the regulation of dimorphism in *M. graminicola*. Furthermore, *MgHog1* mutants are osmosensitive, resistant to phenylpyrrole and dicarboximide fungicides, and do not melanize.

Additional keywords: *Agrobacterium tumefaciens*-mediated transformation, differentiation, transposon, virulence.

The ascomycete *Mycosphaerella graminicola* (Fuckel) J. Schröt. (anamorph *Septoria tritici*), the causal agent of septoria tritici leaf blotch is a major threat to bread and durum wheat in most wheat-growing areas world wide but particularly in areas with high rainfall during the growing season. During the last decades, severe epidemics of the disease have caused substantial yield losses in Western Europe. Recently, the importance of the disease even increased after the rapid evolution of resistance to strobilurin fungicides in this pathogen (Fraaije et al. 2005). Due to its economical importance, *M. graminicola* has been studied extensively, with an emphasis on epidemiology (Royle 1994), population genetics (McDonald et al. 1999), genetics of resistance (McCartney et al. 2002), and host-pathogen interactions (Kema et al. 2000; Stergiopoulos et al. 2003). Recently, we generated over 30,000 expressed sequence tags (EST) from a range of cultural conditions (Kema et al. 2003), and the genome of *M. graminicola* has been sequenced at the Joint Genome Institute of the United States Department of Energy. This is the foundation for an increased understanding of the molecular mechanisms regulating growth and differen-

tiation during the fungal life cycle, essential phases for the accomplishment of successful penetration, colonization, and fructification.

M. graminicola has a unique infection process that makes it an interesting model to study various aspects of pathogenicity. It is a dimorphic pathogen with a 'yeastlike' blastic conidiogenesis and a filamentous form that invades the host. In contrast to many other cereal fungal pathogens, *M. graminicola* does not form appressoria but penetrates wheat leaves through the stomata without differentiating infection structures (Duncan and Howard 2000; Kema et al. 1996). *M. graminicola* is a hemibiotroph with a biotrophic phase that lasts for about ten days, which is followed by a rapid switch to necrotrophy. This results in the typical symptoms of the disease, irregular chlorotic lesions that develop to necrotic blotches bearing the anamorphic and teleomorphic fructifications (Duncan and Howard, 2000; Kema et al. 1996).

We have studied the role of mitogen-activated protein kinase (MAPK)-encoding genes in development and pathogenicity of *M. graminicola* (Cousin et al. 2006; Mehrabi et al. 2006). These genes are involved in many different aspects of fungal development, and the encoded proteins are activated or deactivated by a wide variety of extracellular stimuli through upstream components and, hence, are major components of signal transduction cascades that transduce extracellular signals to the nucleus (Xu 2000). In the budding yeast *Saccharomyces cerevisiae*, five well-characterized MAPK pathways regulate developmental processes such as mating, cell wall integrity, osmoregulation, pseudohyphal growth, and ascospore formation (Gustin et al. 1998).

Here, we describe the MAPK-encoding gene *MgHog1*, the ortholog of *S. cerevisiae* *Hog1* (Brewster et al. 1993). The HOG1 pathway in *S. cerevisiae* (Hohmann 2002; O'Rourke et al. 2002) is regulated by at least two upstream cascades that activate HOG1 MAPK. One branch depends on the SHO1 osmosensor transmembrane protein, while the other branch depends on SLN1, a two component histidine kinase receptor. These two cascades converge to activate the MAPK kinase PBS2 through phosphorylation by STE11 in the SHO1 cascade and by SSK2 and SSK22 in the SLN1 cascade. Activated PBS2 phosphorylates MAPK HOG1, which subsequently transiently accumulates in the nucleus and activates downstream target transcription factors that eventually leads to activation of stress response genes. In turn, this induces a set of osmoadaptive responses and increases the transcription of genes encoding enzymes such as glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase involved in glycerol biosynthesis. Glycerol accumulation eventually leads to increased internal osmo-

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larity and maintains a high cellular turgor. These responses restore the osmotic gradient between cells and their environment required for survival under hyperosmotic stresses (Gustin et al. 1998).

We are interested in the identification of genes regulating the infection process of *M. graminicola* in relation with recognition and differentiation. Recently, we characterized two MAP kinases, *MgFus3* and *MgSlr2*, and demonstrated that *MgFus3* is required for perception of stomata and penetration (Cousin et al. 2006) whereas *MgSlr2* is required for invasive colonization of the mesophyll tissue (Mehrabani et al. 2006). Here, we analyzed the function of the third MAP kinase, *MgHog1*, in *M. graminicola*. Our results indicate that *MgHog1* is required for virulence and has a distinct function in the infection process as it regulates the morphogenic transition from yeastlike to filamentous growth and can therefore be considered a crucial pathogenicity factor with a novel function among plant pathogenic fungi.

RESULTS

DNA and protein sequence analysis and phylogenetic relationships.

Screening of 30,000 *M. graminicola* EST identified twelve EST homologous to *S. cerevisiae* *Hog1*. Assembly of these EST resulted in a contig designated as *MgHog1* with a 1,071-bp open reading frame (ORF) encoding a 357-amino acid (aa) protein sequence. The deduced protein has a calculated molecular weight of 40.77 kDa and shows 93% identity and 97% similarity at protein level with OSM1 of the rice blast pathogen *Magnaporthe grisea*. Results of a multiple sequence alignment of *MgHOG1* with other *HOG1* orthologs indicated that, indeed, *MgHOG1* groups together with *HOG1* orthologs of other filamentous ascomycetes (Fig. 1). Fungal *HOG1* orthologs are highly conserved except for the C-terminus. Furthermore, *MgHOG1* contains a dual phosphorylation motif (TGY; amino acids 171 to 173) characteristic of hyperosmolarity-activated MAP kinases (Cano and Mahadevan 1995). Comparison of the genomic DNA sequence with the EST contig of *MgHog1* showed that the structural region is interrupted by eight introns. A putative polyadenylation signal with a stretch of 65 constitutive A nucleotides is found 389 bp downstream of the stop codon. A GC-rich region with nucleotide A at nucleotide 3 (GCCACCATG), characteristic of Kozak's consensus signature (Kozak 1987), was identified around the putative start codon (ATG).

Isolation of *MgHog1* mutants.

The disruption construct for *MgHog1* (pCGN1589Δ*MgHog1*) was generated using an in vitro transposition system (Mehrabani et al. 2006) (Fig. 2). By polymerase chain reaction (PCR) screening and sequencing, a construct was selected in which the transposon was inserted 507 bp upstream of the stop codon in the ORF of *MgHog1*. We used this construct to disrupt *MgHog1* in *M. graminicola* IPO323 (hereafter referred to as WT) through *Agrobacterium tumefaciens*-mediated transformation (Mehrabani et al. 2006; Zwiers and de Waard 2001). To identify homologous recombinants for *MgHog1*, we used PCR screening, using primers *MgHog1*-F2 and *MgHog1*-R2, which were designed upstream and downstream of the transposon insertion (Table 1). Out of 24 hygromycin-resistant transformants obtained per transformation, we identified eight mutant strains lacking an intact copy of *MgHog1*.

MgHog1 regulates dimorphism in *M. graminicola*.

We noticed that *MgHog1* mutants developed exclusively by yeastlike growth on potato dextrose agar (PDA) and decided to test this phenomenon in more detail on water agar (WA), as this induces filamentous growth and stimulates conidia to germinate and develop a superficial filamentous colony. On WA, the WT and ectopic controls initially germinate from both apical cells of the spores, producing relatively long primary germ tubes within 12 h, which is followed by the development of secondary germ tubes from the same or other cells of the spore. Within 48 h, tertiary hyphal filaments develop from the primary and secondary germ tubes, indicating that a poor medium promotes filamentous growth most probably similar to conditions encountered on the leaf surface of the host (Kema et al. 1996; Mehrabani et al. 2006). Interestingly, the *MgHog1* mutants showed a significantly different germination pattern. Initial elongation of *MgHog1* mutant spores stopped within 12 h and was followed by a process of extensive budding without filamentous growth. This resulted in dense, localized yeastlike colonies, a process that we characterize as micro-conidiation (Fig. 3A).

Early colony development of the WT on PDA is significantly different. On PDA, germ tubes do not grow extensively in length but rather differentiate budding cells that form simultaneously on intermediate cells of the spore and on secondary filaments within 24 h (Fig. 3B). This is followed by a process of intense localized production of additional budding cells that partly elongate in relatively short filamentous hyphae at 36 h and accumulate in the center of the dense colony at 48 h (Fig.

Fig. 1. Phylogenetic comparison of *MgHog1* and its orthologs based on amino acid sequence alignments. The gene name and accession number for each fungus is indicated. The phylogram was constructed with the MEGALIGN program (DNA Star, Madison, WI, U.S.A.).

3B). The budding process in *MgHog1* mutants started immediately, usually from both apical cells of the spores but sometimes also from other cells of the conidia. This process was preceded by a short elongation of the spores but, unlike the WT, these spores did not develop filaments. Instead, budding continued even more intensively as visualized in Figure 3B by the bright spots at 36 and 48 hpi.

MgHog1 is required for virulence.

In order to evaluate the pathogenicity of the mutants, we inoculated the susceptible wheat cv. Obelisk with the WT, ectopic controls, and *MgHog1* mutants. At 8 dpi, plants inoculated with the WT and ectopic controls showed small chlorotic flecks that were expanded into necrotic lesions at 12 dpi. At 16 to 18 dpi, these lesions developed into large necrotic areas containing numerous pycnidia. However, plants inoculated with the *MgHog1* mutants did not develop symptoms until 15 dpi, when limited chlorotic areas started at the tips of the leaves. These chlorotic areas were also observed in water-treated control plants and apparently were due to natural senescence and aging, although it appeared that the chlorotic area was slightly larger on plants inoculated with the mutants (Fig. 4).

In planta filamentation is hampered in *MgHog1* mutants.

Germination, penetration, colonization, and pycnidia formation of *M. graminicola* were cytologically examined in planta over time, using the *MgHog1* mutants as well as the ectopic and WT controls. We observed no significant difference in the germination pattern, penetration, colonization, and pycnidia formation among the WT and the ectopic controls. However, spores of *MgHog1* mutants were unable to develop infectious

germ tubes that are required for penetration (Fig. 5). Some spores were able to generate significantly smaller germ tubes that were generally unable to penetrate the host. Only at very late stages of infection (>16 dpi) did we sometimes observe infectious hyphae of *MgHog1* mutants in the tip of senescent leaves (Fig. 5).

Disruption of *MgHog1* leads to osmotic sensitivity, promotes yeastlike growth, and results in fungicide resistance.

To test whether inactivation of the *MgHog1* gene would result in sensitivity to hyperosmolarity, the *MgHog1* mutants as well as the WT and ectopic controls were grown on PDA plates supplemented with different concentrations of KCl, NaCl, or sorbitol. The growth of *MgHog1* mutants was inhibited under all osmotic stress conditions tested. For instance, on 1.5 M sorbitol, the controls grew normally but the growth of *MgHog1* mutants was reduced to approximately 90% of the growth of the WT (Fig. 6).

When grown on PDA at 20°C, the *MgHog1* mutants exclusively developed as a yeastlike colony and did not melanize until prolonged incubation (>20 days), which was in clear contrast to the phenotype of the WT (Fig. 6C). However, when grown on PDA at 28°C, the melanization of the *MgHog1* mutants was similar to the WT and ectopic strains and filamentous growth was partially restored (Fig. 6D). The mutants and the controls were equally thermosensitive, since development was inhibited at temperatures >29°C (data not shown).

It has been reported that osmosensitive mutants of *Neurospora crassa* and *Colletotrichum lagenarium* are resistant to phenylpyrrole fungicides (Zhang et al. 2002; Kojima et al. 2004). Therefore, we also tested the sensitivity of *MgHog1* mutants to various fungicides, including phenylpyrroles. Compared with WT and ectopic controls, the *MgHog1* mutants were highly resistant to fludioxonil (Fig. 6E), fenpiclonil (>30×), and iprodione (>5×) (Table 2). We did not observe increased sensitivity of either mutant to any of the other fungicides tested.

DISCUSSION

We generated a range of extensive EST libraries as a resource for genomic research and gene identification (Kema et al. 2003), and we used them in a consistent strategy to identify genes involved in pathogenicity in *M. graminicola* (Cousin et al. 2006; Mehrabi et al. 2006). In order to determine the roles of MAPK pathways in the life cycle of the ascomycete *M. graminicola*, we previously characterized the MAPK-encoding genes *MgFus3* and *MgSlr2* and demonstrated that both genes are required for pathogenicity but in different stages of the infection process with no role in dimorphism. In the present study, we showed that disruption of *MgHog1* compromised the ability of *M. graminicola* to infect wheat leaves, which is due to failing filamentous growth. Interestingly, *Hog1* mutants of the rice blast pathogen *Magnaporthe grisea*, the cucumber anthracnose pathogen *C. lagenarium*, and *Bipolaris oryzae* remained fully pathogenic (Dixon et al. 1999; Kojima et al. 2004; Moriwaki et al. 2006). *Cpmk1* mutants of the chestnut blight pathogen *Cryphonectria parasitica* showed only slightly reduced canker areas (Park et al. 2004). The fact that the *Hog1*

Fig. 2. Diagram of disruption strategies used in this study. **A**, cDNA of *MgHog1* was excised from the pSport1 vector using *KpnI/XbaI* restriction enzymes and was inserted into pCGN1589, generating pCGN1589*MgHog1*. **B**, The target construct pCGN1589*MgHog1* was in vitro transposed using the customized donor construct pGPS3HygKan, resulting in **C**, the disruption constructs pCGN1589Δ*MgHog1*. To identify **D**, the homologous recombinants for *MgHog1*, we used a multiplex polymerase chain reaction screening using two gene-specific primers (*MgHog1*-F2 and *MgHog1*-R2) together with primer N, which is located at the right border of the transposon. Homologous recombinants (**D**, lanes 1 to 3) amplified only a small amplicon similar to the amplicon generated from the disruption construct (lane 4), whereas the ectopic controls (lanes 5 and 6) amplified two bands: a small band corresponding to the ectopic controls and a larger band similar to the wild-type amplicon (lane 7). Lane 8 shows the amplicon generated from the cDNA clone. M = 1 Kb plus marker.

Table 1. Primers used in this study

Name	Sequence (5'-3')	Location
<i>MgHog1</i> -F2	ATGGCCGAATTCGTGCG	<i>MgHog1</i>
<i>MgHog1</i> -R2	TCCTGGCCCTCTCCGTT	<i>MgHog1</i>

Fig. 3. Germination, early colony development, and microconidiation of *Mycosphaerella graminicola* IPO323 and the *MgHog1* mutants. **A**, Strains grown on water agar at 20°C in darkness. *M. graminicola* IPO323 (WT) germinates quickly and develops into a superficial filamentous web, whereas *MgHog1* mutants are unable to switch from the yeastlike form to filamentous growth and propagate only by budding. Note the dead spore in the Δ MgHog1-5 panels (star). **B**, Strains grown on potato dextrose agar at 20°C in darkness. The WT strain grows filamentously with localized microconidiation, but the *MgHog1* mutants have a much higher tendency for microconidiation. Ectopic control showed the same phenotype pattern as the WT strain. Composite panels contain images of the same spore at 0, 12, and 24 h. Scale bar = 30 μ m.

orthologs in *Magnaporthe grisea*, *C. lagenarium*, and *B. oryzae* are not involved in pathogenicity, unlike the *Hog1* ortholog in *M. graminicola*, clearly highlights a species-specific role of the *Hog1* pathway. These results suggest that *Hog1* plays an important role in pathogenicity of dimorphic fungal plant pathogens but not in those lacking this phenomenon.

We therefore investigated the role of *Hog1* in the dimorphic life cycle of *M. graminicola* in more detail. Indeed, the *MgHog1* mutants failed to switch from yeastlike to filamentous growth on WA that presumably mimics the nutrient-limiting conditions on the leaf surface. In planta, we hardly observed germinated yeastlike spores for *MgHog1* mutants, which contrasts with reports for the orthologous *Hog1* mutants of the appressorium-forming pathogens *Magnaporthe grisea* and *C. lagenarium*, for which no severe effect on germination and appressorium differentiation was found (Dixon et al. 1999; Kojima et al. 2004).

Our data show that *MgHog1* mutants are completely impaired in filamentation on poor nutrient media and show exclusively yeastlike growth. Even on rich nutrient media, *M. graminicola* *MgHog1* mutants show an elevated yeastlike growth compared with WT and ectopic control strains. Interestingly, *Hog1* does not play a role in dimorphic switching in any other fungus, except in *Candida albicans* (Alonso et al. 1999), but the role of the *Hog1* ortholog in the dimorphic transition of *M. graminicola* is much more pronounced. More importantly, the *Hog1* mutant and WT of *Candida albicans* show a similar phenotype in rich-serum medium, whereas in poor-serum medium the *Hog1* mutants have a higher tendency to grow filamentously. Hence, *Hog1* and *MgHog1* have opposite functions in *Candida albicans* and *M. graminicola*, respectively.

In conclusion, we designate *MgHog1* as a new pathogenicity factor of *M. graminicola* due to the fact that mutants are impaired in filamentous growth and, consequently, neither penetrate nor colonize the host, which clearly distinguishes *MgHog1* from *MgFus3* and *MgSlt2*, which are not required for filamentous growth but are essential for the recognition of the stomata and colonization of mesophyll cells, respectively.

We also showed that *MgHog1* mutants are osmosensitive, similar to orthologous *Hog1* mutants of *S. cerevisiae* and other yeasts and filamentous fungi (Brewster et al. 1993; Degols et al. 1996; Dixon et al. 1999; Iwaki et al. 1999; Moriwaki et al. 2006; Park et al. 2004; San Jose et al. 1996; Zhang et al. 2002;). In contrast, mutants of *hogA* in *Aspergillus nidulans* did not show severe growth defects (Han and Prade 2002), suggesting that, in this fungus, either another mechanism regulates growth under hyperosmotic stress or an additional *Hog1* homolog is present. We also tested the sensitivity of *MgHog1*

mutants to hydrogen peroxide and found that they are slightly more sensitive to hydrogen peroxide as compared with WT and ectopic controls (data not shown). A similar phenotype was reported recently in *S. cerevisiae*, *Candida albicans*, and *B. oryzae* *Hog1* mutants (Alonso et al. 2003; Haghazari and Heyer 2004), indicating a role for the *Hog1* orthologs in response to hydrogen peroxide.

After the initial publication on the effect of phenylpyrroles on glycerol synthesis in *N. crassa* (Pillonel et al. 1997), a negative correlation between osmosensitivity and resistance to phenylpyrrole fungicides was reported when the *os2* and *OSCI* genes in *N. crassa* and *C. lagenarium*, respectively, were disrupted (Irmeler et al. 2006; Kojima et al. 2004; Zhang et al. 2002). We extended studies on the role of *MgHog1* in fungicide sensitivity by testing the effects of different classes of fungicides on the growth of *MgHog1* mutants. Interestingly, we identified that *MgHog1* mutants are highly resistant to phenylpyrrole fungicides. It has been shown that fungicidal activity of the phenylpyrrole fungicide fludioxonil is due to activation of a *Hog1*-related MAPK in the fungal pathogen *C. lagenarium* (Kojima et al. 2004). *N. crassa* *os-2* mutants are resistant to dicarboximide fungicides (Zhang et al.

Fig. 4. Effects of the disruption of *MgHog1* on disease development of *Mycosphaerella graminicola* on the susceptible wheat cv. Obelisk 20 days after inoculation (bottom panel shows magnification). The first leaves were inoculated with water as control (1), the wild-type strain *M. graminicola* IPO323 (2), an ectopic control (3), and three independent mutant strains (4, 5, and 6).

Table 2. Minimal inhibitory concentrations (MIC) of compounds used in toxicity assays with *Mycosphaerella graminicola* IPO323 and *MgHog1* mutants^a

Class	Compounds	MIC ($\mu\text{g ml}^{-1}$)		Q value ^c
		IPO323 ^b	ΔMgHog1	
Azole	Cyproconazole	0.15	0.15	1
	Imazalil	0.5	0.5	1
	Tricyclazole	>60	>60	1
Strobilurin	Kresoxim-methyl	0.3-1	0.3-1	1
	Azoxystrobin	1	1	1
Pyrimidine	Fenarimol	0.25	0.25	1
Dicarboximide	Iprodione	20	>100	>5
	Vinclozolin	>100	>100	1
Aromatic hydrocarbon	Chloroneb	>100	>100	1
	DNOC	10	10	1
Phenylpyrrole	Fludioxonil	1	>30	>30
	Fenpiclonil	1	>30	>30

^a Three *MgHog1* mutants were tested and showed the same MIC value.

^b *M. graminicola* IPO323 and the ectopic transformants showed similar MIC values.

^c Q value = ratio between MIC values of ΔMgHog1 and IPO323

2002), to which *MgHog1* mutants also showed increased resistance ($>5\times$), suggesting a common mechanism against iprodione and fludioxonil in *N. crassa* and *M. graminicola*.

Altogether, these results indicate that interference in cell signaling networks seriously affects various cellular functions, including pathogenicity and sensitivity to fungicides. Detailed studies on these signaling mechanisms are important, as they may provide target sites for novel fungicides.

MATERIALS AND METHODS

Fungal strains and cultural conditions.

We used *M. graminicola* IPO323, which is highly virulent on seedlings of the susceptible wheat cultivar Obelisk, as the WT and recipient strain for gene disruption. Growing conditions, propagation, and maintenance were as described previously (Mehrabi et al. 2006).

Fig. 5. Comparative pathogenesis of *Mycosphaerella graminicola* IPO323 (WT) and *MgHog1* mutants on the susceptible wheat cv. Obelisk at 8, 12, 16, and 20 days postinoculation (dpi). **A**, Spores of the WT strain generate the infectious germ tubes (IGT) that penetrate via stomata but development of infectious filamentation in the *MgHog1* mutants is hampered. **B**, Colonized substomatal cavity by the WT strain. The spores of the *MgHog1* mutants only propagate by budding (B) and are unable to infect the tissue. Rare penetration attempts of *MgHog1* mutants are caused by individual budding cells that are positioned on top of the stomatal slit. **C** and **D**, Fructification of the WT strain. Pycnidia (P) with pycnidiospores exuding in a cyrrhus (C) from the stomatal opening. *MgHog1* mutants only sometimes infect the uncharacteristic tissue at the tip of the leaves. The ectopic and WT strains showed the same pathogenesis. Scale bar = 10 μ m; marked (*) scale bar is 100 μ m.

Fig. 6. Colony morphology of *Mycosphaerella graminicola* IPO323, Δ *MgHog1* and ectopic control strains under different growth conditions. **A**, Layout of the experiment. Approximately 1 μ l of spore suspension (10^7 spore ml^{-1}) was spotted on potato dextrose agar (PDA) and was grown for 10 days at 20°C, except for treatment D, which was incubated at 28°C. **B**, *MgHog1* mutants are osmosensitive on PDA supplemented with 1.5 M sorbitol. **C**, Abolishment of filamentous growth of *MgHog1* mutants on normal PDA. **D**, Partial recovery of the melanization and filamentous growth of *MgHog1* on PDA at 28°C. **E**, *MgHog1* mutants are resistant to the phenylpyrrole fungicide fludioxonil ($30 \mu\text{g ml}^{-1}$) on PDA.

DNA manipulations and analysis.

Standard protocols were used for DNA and RNA manipulation (Sambrook et al. 1989). Plasmid DNA was isolated using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Genomic DNA of *M. graminicola* was extracted using the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN, U.S.A.). The DYEnamic ET dye terminator cycle sequencing kit (Amersham Biosciences, Roosendaal, The Netherlands) was used to sequence DNA on an ABI-prism 3100 capillary automated sequencer according to recommended protocols. Phylogenetic tree generation, DNA and protein sequence alignment, editing, and analysis were performed using MEGALIGN and DNA Star software (Madison, WI, U.S.A.). Homology searches were performed with the BLAST program (Altschul et al. 1997).

Construction of plasmid vectors.

Through in silico analyses of EST databases of *M. graminicola* IPO323 (Kema et al. 2003), we identified cDNA clones of *MgHog1* and used the GPS mutagenesis system (New England Biolabs, Leusden, The Netherlands) to produce disruption constructs, using a homemade customized donor construct, pGPS3HygKan (Mehrabi et al. 2006). The transposition mixture was then cloned to *Escherichia coli* DH10 β , which was subsequently plated on selective gentamycin and kanamycin media. The diagnostic multiplex colony PCR reactions were performed as described previously (Mehrabi et al. 2006). A construct with the transposon inserted in the middle of the gene was selected and designated pCGN1589 Δ *MgHog1*. The exact position of the transposon insertion was determined by sequencing from the right and the left borders of the transposon, using primers N and S (Mehrabi et al. 2006).

Fungal transformation.

The disruption construct pCGN Δ *MgHog1* was cloned into *A. tumefaciens* LBA1100 by electroporation. *A. tumefaciens*-mediated transformation was performed basically according to

Zwiers and de Waard (2001), with minor modifications described previously (Mehrabi et al. 2006).

Phenotyping.

Phenotypic characterizations were always performed with at least two independent disruptants, along with one ectopic and the WT control. Experiments were repeated twice or thrice, with at least two repetitions for each strain. Pathogenicity assays were performed as previously described with minor modifications (Mehrabi et al. 2006). After inoculation, pots were placed in transparent polyethylene boxes ($30 \times 45 \times 30$ cm) and were incubated at 20°C in the dark for 48 h and were subsequently incubated at 16 h of light ($166 \mu\text{E s}^{-1}\text{m}^{-2}$) per day. Disease severity was monitored every four days, and final disease assessment was performed at 20 days postinoculation (dpi).

Cell biology assays were performed by growing spores of two independent mutant strains of *MgHog1* and the WT and ectopic control strains in yeast glucose broth medium at 18°C for five days until they were collected by centrifugation and were washed once with water and adjusted to 10^5 spores per milliliter. The diluted spore suspension ($10 \mu\text{l}$) was spotted on a plug of WA or PDA that was placed on a microscopic slide and covered with a cover slip. The samples were incubated in an empty Petri dish at 20°C in darkness, and at least two spores of each sample were monitored using a Zeiss Axioscop microscope equipped with a Carl Zeiss AxioCam 6.08.0 digital camera (Carl Zeiss, Jena, Germany), and pictures were processed with Axio Vision 4.1 software (Carl Zeiss) every 12 h until 48 h.

To monitor the germination and penetration behavior of *M. graminicola*, we harvested leaves from inoculated plants at 8, 12, 16, and 20 dpi and placed them on filter paper saturated with a mixture of 100% ethanol/glacial acetic acid (3:1, vol/vol), which was refreshed three to four times until the leaves were completely cleared. They were eventually transferred to filter paper saturated with lactoglycerol (1:1:1, lactic acid/glycerol/water, vol/vol/vol) until cytological observation (Shetty et al. 2003). To monitor the colonization of the mesophyll, the harvested leaves were immediately immersed and boiled for 15 min in 20 ml of 0.05% trypan blue dissolved in lactophenol ethanol (1:2, vol/vol). Samples were destained at least overnight in a saturated chloral hydrate solution (5:2, wt/vol) and were then stored in 87% (vol/vol) glycerol until analysis. For all light microscopy observations, specimens (adaxial side up) were placed on a glass slide, covered with a cover slip, observed, and documented using the aforementioned microscopical equipment, camera, and software. For colonization monitoring, samples were additionally stained with 0.1% trypan blue.

Osmosensitivity was determined by growing the *MgHog1* mutants and the WT and ectopic controls at 20°C in darkness on PDA plates supplemented with 1, 1.5, 2.0, and 2.5 M of KCl, NaCl, or sorbitol at 20°C in darkness. Colony development and size were visually examined at 10 dpi. Minimum inhibitory concentrations of fungicides (Table 2) were determined by inoculating PDA plates supplemented with different concentrations of these compounds, with spores harvested from 5-day-old liquid yeast glucose medium in a final concentration of 10^7 spores per milliliter. Plates were evaluated after 10-day incubation at 20°C in darkness.

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