MaHog1, a Hog1-type mitogen-activated protein kinase gene, contributes to stress tolerance and virulence of the entomopathogenic fungus Metarhizium acridum

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Fungal biocontrol agents have great potential in integrated pest management. However, poor efficacy and sensitivity to various adverse factors have hampered their wide application. In eukaryotic cells, Hog1 kinase plays a critical role in stress responses. In this study, MaHog1 (GenBank accession no. EFY85878), encoding a member of the Hog1/Sty1/p38 mitogen-activated protein kinase family in Metarhizium (Me.) acridum, was identified. Targeted gene disruption was used to analyse the role of MaHog1 in virulence and tolerance of adverse factors. Mutants with MaHog1 depletion showed increased sensitivity to high osmotic stress, high temperature and oxidative stress, and exhibited remarkable resistance to cell wall-disturbing agents. These results suggest that Hog1 kinase has a conserved function in regulating multistress responses among fungi, and that MaHog1 might influence cell wall biogenesis in Me. acridum. Bioassays conducted with topical inoculation and intrahaemocoel injection revealed that MaHog1 is required for both penetration and postpenetration development of Me. acridum. MaHog1 disruption resulted in a significant reduction in virulence, likely due to the combination of a decrease in conidial germination, a reduction in appressorium formation and a decline in growth rate in insect haemolymph, which might be caused by impairing fungal tolerance of various stresses during infection.

INTRODUCTION

Fungal biocontrol agents have great potential in integrated pest management because of their environmental safety and the low likelihood of the development of insect resistance (Charnley & Collins, 2007). However, their poor efficacy and sensitivity to various adverse factors have hampered wide application of entomopathogenic fungi for the control of economically important pests (Ekesi et al., 2003; Rangel et al., 2004). The acridid-specific fungal pathogen Metarhizium (Me.) acridum is regarded as one of the most promising agents for biological control of locusts and grasshoppers (Hunter et al., 2001; Peng et al., 2008). Furthermore, Metarhizium has become an excellent model organism to explore a number of questions at the molecular and biochemical level (Gao et al., 2011).

Similar to other entomopathogenic fungi, the conidia of Me. acridum adhere to the insect cuticle, germinate, and penetrate the insect cuticle to enter the blood cavity via cuticle-degrading enzymes and mechanical pressure, and then hyphae proliferate in the insect and exhaust the nutrition of the host (Clarkson & Charnley, 1996). During infection, pathogenic fungi have to encounter various stresses from the environment, such as heat shock and UV radiation (Ekesi et al., 2003; Rangel et al., 2004), and from the host insect, such as the high osmotic stress of the insect (Chapman, 1998), oxidative stress during infection (Bogdan et al., 2000) and behavioural changes, such as behavioural fever in the desert locust (Hunt & Charnley, 2011). Thus, detailed knowledge of the regulatory processes involved in responses to adverse factors from the environment and the host insect is necessary for commercial development and fungal improvement.

In eukaryotic cells, mitogen-activated protein kinase (MAPK) cascades play a critical role in sensing changes in the environment and regulating the expression of genes...
involved in various development and differentiation processes as a reaction to diverse extracellular stimuli (Banuett, 1998; Herskowitz, 1995; Treisman, 1996). Multistresses caused by changes in the environment are sensed via MAPK members of the family of stress-activated protein kinases (SAPKs), such as Saccharomyces (Sa.) cerevisiae Hog1, Schizosacharomyces (Sc.) pombe Sty1, and mammalian p38 and JNK MAPKs (Roux & Blenis, 2004). Previous research has shown that Hog1 responds to various stresses in different fungi. In Sa. cerevisiae, Hog1 is activated in response to a variety of stress stimuli, including high osmolarity, oxidative stress, heat stress, cold stress, arsenite, methlyglyoxal and weak acid stress, and contributes to the regulation of cell wall composition (Alonso-Monge et al., 2001; Bilsland et al., 2004; Brewster et al., 1993; Maeta et al., 2005; Mollapour & Piper, 2006; Panadero et al., 2006; Thorsen et al., 2006; Winkler et al., 2002). In Sc. pombe, the Sty1 pathway is activated in response to diverse stresses, such as osmotic stress, heat shock, oxidative stress, nitrogen limitation, carbon starvation, UV light, methlyglyoxal, cold stress, arsenite and pressure (Degols et al., 1996; Degols & Russell, 1997; George et al., 2007; Millar et al., 1995; Rodriguez-Gabriel & Russell, 2005; Shiozaki & Russell, 1996; Soto et al., 2002; Takatsume et al., 2006). In Candida (Ca.) albicans, CaHog1 is activated in response to osmotic and oxidative stresses, heavy metals, various drugs, cationic peptides and increased glucose levels (Alonso-Monge et al., 2003; Eisman et al., 2006; Rodaki et al., 2009; Smith et al., 2004). Moreover, CaHog1 mutants display significantly attenuated virulence in a mouse model of disease in Ca. albicans (Alonso-Monge et al., 1999). In Trichoderma (Tr.) harzianum, a hog1-silenced strain was sensitive to osmotic and oxidative stress, and showed strongly reduced antagonistic activity against some plant fungal pathogens (Delgado-Jara et al., 2006). In Mycosphaerella (My.) graminicola, the Hog1 kinase acts as the virulence determinant during plant infection (Mehrabi et al., 2006). In Magnaporthe (Ma.) oryzae, the OSM1 Hog1-encoding gene regulates arabitol synthesis in response to high osmolarity, and its inactivation results in growth defects and abnormal hyphal morphology under high osmotic stress, but no impairment in pathogenicity (Dixon et al., 1999). In Cryptonectria (Cr.) parasitica, disruption of the Hog1-homologous gene cpmk1 results in osmosensitivity and reduced virulence on chestnut trees compared with the wild-type (WT) (Park et al., 2004). In Botrytis (Bo.) cinerea, BcSak1 mutants show significant impairment of vegetative and pathogenic development (Segmüller et al., 2007). In Beauveria (Be.) bassiana, BbHog1 mutants are more sensitive to high osmotic stress, high temperature and oxidative stress than the WT, and exhibit greatly reduced virulence to insects (Zhang et al., 2009). These results suggest that Hog1 kinase plays a key role in transducing environmental stress signals and in pathogenicity. However, it is far from clear whether Hog1-type MAPKs in Me. acridum are involved in virulence and tolerance of various stresses.

Here, we report the characterization of Me. acridum MaHog1 and its role in adapting to environmental stresses and host infection. The results demonstrate that the MaHog1 gene encodes a member of the Hog1/Sty1/p38 MAPK family. Disruption of MaHog1 resulted in a significant reduction in virulence and tolerance of various stresses.

**METHODS**

**Strains and culture conditions.** Me. acridum strain CQMa102 from the Genetic Engineering Center of Chongqing University was used in this study. The strain was maintained and cultured as previously described (Xia et al., 2002). Escherichia coli JM109 was employed for DNA manipulations and transformations. Agrobacterium (Ag.) tumefaciens AGL-1 was used for fungal transformations. Spot assays were performed to investigate the stress sensitivity of various strains. Conidia from the WT, MaHog1-disrupted transformant (MaHog1) and complementary transformant (CP) were suspended in double-distilled H2O, and the final concentration was adjusted to 1 × 10⁶ conidia ml⁻¹. Aliquots of 3 μl containing ~3000 conidia were spotted with a micropipette onto potato dextrose agar (PDA) plates supplemented with Congo red (CR; 500 μg ml⁻¹), Calcofluor white (CFW; 50 μg ml⁻¹), H2O2 (6 mmol l⁻¹), sorbitol (1.5 mol l⁻¹) and NaCl (0.5 mol l⁻¹), and incubated at 28 or 35 °C for 3 or 6 days.

**Gene cloning and phylogenetic analysis.** From the whole genomic sequence of Me. acridum (Gao et al., 2011), a gene named MaHog1 (EF185878) was identified, which has a high similarity to the Hog1-homologous protein OSM1 of Ma. oryzae (AAF09475). Fungal total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized with reverse transcription and oligo dT 3-site adaptor primer (Takara). The full cDNA of the homologous protein OSM1 of Ma. oryzae (AAF09475) was amplified using a 3′-full TR and amplified of cDNA ends (RACE kit) (Takara) according to the manufacturer’s instructions, with a pair of primers, the 3-sites adaptor primer and Hog1-3S (Table 1). The PCR product was subcloned into the pMD19-T vector and transformed into E. coli JM109 for determination by GenScript (Nanjing). The program MEGA v4.1 (www.megasoftware.net) was used to construct phylogenetic dendrograms.

**Construction of the targeted gene disruption vector and complementation vector.** To study the biological function of MaHog1 in Me. acridum, the disruption vector pK2-PB-MaHog1/L/R was constructed. Fungal genomic DNA was extracted as described by Raeder & Broda (1985). A 2320 bp DNA fragment, which contained the full-length MaHog1 gene, was cloned with primers Hog1-F and Hog1-R (Table 1) using Agrobacterium (Ag.) tumefaciens AGL-1 for fungal transformation. The selectable marker was constructed. The chorimuron ethyl resistance gene (sur vector containing MaHog1) and the bar vector containing MaHog1 were inserted into Xhol and BglII sites of pMD19-MaHog1 to form pMD19-MaHog1. The selection marker cassette was inserted into Xhol and BglII sites of pMD19-MaHog1 to form pMD19-MaHog1-PB. The disruption vector, pK2-2PB-MaHog1/L/R, was generated by inserting a 3.1 kb fragment containing the disrupted gene excised from the pMD19-MaHog1-PB vector into EcoRI and HindIII sites of the binary vector pPK2 (McCluskey, 2003) in which an lhp cassette was deleted. The resulting gene disruption vector was mobilized into pMD19-T vector and transformed into E. coli JM109 for determination by GenScript (Nanjing).

To complement the ΔMaHog1 mutant, a pK2-sur vector containing the chorimuron ethyl resistance gene sur from Ma. oryzae as a selectable marker was constructed. The sur cassette was cloned from pCB1536 by PCR using primers Sur-F and Sur-R (Table 1) (Zhang et al., 2010). The resulting fragment was then inserted into EcoRI and EcoRI sites of the binary vector pPK2-PB. For construction of the complementation vector for the ΔMaHog1 strain, a 3.9 kb fragment encompassing the MaHog1 ORF and 2.2 kb of the 5′ flanking sequence was amplified by PCR from Me. acridum WT genomic DNA using the primer pair Hog1-RIF/Hog1-RIR (Table 1). The PCR fragment was then inserted in EcoRV and Xhol sites of the pK2-Sur vector.
Table 1. Primers used in this work

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)*</th>
<th>Restriction enzyme site</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hog1-3S</td>
<td>ACCGCAGCAATTGGCTGAATTC</td>
<td></td>
<td>Used to clone MaHog1 by 3′ RACE</td>
</tr>
<tr>
<td>Hog1-F</td>
<td>TACCGGCGCCAATCTTGGCA</td>
<td></td>
<td>Used to clone the DNA fragment containing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>the full-length MaHog1 protein gene</td>
</tr>
<tr>
<td>Hog1-R</td>
<td>CATGAATAGACTGGCATTTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hog1-VF</td>
<td>GCTGGACAAGATTTCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bar-F2</td>
<td>GCTCTAGACCCCACTGTCT</td>
<td>XbaI</td>
<td>Used to clone the DNA fragment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>encompassing the MaHog1 ORF and 2.2 kb</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>of 5′ flanking sequence</td>
</tr>
<tr>
<td>Hog1-RIF</td>
<td>GCTCTAGAGGAAGAGCGTAGTACGC</td>
<td>EcoRV</td>
<td>Used to clone probe 1</td>
</tr>
<tr>
<td>Hog1-PF</td>
<td>ACGCGTTCGCACTGTCAATCTG</td>
<td>EcoRI</td>
<td>Used to clone the sur gene</td>
</tr>
<tr>
<td>Hog1-PR</td>
<td>TGGGCTGATCCAAATCTTC</td>
<td>EcoRV</td>
<td>Used to clone probe 2</td>
</tr>
<tr>
<td>Sur-F</td>
<td>CGGAATTCGTCAGTGCAACTGGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sur-R</td>
<td>GCTATTCGTCAGTGCAACTGGCT</td>
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<tr>
<td>Sur-PF</td>
<td>GTGCAGTGGCAAGCCAGGCGAGTCAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sur-PR</td>
<td>CTCTGTACAGAAATGTCGA</td>
<td></td>
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</tbody>
</table>

*Underlined sequences are restriction sites.

plasmid to yield pK2-Sur-MaHog1. The resulting plasmids were mobilized in Ag. tumefaciens AGL-1 for fungal transformation.

**Fungal transformation and screening.** Agrobacterium-mediated transformation was carried out according to the method of dos Reis et al. (2004). The MaHog1 disruption mutants were initially selected on the basis of herbicide (glufosinate ammonium) resistance. PCR performed with the primer pair Hog1-VF and bar-F2 (Table 1) was used to screen the MaHog1-disrupted transformants. The complementary transformants were selected on the basis of herbicide (chlorimuron ethyl) resistance. Southern blotting was used to confirm successful construction of MaHog1-disruption transformants and the complementary transformants.

**Southern blotting.** Southern blotting was performed with 5 μg of genomic DNA for each sample. The genomic DNA was digested with BglII/EcoRV or EcoRV. The digested DNA was separated on a 1.0% agarose gel and transferred onto an Immobilon-Ny+ transfer membrane (Millipore). Probe labelling, membrane hybridization and visualization were performed using a DIG High Prime DNA Labeling and Detection Starter kit I (Roche).

**Determination of conidial germination, appressorium formation and conidial surface hydrophobicity.** Conidial germination and appressorium formation were examined on locust hind wings using a previously described method (He & Xia, 2009). Samples were stained with CFW buffer (Hoch et al., 2005), then observed and photographed with a fluorescence microscope (model BX51; Olympus). A conidium was considered to have germinated if the length of its germ tube was greater than its width. The frequency of appressorium formation was determined according to the method described by Zhang et al. (2009). Conidial surface hydrophobicity was determined according to the method of Holder et al. (2007).

**Bioassays.** Bioassays of WT, ΔMaHog1 and CP were performed in male adult locusts (Locusta migratoria, 2–3 days after eclosion) as the insect host. Two different assay conditions were tested for each strain. (1) For topical inoculation, locusts were dipped in 5 μl culinary soybean oil suspension containing 1 × 10⁷ conidia ml⁻¹ up to the head–thorax junction. Control locusts were treated with 5 μl blank culinary soybean oil. (2) For intrahaemocoel injection, locusts were injected with 5 μl of an aqueous suspension containing 1 × 10⁸ conidia ml⁻¹ into the haemocoel cavity through the third abdominal segment. Control locusts were injected with 5 μl sterile distilled H₂O. Treated locusts were placed separately in clear plastic cages (20 × 20 × 20 cm) with a hole (2 cm diameter) on the side, and kept at 28 °C with a 16:8 h (light–dark) photoperiod. Each treatment had three replicates with 20 insects each, and the entire experiment was repeated three times with different batches of fungal conidia. Mortality was monitored at 12 h intervals.

**Determination of the differentiation and growth rate of various strains in insect haemolymph in vivo and in vitro.** Hyphal bodies in vivo were observed under a microscope by bleeding infected locusts at 12 h intervals. To investigate fungal differentiation and growth rates in insect haemolymph in vitro, 50 μl of conidial suspension (1 × 10⁷ conidia ml⁻¹) was inoculated into a 2 ml microcentrifuge tube containing 950 μl of fresh locust haemolymph (containing 500 mg cephalosporin ml⁻¹). Samples were maintained at 28 °C on a rotary shaker at 200 r.p.m. Hyphal bodies were observed under a microscope at 12 h intervals.

**Statistical analysis.** All experiments were repeated at least three times, and data from conidial germination, appressorium formation, conidial surface hydrophobicity and insect bioassays were analysed using the one-way analysis of variance (ANOVA) model. Tukey’s honestly significant difference test was used to compare means. The mean 50% lethality time (LT₅₀) was estimated using the Data Processing System program (Tang & Feng, 2007).

**RESULTS**

**Cloning and characteristics of MaHog1**

We cloned a gene homologous to the Osm1 gene of Ma. oryzae, named MaHog1 (EFY85878), from the Me. acridum
genome. Analysis of the cDNA sequence (JQ691634) demonstrated that MaHog1 has an ORF of 1077 bp encoding a predicted protein of 358 amino acids with an estimated molecular mass of 41.1 kDa and pl of 5.65. Eight introns of 76, 65, 67, 53, 56, 68, 61 and 53 bp were found in MaHog1 genomic DNA, located at positions 57, 181, 376, 487, 640, 1053, 1200 and 1445 of the coding region, respectively. All 3' and 5' sequences for the eight introns show close similarity to the internal splicing (G/A)CT(A/G)AC (Gurr et al., 1987). Sequence analysis revealed that the predicted MaHog1 contains all of the conserved characteristic subdomains of other SAPKs, including the TGY activation loop (Robinson & Cobb, 1997) at positions 171–173, a site for threonine and tyrosine phosphorylation required for kinase activation. MaHog1 showed 96, 94, 94, 90, 77 and 79 % amino acid sequence identity with Hog1-type sequences from Ma. oryzae (AAF09475) (Dixon et al., 1999), Be. bassiana (AAS77871) (Zhang et al., 2009), Tr. harzianum (BAE53434) (Delgado-Jarana et al., 2006), Bo. cinerea (AM236311) (Segmüller et al., 2007), Ca. albicans (X90586) (San José et al., 1996) and Sa. cerevisiae (AAA34680) (Brewster et al., 1993), respectively. Using the neighbour-joining method, a phylogenetic tree was constructed based on functionally characterized MAPK proteins from other fungi. The putative MaHog1 protein belongs to the Hog1 clade, but is separated from two other MAPK clades, Fus3/Kss1 and Slt2. On the basis of bioinformatic analysis of the MaHog1 sequence, we conclude that MaHog1 encodes a protein belonging to a member of the Hog1/Sty1/p38 MAPK family (Fig. 1).

**Targeted disruption of MaHog1 and complementation**

Targeted gene disruption was used to determine the molecular function of MaHog1 in fungal stress responses and pathogenicity. The gene disruption vector pK2-PB-MaHog1L/R was constructed to replace a 140 bp genomic region of MaHog1 with a 900 bp cassette of a herbicide resistance gene (Bar) (Fig. 2a). Southern blotting confirmed

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**Fig. 1.** Phylogenetic analysis of fungal MAPKs by the neighbour-joining algorithm of MEGA 4.1. Fungal species were as follows: MaHog1 (Me. acridum), OSM1 (Ma. oryzae), ThHog1 (Tr. harzianum), BbHog1 (Be. bassiana), CpMK1 (Cr. parasitica), MgHog1 (My. graminicola), BcSak1 (Bo. cinerea), CaHog1 (Ca. albicans), Hog1 (Sa. cerevisiae), Sty1 (Sc. pombe), BMP1 (Bo. cinerea), BbMPK1 (Be. bassiana), FMK1 (Fusarium oxysporum), PMK1 (Ma. oryzae), TmkA (Trichoderma virens), CMK1 (Colletotrichum lagenarium), MPS1 (Ma. oryzae), Bmp3 (Bo. cinerea), MgSLT2 (My. graminicola), MPKA (Aspergillus nidulans) and SLT2 (Sa. cerevisiae). Bar, 5 % sequence divergence.
successful construction of ΔMaHog1 and CP. BglII/EcoRV-digested total DNA from ΔMaHog1, WT and CP was blotted and hybridized with a labelled probe, a partial sequence of MaHog1 (Fig. 2a). Whereas WT showed a hybridizing band of ~0.6 kb, in ΔMaHog1 this fragment was replaced by a 1.5 kb fragment, as expected. In CP, the 0.6 and 1.5 kb bands were both observed (Fig. 2b). EcoRV-digested total DNA from ΔMaHog1, WT and CP was also blotted and hybridized with a labelled probe, a partial sequence of the sur cassette (Fig. 2c). The results showed that a 6.3 kb band was detected for CP, but no band was observed for WT or ΔMaHog1 (Fig. 2d).

MaHog1 is required for tolerance of high osmolarity, oxidative stress and high temperature, and sensitivity to cell wall-disturbing agents

MaHog1 contributes to stress tolerance and virulence

MaHog1 is required for not only the penetration but also the postpenetration development of Me. acridum

MaHog1 is required for tolerance of high osmolarity, oxidative stress and high temperature, and sensitivity to cell wall-disturbing agents

To study the role of MaHog1 in multistress responses, spot assays were performed to investigate the stress sensitivity of various strains. On standard PDA medium, no obvious differences in growth rate were observed between the WT and transformants. Under hyperosmotic stress conditions (PDA containing 0.5 M NaCl or 1.5 M sorbitol), growth of ΔMaHog1 was dramatically reduced compared with that of WT and CP. Furthermore, MaHog1 disruption increased sensitivity to high temperature and oxidative stress. At 35 °C or on PDA medium containing 6 mM H₂O₂, ΔMaHog1 showed a significant reduction in growth compared with WT and CP. In addition, ΔMaHog1 exhibited remarkable resistance to the cell wall-disturbing agents CR and CFW (Fig. 3).

MaHog1 is required for not only the penetration but also the postpenetration development of Me. acridum

To investigate the effect of MaHog1 disruption on virulence, both topical inoculation and intrahaemocoel injection bioassays were conducted. After topical inoculation, there were no significant differences in survival rate between WT and CP, but decreased virulence was observed for the ΔMaHog1 mutant. On day 9 after inoculation, locust mortality was <20 % for the ΔMaHog1 mutant but >95 % for WT and CP (Fig. 4a). LT₅₀ values were 6.01 ± 0.69 days for WT and 6.39 ± 0.66 days for CP. Similar results were obtained for the injection assay. On day 7 after injection, locust mortality was <40 % for the ΔMaHog1 mutant but >95 % for WT and CP (P<0.01; Fig. 4b). LT₅₀ values were 5.13 ± 0.57 days for WT, 5.24 ± 0.49 days for CP and 8.93 ± 0.66 days for ΔMaHog1. The LT₅₀ value for ΔMaHog1 was significantly longer than that for WT and CP (P<0.01). These results indicate that MaHog1 determines not only the penetration but also the postpenetration development of Me. acridum.
MaHog1 affects fungal growth in the haemolymph of the host insect in vivo and in vitro

The effects of MaHog1 on hyphal body differentiation and growth in insect haemolymph in vivo and haemolymph cultured in vitro were investigated. On day 5 after injection, numerous hyphal bodies were observed in the haemolymph of locusts injected with WT or CP, but hardly any hyphal bodies were found in locusts infected with the ΔMaHog1 mutant. Up to day 8 after injection, the ΔMaHog1 mutant produced a large number of hyphal bodies in locust haemolymph (Fig. 5a). By day 8, locusts infected with WT or CP had become mycosed cadavers. Similar phenomena were observed for topical inoculation: growth was much quicker for the WT and CP groups than for the ΔMaHog1 group (data not shown). For culture in locust haemolymph in vitro, the number of hyphal bodies at 24 h after inoculation was significantly lower for the ΔMaHog1 mutant compared with WT and CP (Fig. 5b). The observations show that hyphal bodies were morphologically similar for the ΔMaHog1, WT and CP groups, but growth was significantly slower for the ΔMaHog1 mutant than for WT and CP in insect haemolymph in vivo and in vitro. The results demonstrate that MaHog1 affects fungal growth in host haemolymph.

MaHog1 is required for conidial germination and appressorium formation on the locust hind wing, but is not required for conidial surface hydrophobicity

Conidial germination and appressorium formation on locust hind wings were examined 24 h after inoculation. The results show that although germ tubes and appressoria of the ΔMaHog1 mutant were morphologically similar to those of WT and CP (Fig. 6a), the frequency of conidial

**Fig. 3.** Growth characterization of the Me. acridum ΔMaHog1 mutant. Fungal growth was examined on PDA medium or PDA medium supplemented with (per litre) 1.5 mol sorbitol, 0.5 mol NaCl, 50 μg CFW, 500 μg CR or 6 mmol H₂O₂ at 28 or 35 °C. The fungal colonies were photographed after 3 or 6 days of incubation. The strains examined were WT, ΔMaHog1 and CP.

**Fig. 4.** Disruption of MaHog1 reduces the virulence of the fungi. (a) Mortality of locusts after topical application of 5 μl culinary soybean oil suspension containing 1×10⁷ conidia ml⁻¹ into the head–thorax junction (control insects were treated with 5 μl culinary soybean oil). Error bars, sd. (b) Mortality of locusts after injection of 5 μl of an aqueous suspension containing 1×10⁶ conidia ml⁻¹ into the haemocoel cavity through the third abdominal segment (control insects were injected with 5 μl sterile distilled H₂O). Error bars, sd.
Germination and appressorium formation was significantly lower for the ΔMaHog1 mutant compared with the two controls (P<0.01). Approximately 44% of conidia from the ΔMaHog1 mutant had germinated by 24 h after inoculation, compared with ~80% for both WT and CP (Fig. 6b). For the ΔMaHog1 mutant, ~41% of germinated conidia differentiated into appressoria, which was significantly lower than the rate for both controls (~70%) (Fig. 6c). The conidial surface hydrophobicity of the various strains was also examined. The results indicate that MaHog1 disruption did not affect cell surface hydrophobicity in Me. acridum (P>0.05; data not shown).

![Image](https://www.microbiologyresearch.org/article-figs/2019/01/02/MIC-13-2993-g005.png)

**Fig. 5.** Disruption of MaHog1 reduces the fungal growth rate in insect haemolymph both in vivo and in vitro. (a) Hyphal bodies in insect haemolymph in vivo. Hyphal bodies were observed with a microscope by bleeding the locusts at 12 h intervals. (b) Hyphal bodies in insect haemolymph in vitro. Hyphal bodies were observed with a microscope at 12 h intervals. White arrows, haemocytes; black arrows, Me. acridium hyphal bodies.

![Image](https://www.microbiologyresearch.org/article-figs/2019/01/02/MIC-13-2993-g006.png)

**Fig. 6.** Determination of conidial germination and appressorium formation on locust hind wings after 24 h of incubation. (a) Appressorium morphology. Appressorium formation was induced on locust hind wings using a method described previously (He & Xia, 2009). AP, appressorium; CO, conidium. (b) Conidial germination. (c) Frequency of appressorium formation. All experiments were repeated at least three times. Error bars, SD. The strains examined were WT, ΔMaHog1 and CP.
DISCUSSION

Data from earlier research have shown that Hog1 kinases are involved in stress responses (Banuett, 1998; Herskowitz, 1995; Treisman, 1996). In this work, a Hog1 kinase gene was identified in Me. acridum. Bioinformatic analysis suggested that MaHog1 encodes a member of the Hog1/Sty1/p38 MAPK family. Spot assays showed that WT growth was affected by hyperosmotic stress, high temperature and oxidative stress, but the ΔMaHog1 mutant grew even more slowly than WT under these stresses, demonstrating that MaHog1 disruption increased sensitivity to hyperosmotic stress, high temperature and oxidative stress. Thus, Hog1 kinase has a conserved function in regulating multistress responses in fungi.

Spot assays showed that the ΔMaHog1 mutant exhibited remarkable resistance to the cell wall-disturbing agents CR and CFW, indicating the relationship of MaHog1 to cell wall biogenesis. Similar results have been reported for ΔCahog1 mutants of Ca. albicans, and were attributed to higher phosphorylation of Cek1 (Fus3/Kss1 homologue) in ΔCahog1 mutants compared with the WT under CR stimulation (Eisman et al., 2006). It could be reasonably deduced that the resistance to cell wall-disturbing agents of the ΔMaHog1 mutant is possibly due to an increased level of Fus3/Kss1 phosphorylation.

Disruption of Bbhog1 suppresses expression of the hydrophobin-encoding genes hyd1 and hyd2 in Be. bassiana (Zhang et al., 2009). Inactivation of hyd1 and/or hyd2 decreases cell surface hydrophobicity (Zhang et al., 2011). In this study, MaHog1 disruption did not influence cell surface hydrophobicity in Me. acridum. A possible explanation is that relatively low expression of hydrophobin-encoding genes might maintain normal cell surface hydrophobicity.

Earlier research has demonstrated that Hog1 kinases make diverse contributions to pathogenicity in different fungi. In some pathogenic fungi, including the rice blast pathogen Ma. oryzae and the cucumber anthracnose pathogen Colletotricum lagenarium, Hog1 mutants remain fully pathogenic (Dixon et al., 1999; Kojima et al., 2004). In other pathogenic fungi, such as Ca. albicans, My. gramincola, Cr. parasitica, Be. bassiana and Bo. cinerea, Hog1 mutants show significantly reduced pathogenicity (Alonso-Monge et al., 1999; Mehrabi et al., 2006; Park et al., 2004; Segmüller et al., 2007; Zhang et al., 2009).

In the present study, the ΔMaHog1 mutant exhibited greatly reduced pathogenicity, and MaHog1 inactivation influenced both penetration and postpenetration development of Me. acridum during infection of the insect host. The inability of the ΔMaHog1 mutant to penetrate the host cuticle was probably caused by decreases in conidial germination and appressorium formation. With respect to postpenetration development, the ΔMaHog1 mutant showed significantly lower growth rates in vivo and in vitro compared with WT and CP controls. Spot assays showed that MaHog1 disruption decreased fungal tolerance of some stresses in vitro, such as oxidative stress, hyperosmotic stress and high temperature. Earlier research has indicated that pathogenic fungi encounter various stresses from the host insect during infection, such as reactive oxygen intermediates produced by the host (Bogdan et al., 2000), hyperosmotic stress in insect haemolymph (Chapman, 1998) and high temperature due to locust behavioural fever (Hunt & Charnley, 2011). Thus, MaHog1 might play important roles in adapting to various stresses from the insect host during infection. Taken together, the results show that MaHog1 disruption led to a significant reduction in virulence, likely due to the combination of a decrease in conidial germination, a reduction in appressorium formation and a decline in growth rate in insect haemolymph, which might be caused by impairing the fungal tolerance of various stresses during infection.

Knowledge of the signal transduction pathways involved in fungal stress resistance or pathogenesis is very important for mycoinsecticide improvement, and information on the roles and consequences of such genes will be necessary for suitable genetic engineering strategies (St Leger & Wang, 2010). As a regulator, MaHog1 contributes to fungal stress tolerance and virulence in Me. acridum, and it is possible to improve the WT strain by MaHog1 overexpression. Furthermore, genes involved in fungal stress resistance or pathogenesis identified by transcriptome analysis of a hog1 mutant and WT counterparts could be candidates for genetic improvement of entomopathogenic fungi for biocontrol.

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