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The HOG Pathway Plays Different Roles in Conidia and Hyphae During Virulence of *Alternaria alternata*

Olumuyiwa Igbalajobi, Jia Gao, and Reinhard Fischer†

Karlsruhe Institute of Technology (KIT)–South Campus, Institute of Applied Biosciences, Department of Microbiology, Fritz-Haber-Weg 4,D-76131 Karlsruhe, Germany

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The black mold *Alternaria alternata* causes dramatic losses in agriculture due to postharvest colonization and mycotoxin formation and is a weak pathogen on living plants. Fungal signaling processes are crucial for successful colonization of a host plant. Because the mitogen-activated protein kinase HogA is important for the expression of stress-associated genes, we tested a Δ*hogA*-deletion strain for pathogenicity. When conidia were used as inoculum, the Δ*hogA*-deletion strain was largely impaired in colonizing tomato and apple. In comparison, hyphae as inoculum colonized the fruit very well. Hence, HogA appears to be important only in the initial stages of plant colonization. A similar difference between conidial inoculum and hyphal inoculum was observed on artificial medium in the presence of different stress agents. Whereas wild-type conidia adapted well to different stresses, the Δ*hogA*-deletion strain failed to grow under the same conditions. With hyphae as inoculum, the wild type and the Δ*hogA*-deletion strain grew in a very similar way. At the molecular level, we observed upregulation of several catalase (*catA, -B*, and *-D*) and superoxide dismutase (*sodA, -B*, and *-E*) genes in germlings but not in hyphae after exposure to 4 mM hydrogen peroxide. The upregulation required the high osmolarity glycerol (HOG) pathway. In contrast, in mycelia, *catD, sodA, sodB*, and *sodE* were upregulated upon stress in the absence of HogA. Several other stress-related genes behaved in a similar way.

Keywords: *Alternaria*, fungal development, fungus–plant interactions, hyphae, mechanisms of pathogenicity, oxidative stress

Alternaria alternata is a widespread saprotrophic fungus and a weak pathogen (Woudenberg et al. 2015). It causes dramatic losses of food and feed every year, because of the production of a large variety of mycotoxins (Bräse et al. 2009; Rotem 1998). Many of such mycotoxins are polyketide derivatives or small peptides produced at nonribosomal peptide synthases. The genome of *A. alternata* encodes at least 10 different polyketide synthases and five nonribosomal peptide synthetase encoding genes (Dang et al. 2015; Saha et al. 2012; Voß et al. 2020). The most well-known mycotoxin is the polyketide alternariol (AOH), which is also a virulence factor (Graf et al. 2012; Wenderoth et al. 2019). The species *A. alternata* comprises different pathotypes, some of which produce host-specific toxins (HSTs) (Friesen et al. 2008; Meena and Samal 2019). Such HSTs confer the host specificity of the corresponding species, and nonhosts are resistant due to tolerance to the toxin (Scheffer and Livingston 1984).

Plants have different strategies to cope with microbial infections, one of which is the production of reactive oxygen species (ROS), also known as oxidative burst (O'Brien et al. 2012). As a consequence, successful pathogens should be able to inactivate ROS. Many fungi adapt to stressful conditions using signaling cascades, which finally cause activation of corresponding genes coding for catalases or superoxide dismutases (SODs) (Garrido-Bazán et al. 2018). One central module in the fungal stress response is the high osmolarity glycerol (HOG) pathway discovered 20 years ago (Brewster and Gustin 2014). It has been studied extremely well in *Saccharomyces cerevisiae* but also in many filamentous fungi. The module is used to adapt to osmotic stress, but also to other stresses. Therefore, the gene was named *stress-activated kinase A* (*sakA*) in *Aspergillus nidulans* (Jaimes-Arroyo et al. 2015; Kawasaki and Aguirre 2001; Kawasaki et al. 2002). It is also essential for red-light sensing in *Aspergillus nidulans* and in *A. alternata* (Igbalajobi et al. 2019; Yu et al. 2016). Given its central role in many stress-signaling pathways, it is not surprising that it is also required for successful plant colonization (e.g., of *Magnaporthe grisea, Botrytis cinerea*, or *Cryphonectria parasitica*) (Dixon et al. 1999; Liu et al. 2008; Park et al. 2004). Here, we show that the HOG pathway is important for stress adaptation when conidia but not when hyphae are used as inoculum.

RESULTS

Infection of tomato and apple with *A. alternata* conidia or mycelia.

For successful colonization of fruit, *A. alternata* has to cope with the defense mechanisms of the fruit, one of which is ROS resulting from the oxidative burst of the plants (Baker and Orlandi 1995; Heller and Tudzynski 2011; Levine et al. 1994). *A. alternata* is able to react to stressful conditions through activation of the HOG pathway (Graf et al. 2012; Igbalajobi et al. 2019). Here, we tested the colonization potential of *A. alternata* wild-type (WT) (ATCC66981) and Δ*hogA* mutant strains on tomato and apple. Hyphal growth of the Δ*hogA*-deletion strain

Present address of O. Igbalajobi: Michael Smith Laboratories, Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, VGT 1Z4 Canada

†Corresponding author: R. Fischer; reinhard.fischer@KIT.edu

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was impaired when grown on modified Czapek Doth broth (mCDB) medium and largely inhibited on high-osmolarity medium or under oxidative stress (Igbalajobi et al. 2019). Therefore, we anticipated that virulence of the *hogA* mutant strain is also impaired. This hypothesis was tested with tomato and apple as fruit. Both fruit were surface sterilized by immersion in 70% ethanol for 5 min, then rinsed with sterile water and air dried on a clean bench. The air-dried fruit were wounded (approximately 1 cm in depth) with a sterile scalpel prior to inoculation with 10 µl of 1×10^5 fresh conidia of the WT and *hogA* mutant strains. After 7 days at 20°C in darkness, necrotic lesions were monitored. The *hogA* mutant strain was, indeed, strongly impaired in the ability to colonize either of the fruit (Fig. 1). The effect is partly due to a delay in germination. Whereas, in the WT after 3 h, almost 100% of the conidia were germinated, the Δ*hogA*-deletion strain needed 12 h for the same germination rate. Because the virulence experiments were incubated for 7 days, the germination delay at the beginning of the experiment had only a minor contribution.

As an alternative method to perform such infection studies, mycelial plugs can be used as inoculum instead of conidia. We also used this method to compare the virulence of WT and the *hogA*-deletion strain. Tomato and apple were wounded with a cork borer 5 mm in diameter. Wounds were inoculated with mycelial plugs from plates after 36 h of active growth of the WT and mutant strains. All samples were incubated at 20°C for 7 days in darkness for lesion development. WT and *hogA* mutant strains caused the same symptoms and degree of infection.

A. alternata conidia germinate in the absence of nutrients on the plant surface.

The strong difference in virulence when mycelium was used as inoculum rather than conidia raised the question of the relevance of this phenomenon in nature. *A. alternata* disseminates in the environment as conidia, which land on fruit surfaces. If conidia land on a small injury, this will be the entry point for the fungus. We tested whether *A. alternata* ATCC66981 is able to germinate in pure water without any nutrients. In both mCDB liquid medium and on water agar, germination occurred quickly; 30% of the conidia had already germinated after 2 h. After 4 h, approximately 90% had produced a germ tube (Fig. 2A and B). This is in contrast to other fungi, such as *Aspergillus nidulans*, which germinate very poorly without nutrients. We also tested germination on the apple surface. Both WT- and Δ*hogA*-derived conidia were able to germinate on the apple surface, although germination of the Δ*hogA*-deletion strain was delayed (Fig. 2C and D). This shows that *A. alternata* conidia are able to germinate in water on fruit surfaces, suggesting that, under wet climate conditions, hyphae are likely to be the inoculum for small injuries. This may be one important factor to explain the increased susceptibility of fruits toward *A. alternata* in years with long rain periods. Whereas, under dry conditions, injuries can only be infected by conidia landing directly on the wounds, during wet seasons, conidia that land anywhere on the fruit surface can germinate and reach wounds as mycelium.

The HOG pathway is crucial for young germlings but not for mycelial growth.

In order to understand the observed difference in virulence of conidia and hyphae and the role of the HOG pathway, we investigated the effect of osmotic, oxidative, and cell-wall-degrading agents on growth initiated from conidia and from mycelial plugs of WT and *hogA* mutant strains on medium supplemented with these agents. Plates were incubated at 28°C for 4 days in the dark (Fig. 3). The inactivation of *hogA* resulted in high sensitivity of conidia to hydrogen peroxide (H_2O_2) and menadione as well as sorbitol compared with the WT strain (Fig. 3). Interestingly, mycelia of the *hogA* mutant strain was as resistant to oxidative stress as the WT strain (Fig. 3). In the

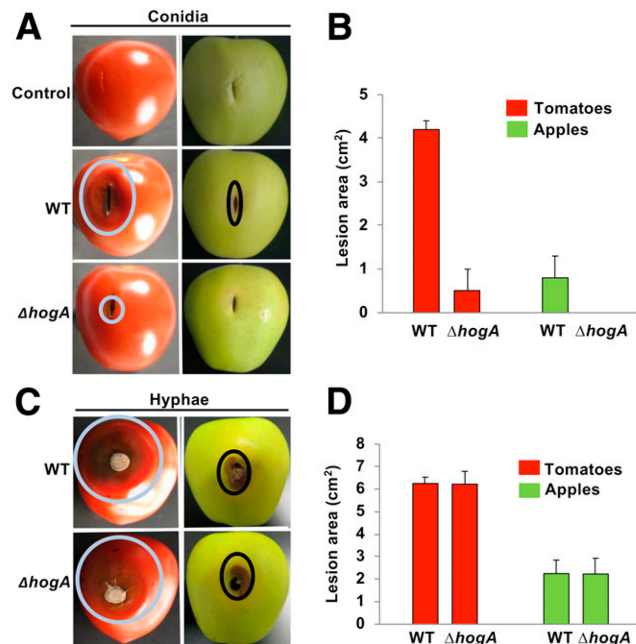

Fig. 1. High osmolarity glycerol A (HogA) is essential for conidial but not hyphal colonization of *Alternaria alternata* on tomato and apple. **A,** Fresh conidia of wild-type (WT) and Δ*hogA* mutant strains were inoculated on tomato and apple and incubated at 20°C for 7 days in the dark. To the control tomato, water was added instead of a conidial suspension. **B,** Quantification of the lesion areas in panel A. **C,** Mycelial plugs from plates after 36 h of active growth of the WT and mutant strains were inoculated in a 0.5-cm-diameter hole and incubated as described above. **D,** Quantification of the lesion areas in panel C. The mean value was calculated from three biological replicates. The arrow bar represents the standard deviation.

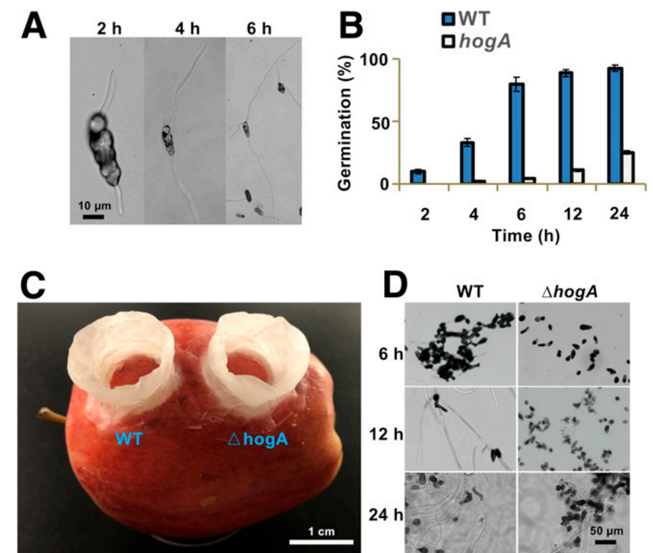

Fig. 2. Analysis of the germination of spores of wild-type (WT) and Δ*hogA*-deletion strains. **A,** Germination of conidia in water agarose at 20°C observed after 2, 6, and 12 h of incubation. **B,** Quantification of the germination rate after incubation for 2, 4, 6, 12, and 24 h. For each time point, 100 conidia (or germlings) were counted. The error bar represents the standard deviation of three replicates. **C,** Apple with two plasticine chambers mounted on the surface. The chambers were filled with 1 ml of water (H_2O) and were inoculated with 1×10^5 fresh conidia of the WT and the Δ*hogA*-deletion strain. **D,** Samples for microscopy were removed after 6, 12, and 24 h.

presence of 1.2 M sorbitol, growth of the *hogA*-deletion strain was still severely impaired. The results indicate that hyphae and conidia of *A. alternata* have different sensitivity toward oxidative stress.

To investigate how the different sensitivities of conidial inoculation as compared with hyphal inoculation are reflected in differential gene expression, we determined the expression level of a number of candidate genes in the presence and absence of 4 mM H_2O_2. In addition, we analyzed the role of HogA in the different mycelial stages. The first candidate was the catalase encoding gene, *catA*. In *Aspergillus nidulans* and *A. fumigatus*, *catA* accumulates during asexual spore formation (Navarro and Aguirre 1998; Navarro et al. 1996; Paris et al. 2003). In small germlings of *A. alternata* treated with H_2O_2, *catA* was induced to approximately 34-fold compared with untreated samples (Fig. 4). The loss of *hogA* resulted in the complete loss of induction of *catA*. Hence, HogA appears to be a positive regulator for the induction of *catA* during germination. The next candidate was *catB*. *catB* is found in the mycelium in *Aspergillus nidulans* and, together with *catA*, though to a lower extent, protects the fungus against oxidative and heat stress (Kawasaki et al. 1997). The regulation of *catB* in *A. alternata* appeared to be very similar to the regulation of *catA* (Fig. 4). The same was true for *catD* (Fig. 4). The *catD* gene of *Aspergillus nidulans* is induced during glucose starvation, the stationary phase, and treatment with H_2O_2 (Kawasaki and Aguirre 2001).

In addition to catalases, SODs also protect fungi from oxidative stress. In *A. alternata*, the expression of three SOD-encoding genes (*sodA, sodB*, and *sodE*) was induced in the presence of H_2O_2 and the induction was dependent on HogA, although to different extents (Fig. 4).

Next, we tested the regulation of *A. alternata srrA* by H_2O_2. In *Aspergillus nidulans* the response regulator and transcription factor SrrA is involved in stress signal transduction (Vargas-Pérez et al. 2007). The expression of *srrA* was induced by H_2O_2 and the induction required HogA (Fig. 4).

Orthologs of the transcription factor Yap1 in *Epichloë festucae*, *A. alternata*, and other filamentous fungi regulate the expression of different sets of genes involved in ROS detoxification (Cartwright and Scott 2013; Guo et al. 2011; Lin et al. 2009; Molina and Kahmann 2007). These may include catalases, SODs, and peroxidases. In *A. alternata*, Yap1 is clearly important for virulence (Lin et al. 2009; Lin et al. 2018). We observed H_2O_2 induction of *yap1* by 12-fold compared with the sample without the treatment. In the *hogA* mutant strain, the induction was reduced to 40%. This suggests a partial crosstalk between the HogA pathway and the transcription factor Yap1 in the modulation of the oxidative stress response in *A. alternata* germlings.

In mycelia, *catA* was expressed in standard medium but the expression level was reduced after addition of H_2O_2 (Fig. 5). All other genes showed low expression, and the expression level did not respond to H_2O_2. In the *hogA mutant* strain, genes

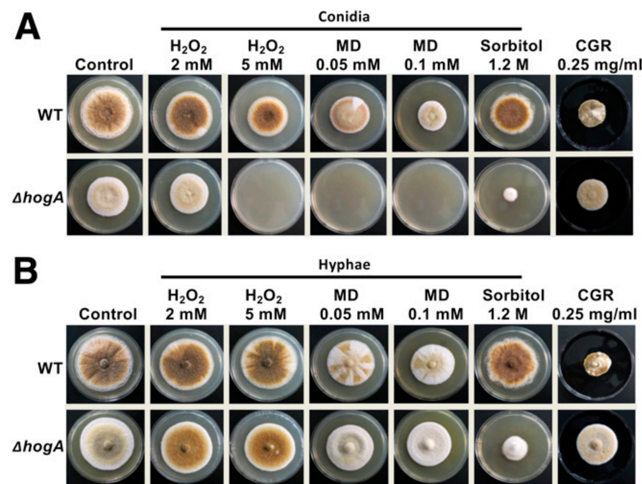

Fig. 3. Role of HogA under different stress conditions using conidia or hyphae as inoculum of agar plates. **A,** Fresh conidia or **B,** mycelial plugs from the edge of 2-day-old colonies of wild-type (WT) and *ΔhogA* mutant strains were inoculated on modified Czapek Doth broth supplemented with different concentrations of hydrogen peroxide (H_2O_2) and menadione (MD), 1.2 M sorbitol, or Congo red (CGR) at 0.25 mg/ml and incubated for 4 days at 28°C in the dark.

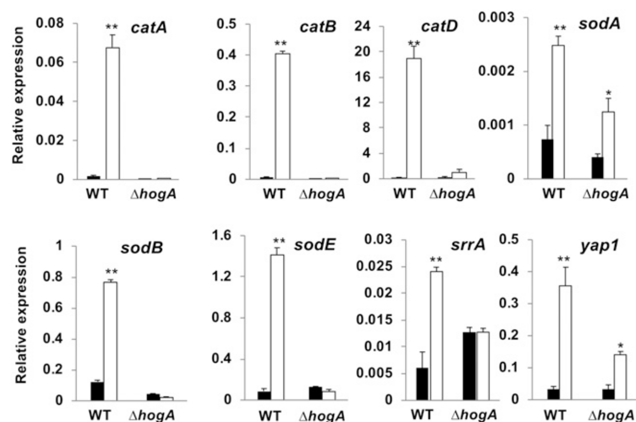

Fig. 4. Expression analysis of oxidative stress-regulated genes in germlings and the role of HogA. RNA was extracted from cultures of wild-type (WT) and *ΔhogA* mutant strains grown on modified Czapek Doth broth medium for 6 h (shaking) at 28°C and thereafter supplemented with 4 mM hydrogen peroxide (H_2O_2) and further incubated for 30 min. mRNA transcript expression levels of the selected genes were normalized using the *H2B* gene. Bars present mean values ± standard deviation of three biological replicates. Statistical analysis was performed with Student's *t* test. Asterisks * and ** indicate $P \leq 0.05$ and 0.01, respectively.

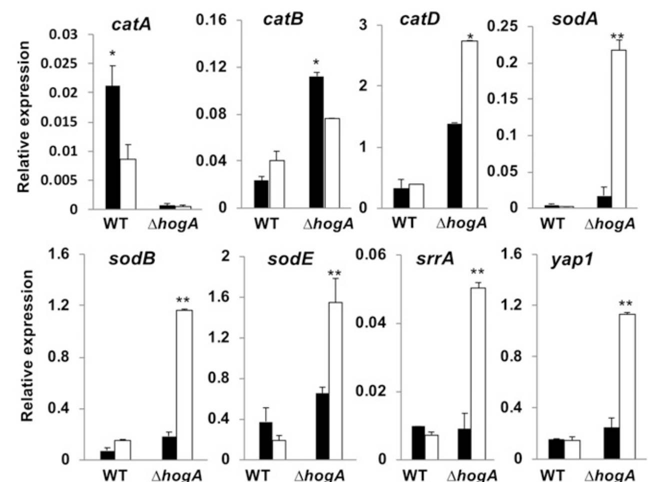

Fig. 5. Transcriptional profiling of oxidative stress-regulated genes in mycelia and the role of HogA. The WT and *ΔhogA* mutant strains were grown on modified Czapek Doth broth liquid medium for 36 h (shaking) at 28°C and then supplemented with 4 mM hydrogen peroxide (H_2O_2) and further incubated for 30 min. mRNA transcript expression levels of the selected genes were normalized using *H2B*. Bars present mean values ± standard deviation of three biological replicates. Statistical analysis was performed with Student's *t* test. Asterisks * and ** indicate $P \leq 0.05$ and 0.01, respectively.

were more highly expressed and the expression of several of them was induced upon exposure to H_2O_2 (Fig. 5).

Taken together, our results suggest that, although the HogA pathway is essential for oxidative stress response in germlings, it is dispensable for the same response in mycelia. This suggests the presence of independent pathways to cope with oxidative response in the mycelia in the absence of HogA.

DISCUSSION

A. alternata is able to colonize many crops and causes large annual losses in agriculture (Rotem 1998). *A. alternata* is not a strong pathogen and usually is pathogenic only on weakened plants. One important external parameter for colonization success is humidity, especially in combination with changing temperatures, when water droplets are formed through condensation. Here, we show that strain ATCC66981, like other *Alternaria* strains, is able to germinate without any nutrients on fruit surfaces. Therefore, intact conidia or hyphae can be the infectious agent in nature. We found a strong difference in the colonization efficiency between the two different inocula, and different roles for the HogA MAP kinase. We present evidence that one parameter for the increased virulence of the *hogA* mutant hyphae is the increased expression of genes encoding enzymes required to cope with oxidative stress.

In WT germlings, many enzymes involved in the oxidative stress response showed low expression but were strongly induced upon exposure to H_2O_2. In the *hogA* mutant, such transcriptional response of those genes was not observed. For the virulence assay, this means that conidia as inoculum are not very effective. In addition, germination of conidia is delayed in *hogA* mutants. In contrast, in mycelia, the genes were still expressed in the WT but were not upregulated anymore upon oxidative stress. The level, however, appears to be sufficient for colonization of tomato and apple. In contrast, most genes were derepressed in mycelia and further stimulated in the presence of H_2O_2. Of course, this is very advantageous for colonization of the fruit. This raises the question of how the genes are induced in the absence of HogA. There is excellent evidence for alternative pathways under stressful conditions, which make the stress response very robust. There are at least three pathways important in fungi to cope with oxidative stress. The indicative transcription factors for each pathway in *Aspergillus nidulans* are AtfA, SrrA, and NapA (Yap1 in *S. cerevisiae* and *A. alternata*) (Lin et al. 2018; Mendoza-Martínez et al. 2017). They control the oxidative response at different stages in the life cycle. We observed that the catalase and other genes encoding antioxidant enzymes are not induced in *A. alternata* WT mycelia after H_2O_2 exposure. If this would be also the case during fruit infection with hyphae as inoculum, other antioxidant systems have to be in place to cope with the stressful conditions during infection. One possibility are thioredoxins, which are important for *B. cinerea* virulence (Viefhues et al. 2014).

In addition to the resistance of hyphae to oxidative stress conditions, other virulence factors may also be important. It was shown that secondary metabolites such as alternariol contribute to the colonization capability of *A. alternata* as well (Graf et al. 2012; Wenderoth et al. 2019). However, it is unlikely that AOH was very important in our experiments, because it was shown that the Δ*hogA* deletion strain does not produce AOH (Igbalajobi et al. 2019). Nevertheless, the Δ*hogA* hyphae colonized tomato and apple well. Of course, other metabolites or virulence factors could be important and could be different in hyphae and conidia. To address this question, genome-wide expression analyses should be performed in future experiments.

Taken together, we show that the expression pattern of several genes involved in the oxidative stress response of *A. alternata* are differently regulated in germlings as compared with mycelia, and that this may contribute to different pathogenic potentials. Oxidative stress is certainly one important factor that plant pathogens have to cope with (Baker and Orlandi 1995; Levine et al. 1994; Ma et al. 2018). However, many other factors (e.g., iron acquisition) (Chen et al. 2014; Voß et al. 2020) are probably equally important, and the next challenge will be to study the observed phenomena at a genome-wide level. There is evidence that AtfA, SrrA, and NapA are controlling a large number of genes, many of which could be important for virulence (Chen et al. 2017; Mendoza-Martínez et al. 2017).

Table 1. Oligonucleotides used in this study

Oligonucleotide	Sequence 5′–3′
catA_RT_fw	GGCATTCTTACCGACACATCG
catA_RT_rv	TGTGTAGAACTTGACGGCGAAA
catB_RT_fw	CCACGGCACCTTTGTTTCTT
catB_RT_rv	AGATCGGTGTCTCCTTTCCT
catD_RT_fw	CAACGTCTCCCTCGACAAG
catD_RT_rv	CAGTGAGAAGCATCAAGTCGG
sodA_RT_fw	ACACCACCATCTCATGGAACAT
sodA_RT_rv	CGTGTGTCTTGTTGTGGGGTT
sodB_RT_fw	GAGGCCAAGCAAAAGGAAGAC
sodB_RT_rv	GAAGAGGCTGTGGTTGATGTG
sodE_RT_fw	GAGGCCAAGCAAAAGGAAGAC
sodE_RT_rv	GAAGAGGCTGTGGTTGATGTG
srrA_RT_fw	GGATCCCAATCAAGCTGGTG
srrA_RT_rv	CCATCGCACAACACTCTCGT
yap1_RT_fw	GCAGGAACTACCAACGACTT
h2B_RT_fw	ACAAGAAGAAGCGCACCAAG
h2B_RT_rv	CGTTGACGAAAGAGTTGAGAA

MATERIALS AND METHODS

Strains, plasmids, and culture conditions.

A. alternata ATC 66981 cultures were grown on mCDB agar. The *hogA*-deletion strain has been described before SOI3 (Igbalajobi et al. 2019). Oligonucleotides used in this study are listed in Table 1.

Virulence assays.

Virulence assays were conducted on prewounded tomato or apple inoculated with conidial suspension as described previously, with little modification (Wenderoth et al. 2019; Zhang et al. 2014). Prior to inoculation, tomato and apple were surface sterilized with 70% ethanol and scratched with a sterile scalpel (1 cm deep). Fresh conidial suspension (10 μl) containing 10^5 conidia of WT and *hogA* mutant strains were inoculated on prewounded tomato and apple fruit. In the case of the mycelial plug test, tomato and apple were wounded with a cork borer 5 mm in diameter. Wounds were inoculated with mycelial plugs from plates after 36 h of active growth of the WT and mutant strains. All samples were incubated at 20°C for 7 days for lesion development. Lesions on the surface of the tomato and apple were measured manually.

Germination assay.

Fresh conidia containing 1×10^5 fresh conidia were incubated in water agarose (1% agarose) on a slide at 20°C in a humid chamber and the number of germinated conidia ($n = 100$) was determined microscopically after 2, 4, 6, 12, and 24 h. For the analysis of germination on an apple surface, small chambers of plasticine were mounted on the surface, filled with H_2O_2, and inoculated with 1×10^5 fresh conidia. Samples were taken for microscopy after 6, 12, and 24 h.

Assays for cellular stress.

Fresh conidia of different strains were collected from cultures grown on mCDB plates incubated at 28°C for 12 days. Drops of conidial suspension containing 5,000 conidia of the WT and Δ*hogA* were inoculated on mCDB supplemented with sorbitol for salt stress and with H_2O_2 (2 mM and 5 mM) or menadione (0.5 and 1 mM) for oxidative stress. To induce cell wall stress, Congo Red (0.25 mg/ml) was added to the medium. To analyze stress responses in hyphae, mycelial plugs from plates after 36 h of active growth of the WT and mutant strains were inoculated on mCDB supplemented with chemical agents as described above. All cultures were incubated at 28°C for 4 days. The experiments were carried out in triplicate.

RNA isolation and quantitative real-time PCR.

In order to investigate oxidative stress responses, transcripts of genes induced by oxidative agents were analyzed. Prior to the addition of the stress agent, fresh conidia were inoculated in a 50-ml flask of liquid mCDB medium and incubated for 6 h at 28°C and 180 rpm. The culture was then supplemented with 4 mM H_2O_2 and further shaken for 30 min. The spores were harvested in complete darkness and frozen immediately in liquid nitrogen. In the case of mycelia, fresh conidia were inoculated in a 50-ml flask with liquid mCDB medium and incubated for 36 h at 28°C and 180 rpm. The cultures were then supplemented with 4 mM H_2O_2 and further shaken for 30 min. The frozen samples were ground into powder, and total RNA was isolated using the E.Z.N.A. fungal RNA minikit (VWR). The isolated RNA was quantified and an aliquot was purified with the RNA TURBO DNA-free KIT. RNA samples were diluted to a final concentration of 50 ng/µl. Quantitative real-time PCR (RT-qPCR) was carried out using gene-specific primers (Table 1), with a SensiFAST SYBR & No-ROX One-Step Kit from Bioline (Luckenwalde, Germany). Each reaction mixture was 25 µl, with 0.2 µM primers and 100 ng of RNA. RT-qPCR was performed as follows: incubation for 10 min at 50°C for the reverse transcription reaction; then 5 min at 95°C for the inactivation of reverse transcription; followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 58°C for 30 s, and extension at 72°C for 15 s; followed by a melting curve analysis in order to check the specificity of fragment amplification. After each PCR, we performed melting curve analyses to show the specific amplification of single DNA segments and the absence of nonspecifically amplified DNA. Transcript levels of the target genes were normalized against histone *H2B* gene expression. All measurements were repeated twice, each with three biological replicates.

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