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The role of AtfA and HOG MAPK pathway in stress tolerance in conidia of *Aspergillus fumigatus*

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ABSTRACT

Aspergillus fumigatus is a life-threatening pathogenic fungus, whose conidium is the infectious agent of aspergillosis. To better understand the mechanism underlying the long-term viability of conidia, we characterized a bZip transcription factor, AtfA, with special reference to stress-tolerance in conidia. The *atfA* deletion mutant conidia showed significant sensitivity to high temperature and oxidative stress. The trehalose content that accumulated in conidia was reduced in the mutant conidia. Transcriptome analysis revealed that AtfA regulated several stress-protection-related genes such as *catA*, *dprA*, *scf1*, and *conJ* at the conidiation stage. The upstream high-osmolarity glycerol pathway was also involved in conferring stress tolerance in conidia because Δ*pbsB* showed stress sensitivity and reduced trehalose in conidia. However, a mutant lacking the SakA mitogen-activated protein kinase (MAPK) produced normal conidia. We investigated another MAPK, MpkC, in relation with SakA, and the double deletion mutant, Δ*sakA,mpkC*, was defective in conidia stress tolerance. We concluded that MpkC is able to bypass SakA, and the two MAPKs redundantly regulate the conidia-related function of AtfA in *A. fumigatus*.

1. Introduction

Aspergillus fumigatus is an important opportunistic pathogen that causes life-threatening invasive aspergillosis. Inhalation of the conidia is the major infection route (Latgé, 1999). Conidia are ubiquitous in the air, and we inhale them daily. The inhaled conidia survive and infect the lungs of immunocompromised patients, whereas they are effectively cleared in a healthy host's lung by professional phagocytes (Latgé, 2001). *A. fumigatus* conidia are 2.0–3.0 μm in size, which is adequate to reach the lower airways. Another notable characteristic is the high hydrophobicity that enables the conidia to disperse efficiently in the air. These properties of conidia are thought to make the fungus one of the most prevalent pathogenic fungi for humans (Kwon-Chung and Sugui, 2013).

Conidia are stress-resistant vehicles that are produced by many fungal species including the genera *Aspergillus* and *Penicillium*. Compatible solutes including trehalose and mannitol confer tolerance to environmental stresses such as heat, desiccation, and oxidative stress to the conidia (Wyatt et al., 2013). *Aspergillus niger* predominantly accumulates mannitol in the conidia, which is required for heat and oxidative stress resistance (Ruijter et al., 2003). The primary biosynthesis pathway for the conidial mannitol involves the reduction of fructose 6-phosphate to mannitol 1-phosphate, followed by dephosphorylation, resulting in mannitol. The trehalose biosynthesis mechanism was investigated in *Aspergillus nidulans* with regard to accumulation in the conidia (Fillinger et al., 2001). The *tpsA* gene, encoding trehalose 6-phosphate (T6P) synthase, is crucial for trehalose production in conidia as well as hyphae in response to heat shock. Conidia of the *tpsA* mutant strain showed reduced viability during prolonged storage and increased sensitivity to high temperature during germination. In *A. fumigatus*, the trehalose biosynthesis genes *tpsA-D* were characterized, and *tpsA* and *tpsB* were found to redundantly contribute to trehalose accumulation in conidia and hyphae (Al-Bader et al., 2010). The *tpsAB* double deletion mutant had reduced trehalose content in both conidia and hyphae. Conidia of the mutant were susceptible to heat and oxidative stress, and they exhibited a delayed germination phenotype. Interestingly, the mutant that was deficient in trehalose metabolism was hypervirulent in a murine model of invasive aspergillosis. Cramer and his colleagues investigated *A. fumigatus orlA* gene encoding a putative T6P phosphatase that converts T6P to trehalose (Puttikamonkul et al., 2010). Although the mutant lacking *orlA* persists trehalose production, high levels of T6P accumulate in the mutant. Infection experiment revealed that lack of *orlA* attenuated *A. fumigatus* virulence.

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Overall, it was suggested that trehalose biosynthesis pathway poses a pathogenic impact on the pathogenic fungus *A. fumigatus*.

The ATF/CREB-type transcription factor AtfA has been characterized as a regulator of conidia stress tolerance in *A. nidulans* and *Aspergillus oryzae*. The *atfA* mutant conidia of *A. nidulans* showed significant sensitivity to oxidative stress and heat stress (Hagiwara et al., 2008). AtfA is required for the expression of *tpsA* in freshly harvested (FH) conidia, suggesting a function in regulating trehalose accumulation in the conidia. In addition to the *tpsA* gene, AtfA is responsible for the expression of the conidia-specific catalase gene *catA*. These regulatory roles seem to reflect the stress-sensitive phenotypes of the *atfA* mutant conidia in *A. nidulans*. *A. oryzae* has three ATF-type proteins, and two of them, AtfA and AtfB, have been characterized (Sakamoto et al., 2008, 2009). The conidia of *atfA* and *atfB* deletion mutants showed an increased sensitivity to several stresses such as oxidative stress, UV irradiation, and heat stress, although the stress-sensitive phenotype was much more severe in the *atfA* mutant conidia. Interestingly, trehalose amounts in the conidia were comparable between these two mutants, which had a level that was approximately half that of the control strain. In line with these observations, the expression of the *tps* genes, *tps1* and *tps3*, was down-regulated in both *atfA* and *atfB* mutant conidia (Sakamoto et al., 2009). Together, these findings suggested a role of the AtfA transcription factor in the stress tolerance of *Aspergillus* conidia. Nonetheless, the ATF-type transcription factors' function remains unknown in *A. fumigatus*. The conidium is an infectious agent that is transmitted from environmental niches to human's lungs. Therefore, a better understanding of the molecular regulatory mechanism underlying conidia stress tolerance would be useful for infection prevention of aspergillosis, the development of new antifungals, and the prevention and elimination of food-contaminating conidia.

In this study, we characterized *A. fumigatus* AtfA with respect to conidia stress tolerance by constructing the deletion mutant. The *atfA* mutant showed significant stress sensitivity in conidia but not hyphae. Genome-wide transcriptome analysis revealed a set of conidia-specific genes associated with stress tolerance that was under the control of AtfA. We further presented an upstream pathway regulating the AtfA function in conidia. This is the first report to suggest that the MpkC mitogen-activated protein kinase (MAPK) is involved in conidia stress tolerance in combination with the paralogous MAPK SakA. Based on our findings, we postulated a new model in which SakA and MpkC play a cooperative role in conferring stress tolerance to the conidia via the AtfA transcription factor, whereas SakA is a predominant stress-activated MAPK in hyphal growth of *A. fumigatus*.

2. Material and methods

2.1. Strains and growth media

A. fumigatus strain AfS35 (FGSC A1159) (*akuA::loxP*) was used to generate the following deletion strains used in this study: Δ*atfA*, Δ*ccg9A*, Δ*ccg9B*, Δ*ccg9A,ccg9B*, Δ*mpkC*, Δ*sakA,mpkC*, and Δ*pbsB* (Krappmann et al., 2006). The sequences of these genes, corresponding to Afu3g11330 (*atfA*), Afu3g12100 (*ccg9A*), Afu5g14780 (*ccg9B*), Afu5g09100 (*mpkC*), and Afu1g15950 (*pbsB*), were retrieved from the AspGD website (http://www.aspgd.org/). The fungal strains used in this study are listed in Table 1. All strains were routinely cultivated in 0.1% yeast extract-containing glucose minimal medium (YGMM) at 37 °C. To collect conidia of each strain, potato dextrose agar (PDA) was used. For plates containing osmotic stress, 1.2 M sorbitol or 1 M NaCl was added. In liquid culture for glycerol quantification, 10 mL 3 M sorbitol or 2.4 M NaCl was added to 20 mL YGMM (final concentration 1 M or 0.8 M, respectively).

Table 1
Fungal strains used in this study.

Strain	Description	Reference
AfS35(FGSC A1159)	Wild type (*akuA::loxP*)	Krappmann et al. (2006)
ΔatfA	*atfA::HygB*^{r}	This study
Co-atfA	*atfA::HygB*^{r} + *atfA*	This study
Δccg9A	*ccg9A::ptrA*^{r}	This study
Δccg9B	*ccg9B::HygB*^{r}	This study
Δccg9A,ccg9B	*ccg9A::ptrA*^{r}, *ccg9B::HygB*^{r}	This study
ΔsskA	*sskA::HygB*^{r}	Hagiwara et al. (2013)
ΔsakA	*sakA::HygB*^{r}	Hagiwara et al. (2013)
ΔmpkC	*mpkC::ptrA*^{r}	This study
ΔmpkC,sakA	*mpkC::ptrA*^{r}, *sakA::HygB*^{r}	This study
ΔpbsB	*pbsB::ptrA*^{r}	This study

For the synchronized induction of asexual development, conidia (10^5 conidia/mL) were cultivated in liquid YGMM for 18 h, and mycelia were harvested using Miracloth (Merck, Frankfurt, Germany), washed with distilled water, and transferred onto YGMM plates. These plates were incubated at 37 °C (this time point was set as 0 h), and the mycelia were harvested at appropriate times.

2.2. Construction of the gene deletion and reconstituted strains

To construct the *A. fumigatus* deletion and reconstituted strains, plasmids for each purpose were generated. DNA manipulation was performed according to standard laboratory procedures for recombinant DNA. To amplify DNA fragments from the genome and prepare gene replacement cassettes for transformation, Prime STAR HS (Takara BIO, Otsu, Japan) was used. Plasmids for the preparation of the cassettes or transformation were constructed by GeneArt Seamless Cloning and Assembly Kit (Invitrogen). Primers used in this study are listed in Table S1.

To generate a replacement cassette for the *atfA* gene, the 5′- and 3′-flanking regions were obtained using the primers atfA-F(pUC119B) and atfA-U-R(HygBr) (for the 5′-region) and atfA-D-F(HygBr) and atfA-R(pUC119E) (for the 3′-region). These flanking regions and a *hygB*^{r} fragment, a hygromycin B resistant marker that was amplified from pCB1004 plasmid, were fused into pUC119 using the GeneArt system, resulting in plasmid pUC119-atfA::hygB, from which the cassette for transformation was amplified.

To generate replacement cassettes for the *ccg9A* and *ccg9B* genes, the 5′- and 3′-flanking regions for each gene were obtained using the primers ccg9A-U-F(pUC119B) and ccg9A-U-R(ptrA) (for the *ccg9A* 5′-region) and ccg9A-D-F(ptrA) and ccg9A-R(pUC119E) (for the *ccg9A* 3′-region) or ccg9B-U-F(pUC119B) and ccg9B-U-R(HygBr) (for the *ccg9B* 5′-region) and ccg9B-D-F(HygBr) and ccg9B-R(pUC119E) (for the *ccg9B* 3′-region). These flanking regions and *ptrA* or *hygB*^{r} fragments were fused into pUC119 using the GeneArt system, resulting in plasmids pUC119-ccg9A::ptrA and pUC119-ccg9B::hygB, from which the cassettes for transformation were amplified.

To generate replacement cassettes for the *mpkC* and *pbsB* genes, the 5′- and 3′-flanking regions for each gene were obtained using the primers mpkC-U-F(pUC119B) and mpkC-U-R(ptrA) (for the *mpkC* 5′-region) and mpkC-D-F(ptrA) and mpkC-R(pUC119E) (for the *mpkC* 3′-region) or pbsB-U-F(pUC119B) and pbsB-U-R(ptrA) (for the *pbsB* 5′-region) and pbsB-D-F(ptrA) and pbsB-R(pUC119E) (for the *pbsB* 3′-region). These flanking regions and a *ptrA* fragment were fused into pUC119 using the GeneArt system, resulting in plasmids pUC119-mpkC::ptrA and pUC119-pbsB::ptrA, from which the cassettes for transformation were amplified.

To generate plasmid pPRTI-atfA, the *atfA* fragment containing the 5′- and 3′-flanking regions were obtained using the primers atfA + P1100-F(pPTR-p) and atfA + T600-R(pPTR-k). This fragment