AbaA and WetA govern distinct stages of *Aspergillus fumigatus* development

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The opportunistic human pathogen *Aspergillus fumigatus* produces a massive number of asexual spores (conidia) as the primary means of dispersal, survival, genome protection and infection of hosts. In this report, we investigate the functions of two developmental regulators, *AfuAbaA* and *AfuWetA*, in *A. fumigatus*. The *AfuAbaA* gene is predicted to encode an ATTS/TEA DNA-binding domain protein and is activated by *AfuBrLA* during the middle stage of *A. fumigatus* asexual development (conidiation). The deletion of *AfuAbaA* results in the formation of aberrant conidiophores exhibiting reiterated cylinder-like terminal cells lacking spores. Furthermore, the absence of *AfuAbaA* causes delayed autolysis and cell death, whereas the overexpression of *AfuAbaA* accelerates these processes, indicating an additional role for *AfuAbaA*. The *AfuWetA* gene is sequentially activated by *AfuAbaA* in the late phase of conidiation. The deletion of *AfuWetA* causes the formation of defective spore walls and a lack of trehalose biogenesis, leading to a rapid loss of spore viability and reduced tolerance to various stresses. This is the first report to demonstrate that *WetA* is essential for trehalose biogenesis in conidia. Moreover, the absence of *AfuWetA* causes delayed germ-tube formation and reduced hyphal branching, suggesting a role of *AfuWetA* in the early phase of fungal growth. A genetic model depicting the regulation of conidiation in *A. fumigatus* is proposed.

**INTRODUCTION**

*Aspergillus fumigatus* is a saprophytic soil fungus that causes more infections worldwide than any other mould. Inhalation of asexual spores (conidia) by immunocompromised individuals may lead to germination of spores and growth of the fungal cells in the lung, followed by progression to a serious disease called invasive pulmonary aspergillosis (IPA; reviewed by Cockrill & Hales, 1999; Dagenais & Keller, 2009; Rhodes & Askew, 2010). Recently, despite advances in antifungal therapy, the incidence of IPA has increased significantly and the mortality of patients has risen to approximately 35% (Ejzykowicz et al., 2009; Gao et al., 2010; Agarwal et al., 2010). As the infectious life cycle of *A. fumigatus* usually begins with the production of conidia that are small enough to be deposited in the alveoli after being inhaled (Wasylka & Moore, 2003), it is important to understand the underlying mechanisms of sporulation in this fungus. This may contribute to the development of new prevention strategies for *Aspergillus* infection.

Asexual sporulation (conidiation) in *A. fumigatus* involves the elaboration of a multicellular reproductive structure called a conidiophore that requires activity of the key regulator BrLA (Fig. 1a; Mah & Yu, 2006; reviewed by Yu et al., 2006). The developmental mechanisms in *Aspergillus nidulans* have been studied intensively and the findings have provided important clues for understanding conidiation in other aspergilli (reviewed by Adams et al., 1998; Ni et al., 2010; see Fig. 1b). In *A. nidulans*, early conidiophore formation begins with the specification of thick-walled foot cells that branch to form aerial conidiophore stalks (Mims et al., 1988). BrLA, a C\(_2\)H\(_2\) zinc-finger transcription factor that is expressed during the early phase of conidiation, mediates the switch from polarized stalk elongation to vesicle formation and budding-like cell growth (Adams et al., 1988). Then, a layer of specialized cells (metulae) bud from the vesicle, and two phialides bud from the tip of the metulae and subsequently differentiate into spore-producing (conidiogenous) cells. Loss-of-function *brLA* mutants form structures that resemble conidiophore stalks (‘bristle’ phenotype), except that they grow indeterminately and fail to produce vesicles, metulae, phialides and conidia, indicating that BrLA controls the commencement of conidiophore development (Adams et al., 1988).

The *abaA* gene, activated by BrLA during the middle stages of conidiation when metulae and phialides are being actively formed, has been reported to be involved in the differentiation and functionality of phialides. Mutational
inactivation of AbaA results in the formation of supernumerary tiers of cells with properties of metulae, not phialides, i.e. the ‘abacus’ phenotype (Sewall et al., 1990a; Andrianopoulos & Timberlake, 1994). The wetA gene is activated by AbaA during the late phase of conidiation and plays a role in the synthesis of a crucial conidial wall component, the inner C4 layer, which makes conidia impermeable and mature (Marshall & Timberlake, 1991; Sewall et al., 1990b). Together, the brlA→abaA→wetA cascade has been proposed to define a central regulatory pathway that controls temporal and spatial expression of conidiation-specific genes during conidiophore development and spore maturation (Boylan et al., 1987; Mirabito et al., 1989; Ni et al., 2010; see Fig. 1b). Loss-of-function mutations in any one of these three genes blocks a specific stage in conidiogenesis, resulting in defective conidiophores that fail to produce mature conidia. Recently, the novel regulator VosA has been identified by a genetic screen. VosA is a multifunctional regulator that acts in concert with the central regulatory genes, couples trehalose biogenesis and conidial maturation, and exerts negative feedback regulation on brlA (Ni & Yu, 2007; Fig. 1b).

Based on this framework from the study of A. nidulans conidiation, we have been dissecting the mechanisms underlying the regulation of conidiation in A. fumigatus, primarily focusing on characterization of key developmental regulators (reviewed by Yu, 2011). Our previous study showed that the role of the core downstream transcription factor BrlA is conserved in both species (Mah & Yu, 2006; Yu et al., 2006). In the present study, we hypothesize that AfuBaaA and AfuWetA play distinct roles in A. fumigatus conidiogenesis, and test this by studying sets of AfuBaaA and AfuWetA deletion and overexpression (OE) mutants for hyphal growth and conidiogenesis. Our results demonstrate that AfuBaaA and AfuWetA direct distinct steps of conidiogenesis, and that the central regulatory pathway is conserved between A. fumigatus and A. nidulans. In addition, we further demonstrate that AfuBaaA participates in the regulation of cell death and autolysis, and that AfuWetA is required for normal germ-tube formation and hyphal branching. A genetic model depicting regulation of conidiogenesis in A. fumigatus is presented.

METHODS

Strains, media and growth conditions. Escherichia coli DH5α was used for general cloning. Aspergillus strains used in this study are listed in Table 1. The fungal strains were grown on minimal solid or liquid medium (MM) with 0.1% yeast extract and appropriate supplements (e.g. 1 g uracil l⁻¹ + 1 g uridine l⁻¹, MMG; Kafer, 1977) and incubated at 37 °C. For liquid submerged culture, about 1 x 10⁶ conidia ml⁻¹ were inoculated into liquid MMG and incubated at 250 r.p.m. at 37 °C. To induce AfuabaA overexpression, solid or liquid MM with 100 mM threonine as sole carbon source (MNT) was used. Conidiation induction was performed as described by Seo et al. (2003).

Construction of A. fumigatus strains. The oligonucleotides used in this study are listed in Table S1 (available with the online version of this paper). Double-joint PCR was used to generate the AfuBaaA and AfuWetA deletion constructs (Yu et al., 2004). The deletion constructs containing A. nidulans pyrG (amplified with primer pair OBS8/OSB9) with approximately 1 kb each of the 5' and 3' flanking regions of AfuBaaA or AfuWetA were introduced into the recipient strain Af293.1 (AfypyrG1) (Xue et al., 2004). Primer pairs OS1/OS2 and OS7/OS8 were used to amplify the 5'-flanking regions of AfuBaaA and AfuWetA, respectively. The 3'-flanking regions of the two genes were amplified with primer pairs OS3/OS4 (AfuBaaA) or OS9/OS10 (AfuWetA). Transformation was performed as described by Szewczyk et al. (2006). Solid medium without uracil and uridine was used to select transformants, and deletion candidates were confirmed by PCR followed by enzyme digestion as described previously (Yu et al., 2004). Three AfuBaaA and three AfuWetA deletion mutants (ΔAfuBaaA and ΔAfuWetA) were isolated from 20 and 14 transformants examined, respectively. The alcA(p)::AfuBaaA construct was generated by inserting 2518 bp of the AfuBaaA ORF (amplified using OLT3 and OLT4) between the Kan and NotI sites of pN125 (Shin et al., 2009b), giving rise to pLT8. Multiple AfuBaaA OE strains were generated by transforming Af293.1 with pLT8, where a single copy of alcA(p)::AfuBaaA is integrated into the AfypyrG1 locus. OE candidates were isolated and confirmed by PCR and Northern blot analyses. To generate complemented strains, the AfuBaaA(p)::AfuBaaA and AfuWetA(p)::AfuWetA constructs amplified with OLT13/OLT14
(AfuabaA), and OLT15/OLT16 (AfuwetA), were cloned into pUCH2-8 (HygR), resulting in pLT9 and pLT10, respectively. The AfuupaG(p): AfuAbAC: HygR and AfuwetA(p): AfuWetAC: HygR ampiclons were introduced into strains TSGa17 (AfupyrG1 ΔAfuabaA: ΔAnipyrrG+) and TSGw4 (AfupyrG1 ΔAfuwetA: ΔAnipyrrG+) (AfupyrG+), respectively. Six AfuabaA(p): AfuAbAC (e.g. TLI6 in Table 1, designated C AfuabaA in this study) and five AfuwetA(p): AfuWetAC (e.g. TLI4, designated C AfuwetA strains exhibiting wild-type (WT) phenotype were isolated and confirmed.

Nucleic acid isolation and manipulation. Genomic DNA isolation, total RNA preparation and Northern blot analyses were carried out as described previously (Yu et al., 2004). Total RNA was isolated from conidia and mycelia collected at various time points. Briefly, 107 conidia from relevant strains were inoculated into 100 ml liquid MMG in 250 ml flasks and incubated at 37°C, 250 r.p.m. For vegetative growth phases, samples were collected at designated time points from 2-day-old cultured WT (Af293), and TSGa17 (AfupyrG1 ΔAfuabaA: ΔAnipyrrG+) and TSGw4 (AfupyrG1 ΔAfuwetA: ΔAnipyrrG+) strains described previously (Yu et al., 2009a; Tao et al., 2010). The results are representative of triplicate samples of individual cultures.

Spore viability test. To check the viability of spores, fresh conidia from 2-day-old cultured WT (AF293), ΔAfuwetA and complemented strains on solid MMG were collected and spread onto solid MMG (105 spores per plate) and incubated at 37°C. Then, conidia from 2-, 5-, 10- and 20-day-old cultures were collected, approximately 150 (expected number) conidia were inoculated onto solid MMG and incubated at 37°C until colonies appeared. Survival rate (measured in triplicate for each sample) was calculated as the ratio of the number of growing colonies to the expected number of spores inoculated.

Trehalose assay and stress tolerance test. Trehalose was determined as described by d’Enfert & Fontaine (1997), Fillinger et al. (2001) and Ni & Yu (2007) with a slight modification. Two-day-old spores (2 × 105) were washed with ddH2O three times, resuspended in 200 μl ddH2O and incubated at 100°C for 20 min. The suspension was then centrifuged for 10 min at 11 000 g, and supernatant containing trehalose was collected. Fifty microlitres of supernatant was mixed with 50 μl 6.2 M sodium citrate (pH 5.5) and samples were incubated at 37°C for 8 h with or without 3 mM trehalase (Sigma, T8778). The amount of glucose liberated by the activity of trehalase was assayed using a glucose (GO) assay kit for the AfupaG ORF, OLT63 (forward primer for the AfuwetA ORF), and OLT61 and OLT62 (reverse primers for the AfuwetA ORF) were used. Direct sequencing analyses of the PCR products led us to verify the introns of the AfupaG and AfuwetA genes by comparing the cDNA sequences with the ORF sequences.
(Sigma, GAGO-20) and converted into trehalose per conidium (measured in triplicate). Each sample without trehalase treatment served as a negative control. To examine thermal tolerance, WT, mutant and complemented strain conidia were incubated at 50 °C for 0, 1, 2.5 and 5 min. To examine oxidative tolerance, WT, mutant and complemented strain conidia were treated with varying concentrations (0, 0.025, 0.05, 0.1 and 0.2 M) of H$_2$O$_2$ and incubated for 10 min at room temperature (Ni & Yu, 2007). Osmotic tolerance of WT, mutant and complemented strain conidia was examined by incubating conidia on MMG with different concentrations (0, 0.2, 0.5, 1 and 1.5 M) of NaCl. For each treatment, the spores were inoculated onto solid MMG and incubated at 37 °C for 2 days. Colony numbers were counted and calculated as a percentage of the untreated control.

Microscopy. Photomicrographs were taken by using a Zeiss Axioplan 2 microscope with AxioVision digital imaging software (Zeiss). Culture plate and Northern blot photographs were taken using a SONY DSC-F828 digital camera. TEM was performed as described by Ni & Yu (2007).

RESULTS

Identification and expression of AfuabaA and AfuwetA

Our previous study (Yu et al., 2006) identified AfuabaA (EAL88194, 60 % identity, 74 % similarity with AbaA in A. nidulans) and AfuwetA (EAL89470, 58 % identity, 68 % similarity) through genome searches (Nierman et al., 2005). Here, we further confirmed the ORFs of AfuabaA and AfuwetA by PCR amplification of the coding regions from a cDNA library followed by sequence analyses (see Methods). In summary, the AfuabaA ORF is composed of 2518 bp with three exons and two introns, and is predicted to encode a 797 aa-length protein containing an ATTS/TEA DNA-binding domain (see Fig. S1a, available with the online version of this paper; Andrianopoulos & Timberlake, 1991). The AfuwetA ORF comprises 1701 bp with no intron, and is predicted to encode a 566 aa-length protein with a conserved C-terminal domain (see Fig. S1b).

Levels of AfuabaA and AfuwetA transcripts throughout the life cycle were examined by Northern blotting. AfuabaA and AfuwetA genes were highly expressed during conidiation. AfuabaA mRNA started to accumulate at 6 h post-developmental induction, reached the highest level at 12 h and decreased at 48 h (Fig. 1c). Accumulation of AfuwetA mRNA was observed at 12 h post developmental induction, reached the highest level at 48 h and was also observed in conidia (Fig. 1d).

AfuAbAA and AfuWetA control distinct stages of conidiogenesis

To investigate the functions of AfuabaA and AfuwetA, deletion mutants were generated and their phenotypes were examined. Colonies and micrographs of the ΔAfuabaA and ΔAfuwetA mutants are shown in Fig. 2(a), and transmission electron micrographs of abaA and wetA mutant conidiophores in both A. fumigatus and A. nidulans are shown in Fig. 2(b, c). These results indicate that while WT and complemented strains produce a large number of conidia, the ΔAfuabaA and ΔAfuwetA mutants exhibit aberrant condiation phenotypes. The ΔAfuabaA mutant formed defective conidiophores that failed to generate conidia. The ΔAfuwetA mutant produced colourless conidia and formed droplets of liquid on the surface of the colony. As shown in Fig. 2(b, c), when WT strains developed normal conidia, loss of function of abaA in both species leads to the formation of aberrant conidio- phores exhibiting reiterated cylinder-like terminal cells (elongated phialides) without conidia, indicating that AfuAbAA is essential for differentiation and functionality of A. fumigatus phialides as conidiogenous cells. Furthermore, TEM of the wetA mutants in both A. nidulans and A. fumigatus revealed that wetA conidiophores exhibited the inter-connected conidia phenotype, i.e. incomplete conidial separation and maturation (Fig. 2b, c), implying that AfuWetAA plays an essential role in the completion of conidial maturation.

A role of AfuAbAA in cell death and autolysis

Previous studies reported that A. nidulans abaA also functions in the regulation of autolysis (Emri et al., 2008). To investigate a potential role for AfuAbAA in autolysis and cell death, aberrant ΔAfuabaA conidiophores were inoculated into liquid MMG (~10$^4$ conidiophores ml$^{-1}$), and the morphology and dry weight of mycelium were examined. Microscopic analyses revealed that the most distal cells of the aberrant ΔAfuabaA conidiophores were capable of apical hyphal growth, whereas the fractions of cylinder-like elongated phialides were not able to undergo vegetative growth (Fig. 3a). Determination of dry weight indicated that the absence of AfuabaA caused a delay in the normal reduction of hyphal mass seen in WT (Fig. 3b). Moreover, even after 5 days incubation, when WT and complemented strains exhibited clear hyphal disintegration, the ΔAfuabaA mutant still retained its intact and compact hyphal morphology (Fig. 3b). To quantify mycelium fragmentation, the numbers of fragmented hyphae per 100 microscopic fields were counted. The values of WT, ΔAfuabaA and complemented strains were 401.43, 72.00 and 363.08, respectively, demonstrating an approximately fivefold reduction in fragmentation levels in the ΔAfuabaA mutant. These results indicate that AfuAbAA is necessary for normal progression of autolysis.

We then examined whether AfuAbAA plays a role in cell death by determining percentage AB reduction, which corresponds to the activity of living cell mitochondria. While both WT and complemented strains displayed dramatically reduced cell viability at day 4, no reduction in cell viability was observed in the ΔAfuabaA mutant (Fig. 3c). Furthermore, whereas WT and complemented strains displayed a complete loss of cell viability at days 5 and 6, the ΔAfuabaA mutant retained a certain level of viability...
even at day 6, indicating that AfuAbaA is required for controlled progression of cell death.

To further confirm the role of AfuAbaA in autolysis and cell death, we tested the effects of overexpression of AfuAbaA. As AfuAbaA plays a positive role, upon induction in MMT, OE AfuAbaA caused accelerated hyphal disintegration and a dramatic reduction in mycelial mass. As shown in Fig. 4(a, b), while the control strain exhibited normal levels of hyphal extension and conidiation, OE AfuAbaA resulted in clear hyphal disintegration and complete lysis at 72 h. Furthermore, on day 2 the OE AfuAbaA strain exhibited clear acceleration of cell death compared to the control strain (Fig. 4c), corroborating the idea that AfuAbaA functions positively in autolysis and cell death.

In addition, we found that OE AfuAbaA caused adverse effects on hyphal growth and development. First, hyphal tip growth stopped when AfuAbaA was overexpressed (Fig. 4a, b). Second, OE AfuAbaA strains exhibited reduced levels of conidiation when grown on solid (Fig. 4d) or in liquid (Fig. 4a) MMT. To quantify the conidiation level, the number of conidia mm$^{-2}$ of control and OE AfuAbaA strains grown in solid MMG+0.5% yeast extract (MMGY) or MMT+0.5% yeast extract (MMGT) was counted. When grown in inducing MMTY, the OE AfuAbaA strain formed a lower number of conidia at any position of the colony than that produced by the control strain (Fig. 4d), whereas no phenotypic differences were observed when they were grown in MMGY. These results suggest that overexpression of AfuAbaA inhibits both hyphal extension and conidiation, and that proper expression of AfuAbaA is crucial for normal growth and development in A. fumigatus.

Complex roles of AfuWetA

As AfuWetA plays an essential role in the completion of the conidial wall, we hypothesized that the absence of AfuWetA may affect viability and/or integrity of conidia. To test this, the abilities of 2-, 5-, 10- and 20-day-old WT, ΔAfuwetA
and complemented strain conidia to generate colonies were compared. As shown in Fig. 5(a), the ΔAfuwetA mutant exhibited dramatically reduced viability starting from day 10, while WT and complemented strain conidia maintained high viability until day 20. Moreover, TEM studies revealed that, even on day 2, about 20% of the ΔAfuwetA conidia appeared to lack cytoplasm and exhibited sheared conidial walls (Fig. 5b). These results indicate that AfuWetA is essential for both viability and integrity of conidia.

We then examined the effects of ΔAfuwetA on spore tolerance to various stresses and found that ΔAfuwetA mutant conidia exhibited drastically reduced tolerance to thermal (Fig. 5c), oxidative (Fig. 5d) and osmotic (Fig. 5e) stresses. As trehalose is essential for long-term spore viability and stress resistance (Fillinger et al., 2001; Ni & Yu, 2007), we asked whether the ΔAfuwetA conidia contained a sufficient amount of trehalose, and therefore examined the amount of trehalose in 2-day-old conidia of WT, ΔAfuwetA and complemented strains. As shown in Fig. 5(f), ΔAfuwetA conidia did not contain any trehalose, whereas WT and complemented strains harboured about 3.4 pg trehalose per conidium, indicating that AfuWetA plays an essential role in trehalose biogenesis in conidia, which probably affects the viability and stress tolerance of conidia.

**AfuWetA functions in the completion of the conidial wall**

To examine the role of AfuWetA in conidial wall formation, WT and wetA mutant conidia in both A.
AfuWetA is required for proper hyphal branching and timely conidiation

Given that the AfuwetA gene is activated during the late stage of conidiation, and that its mRNA accumulates preferentially in mature conidia, we asked whether the absence of AfuwetA has any effect on conidial germination and early hyphal growth. We found that after 8 h incubation in liquid submerged culture, conidia of WT and complemented strains produced bi- or multi-directional germ tubes that formed branches which eventually turned into compact mycelial aggregates. However, in the ΔAfuwetA mutant, each conidium formed only one unidirectional germ tube without branching, which resulted in loosened mycelial aggregates (Fig. 6b). Moreover, after 14 h incubation, the ΔAfuwetA mutant clearly showed reduced branch density compared to the WT and complemented strains. The branching rates of these strains were defined by peripheral growth unit (PGU) values (Ettebeste et al., 2009; see Fig. 6b). The PGU values of WT, ΔAfuwetA and complemented strain hyphae were 18.75 ± 1.56 μm, 86.18 ± 9.23 μm and 19.27 ± 2.39 μm, respectively, demonstrating an approximately fivefold reduction in hyphal branching in the ΔAfuwetA mutant. These results suggest that AfuWetA is associated with proper germ-tube formation and vegetative growth. This is consistent with the finding that over-expression of wetA in hyphae causes excessive branching in A. nidulans (Marshall & Timberlake, 1991).

We also investigated the effects of ΔAfuwetA on development. Conidia of WT, ΔAfuwetA and complemented strains were inoculated into liquid MMG, incubated for 18 h at 37 °C, 250 r.p.m., and the mycelium was collected and transferred into liquid (L) and solid (S) MMG and MMG + 0.6 M KCl and further incubated at 37 °C (250 r.p.m. for liquid culture). Developmental progression under each culture condition was examined. As shown in Fig. 6(c), while the WT and complemented strains readily produced abundant conidiophores when exposed to air (solid MMG) and/or high salt (solid/liquid MMG + KCl), the ΔAfuwetA mutant exhibited delayed and reduced conidiation in all three inducing conditions. These results suggest that, although activated during the late phase of conidiation, AfuWetA is necessary for the proper initiation and progression of development.

Genetic interactions between conidiation genes in A. fumigatus

To dissect the genetic interactions between the central regulatory genes in A. fumigatus, a series of Northern blot analyses were carried out. As shown in Fig. 7(a), the absence of AfubrlA eliminated the expression of all conidiation-specific genes tested, indicating that AfubrlA functions upstream of AfuabaA, AfuwetA, AfuvosA and AfurodA. As AfuabaA is necessary for the activation of AfuwetA, AfuwetA mRNA accumulation was not detected in the ΔAfubrlA mutant, confirming the AfubrlA→AfuvosA→AfuwetA regulatory cascade in A. fumigatus. Moreover, levels of AfuvosA mRNA decreased considerably in both the ΔAfubrlA and ΔAfuwetA mutants, indicating the requirement for both genes in proper AfuvosA expression. However, as the activation of AfuvosA occurs before the activation of AfuwetA in WT, it is proposed that AfuabaA primarily activates AfuvosA with the assistance of AfuWetA. Importantly, AfubrlA is highly upregulated in ΔAfubrlA and ΔAfuwetA mutants, particularly in conidia (aberrant conidiophores in ΔAfubrlA) and early (6 h) and late (24 h) phases of vegetative growth, suggesting that AfuabaA and AfuWetA are necessary for proper negative feedback regulation of AfubrlA upon completion of conidiation and during certain phases of vegetative growth. AfurodA, a small, secreted, moderately hydrophobic polypeptide, is involved in the formation of the outermost rodlet layer of conidia, and the conidia of AfurodA mutants lack their external rodlet layer and are hydrophilic (Thau et al., 1994). The observations that no AfurodA mRNA was detected in the ΔAfubrlA mutant and that levels of AfurodA were low in the ΔAfubrlA mutant suggest that AfubrlA and AfuabaA activated AfurodA in a supportive manner. Taking all these observations together, a genetic model regulating asexual development in A. fumigatus is proposed (Fig. 7b).

DISCUSSION

While a number of key developmental regulators have been identified and characterized in A. nidulans, they remain to be studied in other aspergilli. In this study, we have characterized how AfuabaA and AfuwetA control growth and development of the opportunistic pathogen A. fumigatus and have demonstrated that central regulatory components are conserved in both Aspergillus species: AfuAbA functions in the differentiation of phialides (the conidiogenous cells which are required for the generation of conidia), and AfuWetA plays a role in the completion of the conidial wall and is essential for trehalose biosynthesis, conidial viability, integrity and stress tolerance.
Fig. 4. Overexpression of *Afuaba*A accelerates autolysis and cell death, but inhibits growth and development. (a) Phenotypes of control (TU11) and OEAfuabaA (TU9) strains in liquid MMT submerged culture, and Northern blot analyses for levels of *Afuaba*A mRNA. Micrographs were taken at 0, 24 and 48 h of incubation after transferring 18 h-old vegetative cells into MMT. The close-up views show the conidiation defects in the OEAfuabaA strain. Arrows indicate the simplified conidiophores formed by the control strain at 24 h. (b) Mycelial dry weight of control (black bars) and OEAfuabaA (white bars) strains grown in liquid MMG or MMT. (c) Cell viability of control and OEAfuabaA strains determined by AB reduction rates. Data are the mean values of three individual cultures. Note that the OEAfuabaA strain exhibits reduced viability at 24 h. Black bars, control in MMG; dotted bars, OEAfuabaA in MMG; grey bars, control in MMT; white bars, OEAfuabaA in MMT. (d) Phenotypes of control and OEAfuabaA strains grown on solid inducing medium (MMT) at 37 °C for 3 days, and the number of conidia per mm² of control and OEAfuabaA colonies. Note that the OEAfuabaA strain formed small colonies with reduced numbers of conidia. ■, Control in MMGY; ●, OEAfuabaA in MMGY; □, control in MMTY; ●, OEAfuabaA in MMTY.
Importantly, AfuAbaA also plays a crucial role in cell death and autolysis in A. fumigatus. Autolysis can be defined as a natural process of self-digestion of aged hyphal cultures, occurring as a result of hydrolase activity, causing vacuolization and disruption of organelle and cell-wall structure (White et al., 2002). Previously, it has been reported that loss-of-function mutations in abaA of A. nidulans result in reduced and delayed hydrolase (chitinase) production (Emri et al., 2008). Thus it can be speculated that AfuAbaA affects the autolysis process potentially by activating production of various hydrolases. As the regulatory pathways of autolysis and apoptotic cell death are tightly coupled, the absence of AfuAbaA also results in delayed cell death via an unknown mechanism. For the development of novel antifungal targets, it would be interesting to investigate the molecular mechanisms of AfuAbaA-mediated regulation of cell death and autolysis and identify its potential target genes.

Probably the most important finding in our present study is that AfuwetA is essential for trehalose biogenesis in conidia. Trehalose is an important osmolyte produced by bacteria, fungi, insects and plants to protect the integrity of cells against various environmental stresses (reviewed by Elbein et al., 2003; Ni & Yu, 2007). Thus, the absence of trehalose in fungal spores results in rapid loss of viability and extreme sensitivity to various stresses. In fact, as reported above, ΔAfuwetA conidia are highly sensitive to heat, salt and oxidative stresses. While our previous study reported that the deletion of AfuvosA resulted in a 50% reduction in trehalose in A. fumigatus conidia (Ni & Yu, 2007), this is the first report of the essential role of WetA in trehalose biogenesis in fungal conidia.

TEM analyses indicate that ΔAfuwetA conidia exhibit unclear and aberrant wall layers compared to those of WT, i.e. AfuWetA is required for the modification of conidial wall and the integrity of conidia, as reported in A. nidulans (Sewall et al., 1990b). In A. nidulans, conidium maturation (a differentiation process occurring after the formation of the septum between the phialide and the newly formed conidium) can be divided into three stages (Sewall et al., 1990b). At stage I, conidia are completely separated from the phialide and they contain two cell-wall layers, C1 (outer) and C2 (inner). At stage II, C2 condenses and C1 becomes crenulated. In the third stage, two new cell-wall layers, C3 and C4, are formed and render the conidia impermeable and mature. Loss-of-function mutations in the wetA locus lead to defects in the formation of the C3 and C4 wall layers and in condensation of the C2 wall layer, resulting in incomplete maturation and consequent lysis of conidia (Sewall et al., 1990b). The absence of AfuwetA results in almost identical defects in the formation of layers C3 and C4 and condensation of the C2 wall layer, suggesting that the spore-wall components affected by WetA might be conserved in both aspergilli.

Microscopical analyses of conidial germination in the ΔAfuwetA mutant revealed that most conidia form only...
one germ tube, and that the branching of the germlings is reduced. Due to the inhibited hyphal branching, the ΔAfuwetA mutant growing in submerged culture formed less compact mycelial aggregates than those of the WT. A previous study has proposed that branch formation involves hyphal wall remodelling accompanied by the accumulation of vesicles at the lateral wall (reviewed by Adams, 2004). Importantly, Marshall & Timberlake (1991) reported that overexpression of A. nidulans wetA in hyphae inhibited growth and caused excessive branching. This is somewhat consistent with our finding that ΔAfuwetA mutant hyphae show an approximately fivefold reduction in branching (Fig. 6b). These results imply that the absence of AfuwetA not only affects the modification of the conidial wall, but also causes hyphal wall alterations, which in turn affect hyphal branching.

A genetic model depicting the regulation of A. fumigatus conidiation is presented (Fig. 7b). In this model, upstream regulator AfuFluG is proposed to activate Afuflb (e.g. AfuflbB and AfuflbE; Yu et al., 2006; Kwon et al., 2010; Xiao et al., 2010; Yu, 2011) and AfuBrlA, thereby triggering the
central regulatory pathway ($AfubrlA \rightarrow AfuabaA \rightarrow AfuwetA$) of conidiation. Subsequently, $AfuvosA$ (Ni & Yu, 2007) is activated by $AfuAbaA$ with the assistance of $AfuWelA$. $AfuWelA$ and $AfuVosA$ coordinately complete spore maturation, including trehalose biogenesis. As found in $A. nidulans$, both $AfubrlA$ and $AfuAbaA$ are required for

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Fig. 6. Additional phenotypes resulting from $\Delta AfuwetA$. (a) TEM images of 2-day-old conidia wall structures of $A. fumigatus$ WT (Af293) and the $\Delta AfuwetA$ mutant (TSGw4), and $A. nidulans$ WT (FGSC4) and the wet$^{-}$ mutant (AJC472.1). Arrowheads indicate the wall thickness in four conidia. C1 (outer) and C2 (inner) indicate the wall layers of $A. nidulans$ conidia as reported by Sewall et al. (1990b). (b) Conidia of WT, $\Delta AfuwetA$ and complemented strains were cultured in liquid MMG. Photomicrographs were taken at 8 and 14 h. The PGU values are indicated below the photograph. (c) WT, $\Delta AfuwetA$ and complemented strains grown under conditions that induce development. After 18 h incubation in liquid MMG, mycelia were transferred to both solid (S) and liquid (L) MMG and/or MMG + KCl. Photomicrographs were taken after 6 h (solid medium, S) and 12 h (liquid medium, L) incubation. Note the delayed conidiation caused by the absence of $AfuwelA$. 

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proper expression of AfurodA (see Adams et al., 1998). The repressive roles of AfuAbA and AfuWetA on expression of AfubrlA may be attributed to AfuVosA (or other velvet regulators) functioning in a negative-feedback loop (Ni & Yu, 2007; Yu, 2011; H.-S. Park & J.-H. Yu, unpublished data). The observations that AfuWetA may negatively regulate AfuabaA and that AfuAbA may directly repress AfubrlA are indicated. AfuAbA plays a key role in autolysis and cell death.

Previous studies have identified the consensus binding sites for A. nidulans BrlA and AbaA [BrlA response elements (BREs), 5'-(C/A)(G/A)AGGG(G/A)-3'; AbaA response elements (AREs), 5'-CATTCCY-3', where Y is a pyrimidine] (Chang & Timberlake, 1993; Andrianopoulos & Timberlake, 1994). We analysed the promoter regions of several A. fumigatus conidiation-related genes for the presence of the predicted A. nidulans BREs or AREs. As shown in Fig. 7(c), AREs are present in all genes tested, including AfubrlA, AfuwetA, AfuvosA, AfurodA, AfuveA, AfuvelB and AfuvelC, suggesting that AfuAbA might bind to these genes and affect their expression. Among those tested, AfuVeA, AfuVelB and AfuVelC are the velvet proteins that are highly conserved in filamentous fungi (Ni & Yu, 2007; Bayram et al., 2008). In A. nidulans, the velvet complex has been shown to control development and secondary metabolism (Bayram et al., 2008). AfuAbA may also influence secondary metabolism by regulating expression of AfuveA,
AfuwelB and AfuwelC. As one, two and three BREs are found in the AfuwetA, AfuabaA and AfuvasoA genes, respectively, and one is found in AfuroidA, AfubriaA may control AfuwetA, AfuabaA, AfuvasoA and AfuroidA expression. Further studies to identify AfuAbA and AfuWetA target genes in A. fumigatus are in progress.

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