Microbiology (2011), **157**, 313–326 DOI 10.1099/mic.0.044271-0

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

Li Tao[1,2] and Jae-Hyuk Yu[1]

Correspondence
Jae-Hyuk Yu
jyu1@wisc.edu

[1]Departments of Bacteriology and Genetics, University of Wisconsin, Madison, WI 53706, USA
[2]State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing, PR China

Received 30 July 2010
Revised 6 October 2010
Accepted 14 October 2010

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, *Afu*AbaA and *Afu*WetA, in *A. fumigatus*. The *AfuabaA* gene is predicted to encode an ATTS/TEA DNA-binding domain protein and is activated by *Afu*BrlA 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 *Afu*AbaA. The *AfuwetA* gene is sequentially activated by *Afu*AbaA 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 *Afu*WetA 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 (Wasylnka & 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_2H_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

Abbreviations: AB, Alamar Blue; ARE, AbaA response element; BRE, BrlA response element; OE, overexpression; PGU, peripheral growth unit.

Supplementary data are available with the online version of this paper.

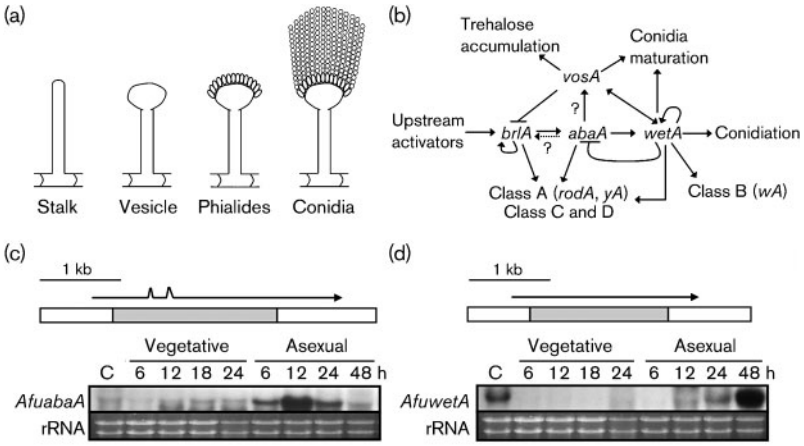


Fig. 1. Background information and expression of *AfuabaA* and *AfuwetA*. (a) Formation of the *A. fumigatus* conidiophore. (b) The current model for downstream regulation of conidiation in *A. nidulans* (adapted and modified from Ni & Yu, 2007). (c) and (d) Northern blot analyses. Levels of *AfuabaA* (c) and *AfuwetA* (d) mRNA throughout the life cycle of wild-type *A. fumigatus* (Af293) are presented. Numbers indicate incubation time (h) in liquid MMG (vegetative) and post-asexual developmental (asexual) induction. C represents conidia. Equal loading of total RNA was evaluated by ethidium bromide staining of rRNA.

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 conidia 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 *Afu*AbaA and *Afu*WetA play distinct roles in *A. fumigatus* conidiogenesis, and test this by studying sets of *AfuabaA* and *AfuwetA* deletion and overexpression (OE) mutants for hyphal growth and conidiogenesis. Our results demonstrate that *AfuabaA* 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 *Afu*AbaA participates in the regulation of cell death and autolysis, and that *Afu*WetA 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; Käfer, 1977) and incubated at 37 °C. For liquid submerged culture, about 1×10^6 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 (MMT) 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 *AfuabaA* and *AfuwetA* deletion constructs (Yu *et al.*, 2004). The deletion constructs containing *A. nidulans pyrG* (amplified with primer pair OBS8/OBS9) with approximately 1 kb each of the 5′ and 3′ flanking regions of *AfuabaA* or *AfuwetA* were introduced into the recipient strain Af293.1 (*AfupyrG1*) (Xue *et al.*, 2004). Primer pairs OSG1/OSG2 and OSG7/OSG8 were used to amplify the 5′-flanking regions of *AfuabaA* and *AfuwetA*, respectively. The 3′-flanking regions of the two genes were amplified with primer pairs OSG3/OSG4 (*AfuabaA*) or OSG9/OSG10 (*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 *AfuabaA* and three *AfuwetA* deletion mutants (Δ*AfuabaA* and Δ*AfuwetA*) were isolated from 20 and 14 transformants examined, respectively. The *alcA*(p)::*AfuabaA* construct was generated by inserting 2518 bp of the *AfuabaA* ORF (amplified using OLT3 and OLT4) between the *Kpn*I and *Not*I sites of pNJ25 (Shin *et al.*, 2009b), giving rise to pLT8. Multiple *AfuabaA* OE strains were generated by transforming Af293.1 with pLT8, where a single copy of *alcA*(p)::*AfuabaA* is integrated into the *AfupyrG* locus. OE candidates were isolated and confirmed by PCR and Northern blot analyses. To generate complemented strains, the *AfuabaA*(p)::*AfuabaA* and *AfuwetA*(p)::*AfuwetA* constructs amplified with OLT13/OLT14

Table 1. *Aspergillus* strains used in this study

Strain	Genotype	Source*
A. fumigatus		
Af293	Wild-type	Brookman & Denning (2000)
Af293.1	*AfupyrG1*	Xue *et al.* (2004)
A1176	*AfupyrG1* Δ*AfubrlA*::*AfupyrG*$^+$	FGSC
TSGa17†	*AfupyrG1* Δ*AfuabaA*::*AnipyrG*$^+$	This study
TSGw4†	*AfupyrG1* Δ*AfuwetA*::*AnipyrG*$^+$	This study
TLI4†	*AfupyrG1* Δ*AfuwetA*::*AnipyrG*$^+$ *AfuwetA*(p)::*AfuwetA*	This study
TLI6†	*AfupyrG1* Δ*AfuabaA*::*AnipyrG*$^+$ *AfuabaA*(p)::*AfuabaA*	This study
TLI9†	*AfupyrG1*‡ *alcA*(p)::*AfuabaA*::*AfupyrG*$^+$	This study
TLI11†	*AfupyrG1*::pNJ25::*AfupyrG*+	This study
A. nidulans		
FGSC4	Wild-type	FGSC
AJC4641.4	*bioA1 abaA14 trpC801 veA1*	Clutterbuck (1969)
AJC472.1	*bioA1 yA2 wetA6 trpC3998 veA1*	Clutterbuck (1969)

*FGSC, Fungal Genetics Stock Center, School of Biological Sciences, University of Missouri, Kansas City, MO 64110, USA.
†Multiple isogenic strains (all behaved identically).
‡The 3/4 *AfupyrG* marker in pNJ25 causes targeted integration at the *AfupyrG1* locus.

(*AfuabaA*), and OLT15/OLT16 (*AfuwetA*), were cloned into pUCH2-8 (HygR), resulting in pLT9 and pLT10, respectively. The *AfuabaA*(p)::*Afu*AbaA::HygR and *AfuwetA*(p)::*Afu*WetA::HygR amplicons were introduced into strains TSGa17 (*AfupyrG1* Δ*AfuabaA*::*AnipyrG*$^+$) and TSGw4 (*AfupyrG1* Δ*AfuwetA*::*AnipyrG*$^+$), respectively. Six *AfuabaA*(p)::*Afu*AbaA (e.g. TLI6 in Table 1, designated C′ *AfuabaA* in this study) and five *AfuwetA*(p)::*Afu*WetA (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, 10^8 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 liquid submerged cultures. For asexual developmental induction, 18 h-old vegetatively grown mycelia were harvested and transferred to solid MMG (Seo *et al.*, 2006) and samples were collected at the indicated time points. All samples were squeeze-dried, quick-frozen in liquid N_2 and stored at −80 °C until required for RNA isolation. For total RNA isolation, around 10 mg mycelium was homogenized using a Mini Bead beater in the presence of 1 ml Trizol reagent (Invitrogen) and 0.6 ml silica/zirconium beads (Biospec). RNA extraction from ground hyphae was performed according to the manufacturer's instructions (Invitrogen). A Hybond-N membrane (Amersham) was used for blotting nucleic acids after separation of 10 μg total RNA in 1.1 % agarose gel containing 3 % (v/v) formaldehyde. DNA probes were prepared by amplification of the coding regions of individual genes (*AfubrlA*, *AfuabaA*, *AfuwetA*, *AfuvosA* and *AfurodA*) using the appropriate primer pairs with Af293 genomic DNA as template (Table S1).

Confirmation of *AfuabaA* and *AfuwetA* ORFs. PCRs were performed to amplify the ORFs of *AfuabaA* and *AfuwetA* using the UniZAP *A. fumigatus* cDNA library as template (Reyes *et al.*, 2006; kindly provided by Dr G. S. May, University of Texas MD Anderson Cancer Center). The sequencing primers OLT59 and OLT60 (forward primers for the *AfuabaA* ORF), OLT57 and OLT58 (reverse primers for the *AfuabaA* 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 *AfuabaA* and *AfuwetA* genes by comparing the cDNA sequences with the ORF sequences.

Determination of mycelial mass and cell viability. Dry weight was determined as described previously (Yamazaki *et al.*, 2007; Shin *et al.*, 2009a). Cell viability was determined by the percentage reduction in Alamar Blue (AB). Briefly, the AB assay reagent (AbD Serotec) was placed into each test well of a 24-well plate (Nunc) containing 1 ml fresh medium and 0.5 ml individual cultures, at a final amount equal to 10 % of the volume in the well. After 6 h incubation at 37 °C, the absorbance of the contents of each well was measured at 570 and 600 nm. The percentage reduction of AB was calculated as described previously (Al-Nasiry *et al.*, 2007; Shin *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), Δ*AfuwetA* and complemented strains on solid MMG were collected and spread onto solid MMG (10^5 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 (2×10^8) were washed with ddH_2O three times, resuspended in 200 μl ddH_2O 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 0.2 M sodium citrate (pH 5.5) and samples were incubated at 37 °C for 8 h with or without 3 mU trehalase (Sigma, T8778). The amount of glucose liberated by the activity of trehalase was assayed using a glucose (GO) assay kit

(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_2O_2 and incubated for 10 min at room temperature (Ni & Yu, 2007). Osmotic tolerance of WT, mutant and complemented strain conidia was examined by inoculating 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 *Afu*AbaA (EAL88194, 60 % identity, 74 % similarity with AbaA in *A. nidulans*) and *Afu*WetA (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).

*Afu*AbaA and *Afu*WetA 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 Δ*AfuabaA* and Δ*AfuwetA* 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 Δ*AfuabaA* and Δ*AfuwetA* mutants exhibit aberrant conidiation phenotypes. The Δ*AfuabaA* mutant formed defective conidiophores that failed to generate conidia. The Δ*AfuwetA* 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 conidiophores exhibiting reiterated cylinder-like terminal cells (elongated phialides) without conidia, indicating that *Afu*AbaA 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 *Afu*WetA plays an essential role in the completion of conidial maturation.

A role of *Afu*AbaA 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 *Afu*AbaA in autolysis and cell death, aberrant Δ*AfuabaA* 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 Δ*AfuabaA* 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 Δ*AfuabaA* 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, Δ*AfuabaA* and complemented strains were 401.43, 72.00 and 363.08, respectively, demonstrating an approximately fivefold reduction in fragmentation levels in the Δ*AfuabaA* mutant. These results indicate that *Afu*AbaA is necessary for normal progression of autolysis.

We then examined whether *Afu*AbaA 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 Δ*AfuabaA* mutant (Fig. 3c). Furthermore, whereas WT and complemented strains displayed a complete loss of cell viability at days 5 and 6, the Δ*AfuabaA* mutant retained a certain level of viability

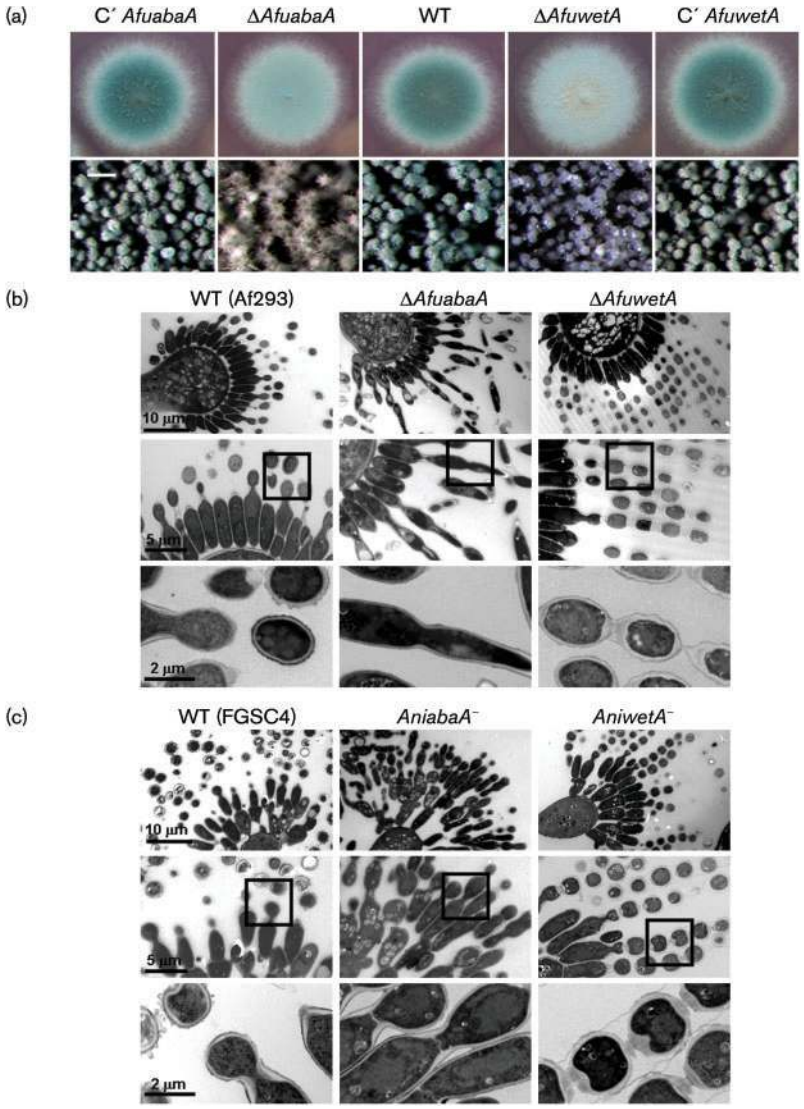


Fig. 2. Requirement for *Afu*AbaA and *Afu*WetA in distinct steps of conidiation. (a) Phenotypes of WT (Af293), Δ*AfuabaA* (TSGa17), Δ*AfuwetA* (TSGw4) and complemented strains TLI6 (for *AfuabaA*=C′ *AfuabaA*) and TLI4 (for *AfuwetA*=C′ *AfuwetA*) grown on solid MMG at 37 °C for 3 days. Bar,100 μm. (b, c) Effects of *abaA* and *wetA* mutations on conidiogenesis in *A. fumigatus* (b) and *A. nidulans* (c). TEM images of 2-day-old conidiophores or conidia of the following strains are shown: *A. fumigatus*: WT (Af293), Δ*AfuabaA* (TSGa17), Δ*AfuwetA* (TSGw4); *A. nidulans*: WT (FGSC4), *abaA*$^{-}$ (AJC4641.4), *wetA*$^{-}$ (AJC472.1). Note the cylinder-like terminal cells formed by the Δ*AfuabaA* mutant and the defective conidia produced by the Δ*AfuwetA* mutant.

even at day 6, indicating that *Afu*AbaA is required for controlled progression of cell death.

To further confirm the role of *Afu*AbaA in autolysis and cell death, we tested the effects of overexpression of *AfuabaA*. As *Afu*AbaA 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 *Afu*AbaA 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 *Afu*WetA

As *Afu*WetA 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, Δ*AfuwetA*

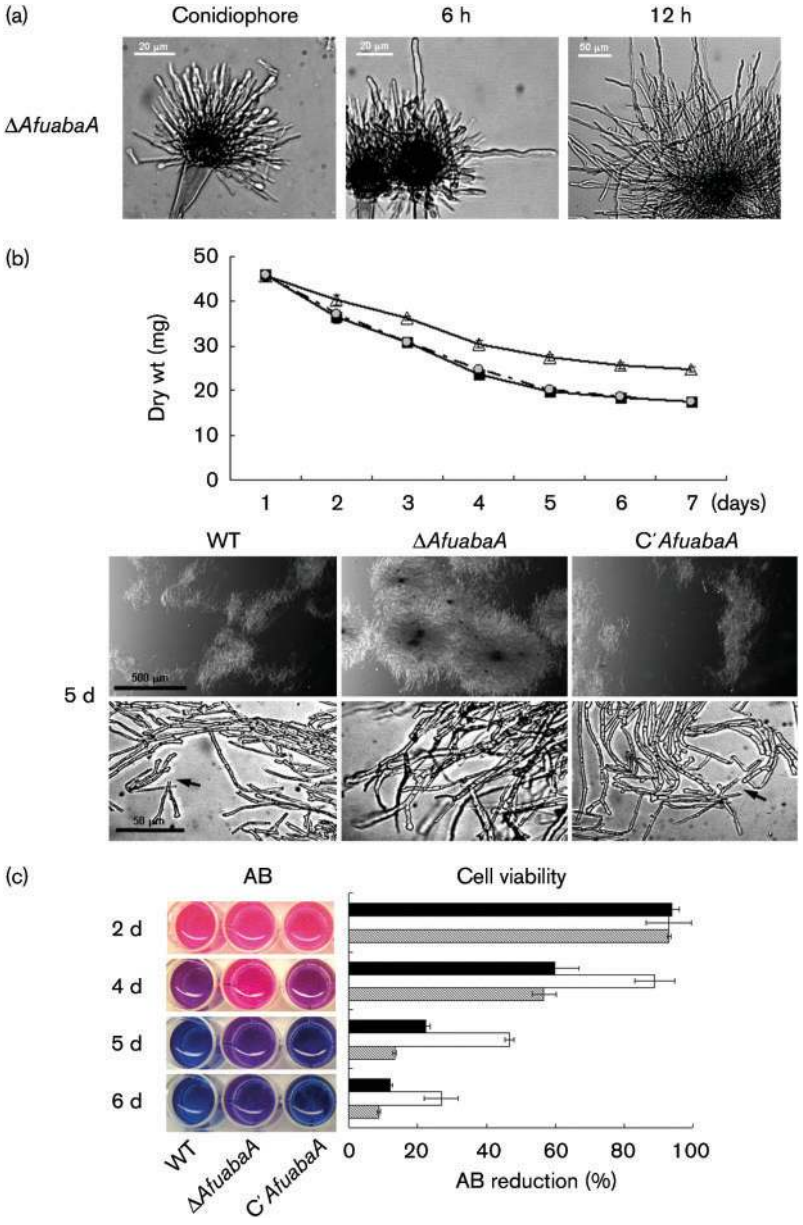


Fig. 3. The role of *Afu*AbaA in autolysis and cell death. (a) Δ*AfuabaA* conidiophores (0 h) were inoculated into liquid MMG and incubated at 37 °C, 250 r.p.m. Photomicrographs were taken at 6 and 12 h. Note that cylinder cells underwent vegetative growth starting from 6 h incubation. (b) Mycelial morphology and dry weight of WT (Af293, ■), Δ*AfuabaA* (TSGa17, ▲) and complemented (TLI6, ●) strains grown in liquid submerged culture. Note that, compared to WT and complemented strains, the Δ*AfuabaA* mutant exhibited delayed hyphal disintegration. Arrows indicate the hyphal fragmentation observed in WT and complemented strains. (c) Cell viability of WT (black bars), Δ*AfuabaA* (white bars) and complemented strains (hatched bars) determined by AB reduction rates. Data are representative of triplicates of individual cultures. Note that at days 4 and 5, the Δ*AfuabaA* mutant showed high levels of cell viability.

and complemented strain conidia to generate colonies were compared. As shown in Fig. 5(a), the Δ*AfuwetA* 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 Δ*AfuwetA* conidia appeared to lack cytoplasm and exhibited sheared conidial walls (Fig. 5b). These results indicate that *Afu*WetA is essential for both viability and integrity of conidia.

We then examined the effects of Δ*AfuwetA* on spore tolerance to various stresses and found that Δ*AfuwetA* 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 Δ*AfuwetA* conidia contained a sufficient amount of trehalose, and therefore examined the amount of trehalose in 2-day-old conidia of WT, Δ*AfuwetA* and complemented strains. As shown in Fig. 5(f), Δ*AfuwetA* conidia did not contain any trehalose, whereas WT and complemented strains harboured about 3.4 pg trehalose per conidium, indicating that *Afu*WetA plays an essential role in trehalose biogenesis in conidia, which probably affects the viability and stress tolerance of conidia.

*Afu*WetA functions in the completion of the conidial wall

To examine the role of *Afu*WetA in conidial wall formation, WT and *wetA* mutant conidia in both *A.*

nidulans and *A. fumigatus* were compared by TEM (Fig. 6a). The *A. fumigatus* WT strain exhibited similar conidial wall structure to *A. nidulans* (Sewall *et al.*, 1990b). As shown in Fig. 6(a), WT conidia form the crenulated electron-dense C1 layer and the condensed electron-light C2 layer. In the *wetA* mutants, although the C1 and C2 layers were formed, the C1 layer was not crenulated and the C2 layer failed to condense, resulting in a thicker conidial wall than in the WT strain. We then measured the conidial wall thickness of the test strains. The *A. nidulans* and *A. fumigatus wetA* mutant conidial walls had a mean thickness of 0.2 and 0.18 μm, respectively, while both WT conidia exhibited a mean thickness of 0.13 μm. These data indicate that the Δ*AfuwetA* mutant exhibits conidial wall defects similar to those found in *A. nidulans*, and that *Afu*WetA plays an essential role in conidial wall completion and spore maturation.

*Afu*WetA 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 Δ*AfuwetA* 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 Δ*AfuwetA* 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 (Etxebeste *et al.*, 2009; see Fig. 6b). The PGU values of WT, Δ*AfuwetA* 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 Δ*AfuwetA* mutant. These results suggest that *Afu*WetA is associated with proper germ-tube formation and vegetative growth. This is consistent with the finding that overexpression of *wetA* in hyphae causes excessive branching in *A. nidulans* (Marshall & Timberlake, 1991).

We also investigated the effects of Δ*AfuwetA* on development. Conidia of WT, Δ*AfuwetA* 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 Δ*AfuwetA* mutant exhibited delayed and reduced conidiation in all three inducing conditions. These results suggest that, although activated during the late phase of conidiation, *Afu*WetA 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 *Afu*BrlA functions upstream of *AfuabaA*, *AfuwetA*, *AfuvosA* and *AfurodA*. As *Afu*AbaA is necessary for the activation of *AfuwetA*, *AfuwetA* mRNA accumulation was not detected in the Δ*AfuabaA* mutant, confirming the *AfubrlA*→*AfuabaA*→*AfuwetA* regulatory cascade in *A. fumigatus*. Moreover, levels of *AfuvosA* mRNA decreased considerably in both the Δ*AfuabaA* and Δ*AfuwetA* 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 *Afu*AbaA primarily activates *AfuvosA* with the assistance of *Afu*WetA. Importantly, *AfubrlA* is highly upregulated in Δ*AfuabaA* and Δ*AfuwetA* mutants, particularly in conidia (aberrant conidiophores in Δ*AfuabaA*) and early (6 h) and late (24 h) phases of vegetative growth, suggesting that *Afu*AbaA and *Afu*WetA are necessary for proper negative feedback regulation of *AfubrlA* upon completion of conidiation and during certain phases of vegetative growth. *Afu*RodA, 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 Δ*AfubrlA* mutant and that levels of *AfurodA* were low in the Δ*AfuabaA* mutant suggest that *Afu*BrlA and *Afu*AbaA 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: *Afu*AbaA functions in the differentiation of phialides (the conidiogenous cells which are required for the generation of conidia), and *Afu*WetA plays a role in the completion of the conidial wall and is essential for trehalose biogenesis, conidial viability, integrity and stress tolerance.

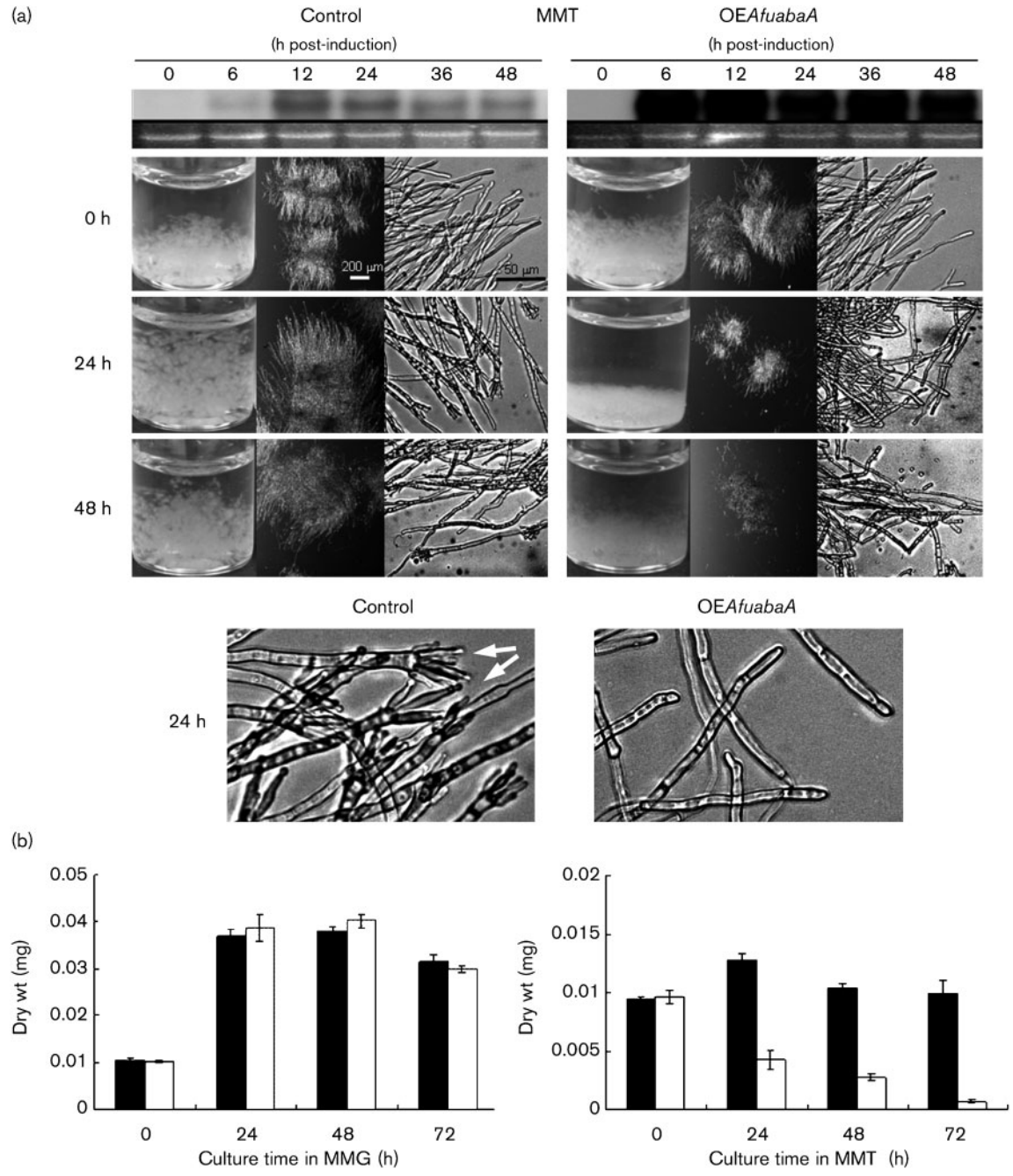
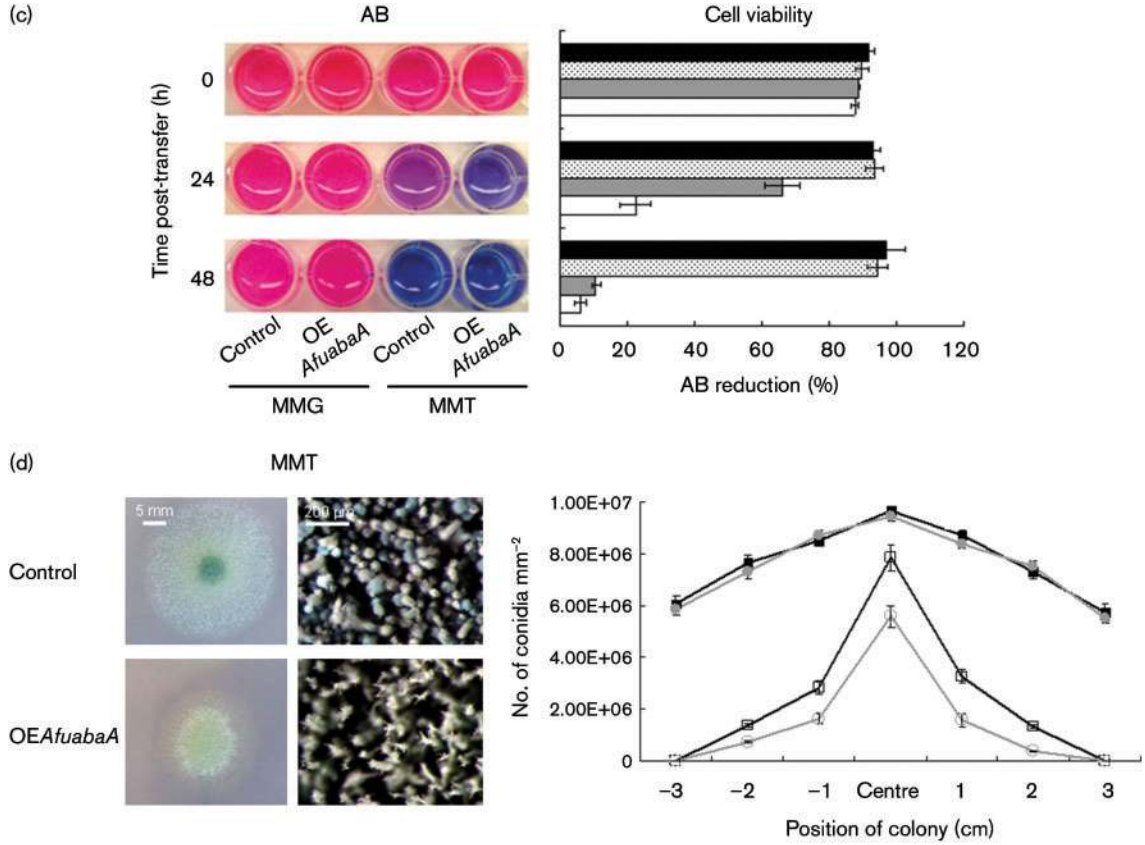


Fig. 4. Overexpression of *AfuabaA* accelerates autolysis and cell death, but inhibits growth and development. (a) Phenotypes of control (TLI11) and OE*AfuabaA* (TLI9) strains in liquid MMT submerged culture, and Northern blot analyses for levels of *AfuabaA* mRNA. Micrographs were taken at 0, 24 and 48 h of incubation after transferring 18 h-old vegetative cells into MMT. The close-up views show the conidiation defects in the OE*AfuabaA* strain. Arrows indicate the simplified conidiophores formed by the control strain at 24 h. (b) Mycelial dry weight of control (black bars) and OE*AfuabaA* (white bars) strains grown in liquid MMG or MMT. (c) Cell viability of control and OE*AfuabaA* strains determined by AB reduction rates. Data are the mean values of three individual cultures. Note that the OE*AfuabaA* strain exhibits reduced viability at 24 h. Black bars, control in MMG; dotted bars, OE*AfuabaA* in MMG; grey bars, control in MMT; white bars, OE*AfuabaA* in MMT. (d) Phenotypes of control and OE*AfuabaA* strains grown on solid inducing medium (MMT) at 37 °C for 3 days, and the number of conidia per mm^2 of control and OE*AfuabaA* colonies. Note that the OE*AfuabaA* strain formed small colonies with reduced numbers of conidia. ■, Control in MMGY; ●, OE*AfuabaA* in MMGY; □, control in MMTY; ●, OE*AfuabaA* in MMTY.



Importantly, *Afu*AbaA 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 *Afu*AbaA 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 *Afu*AbaA-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, Δ*AfuwetA* 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 Δ*AfuwetA* conidia exhibit unclear and aberrant wall layers compared to those of WT, i.e. *Afu*WetA 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 Δ*AfuwetA* mutant revealed that most conidia form only

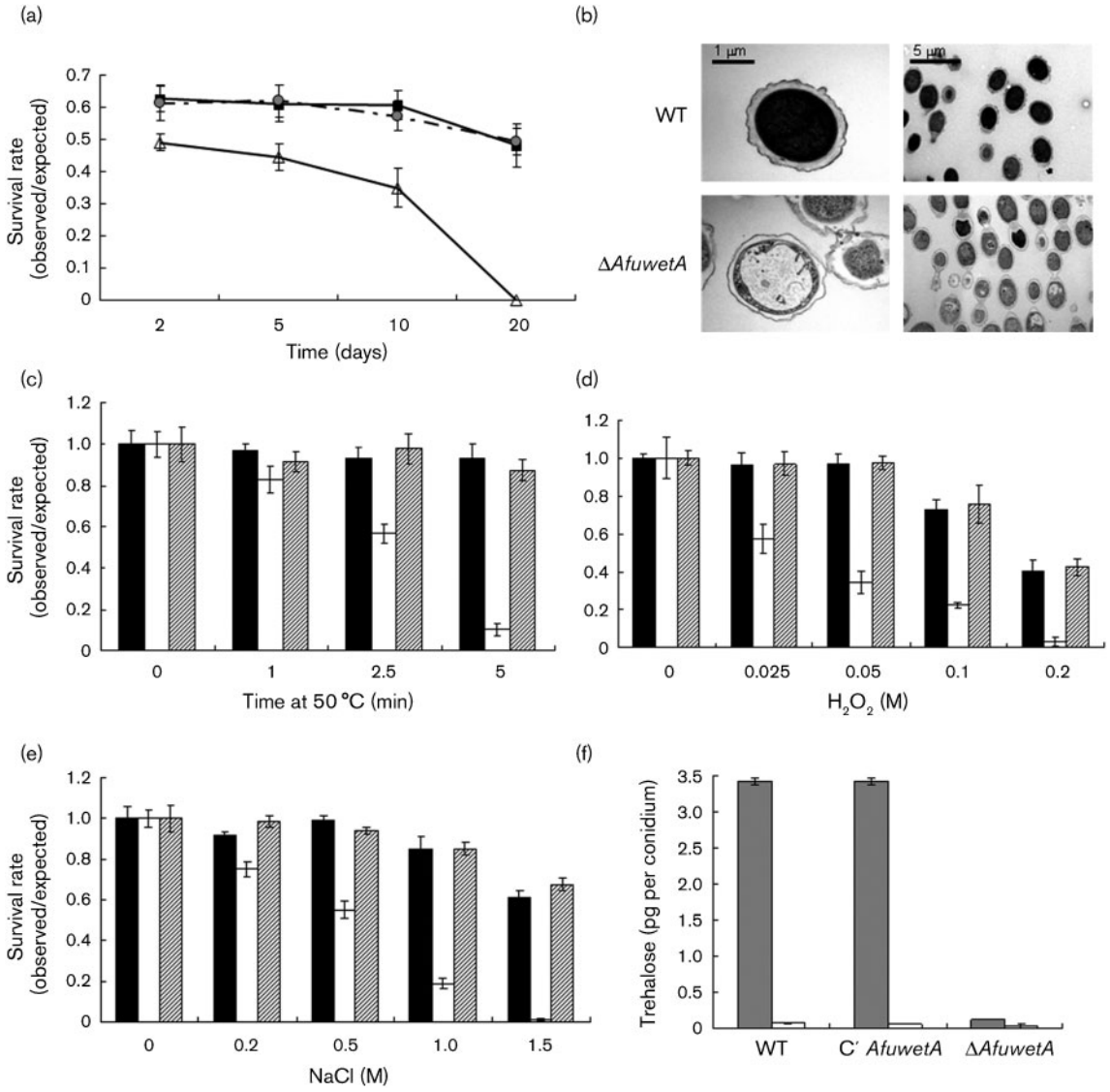


Fig. 5. Requirement of *Afu*WetA for viability and stress tolerance of conidia. (a) Viability of conidia of WT (Af293, ■), Δ*AfuwetA* (TSGw4, △) and complemented (TLI4, ●) strains grown at 37 °C for 2, 5, 10 and 20 days. (b) TEM images of 2-day-old conidia of WT and Δ*AfuwetA* strains. Note that even at day 2, about 20 % of the Δ*AfuwetA* conidia have lost their cytoplasm. (c–e) Tolerance of WT (black bars), Δ*AfuwetA* (white bars) and complemented strain (hatched bars) conidia to heat (c), oxidative (d) and salt (e) stresses. (f) The amount of trehalose (pg per conidium) in 2-day-old conidium of WT, Δ*AfuwetA* and complemented strains. No trehalase treatment (white bars) served as a negative control.

one germ tube, and that the branching of the germlings is reduced. Due to the inhibited hyphal branching, the Δ*AfuwetA* 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 Δ*AfuwetA* 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 *Afu*FluG 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 *Afu*BrlA, thereby triggering the

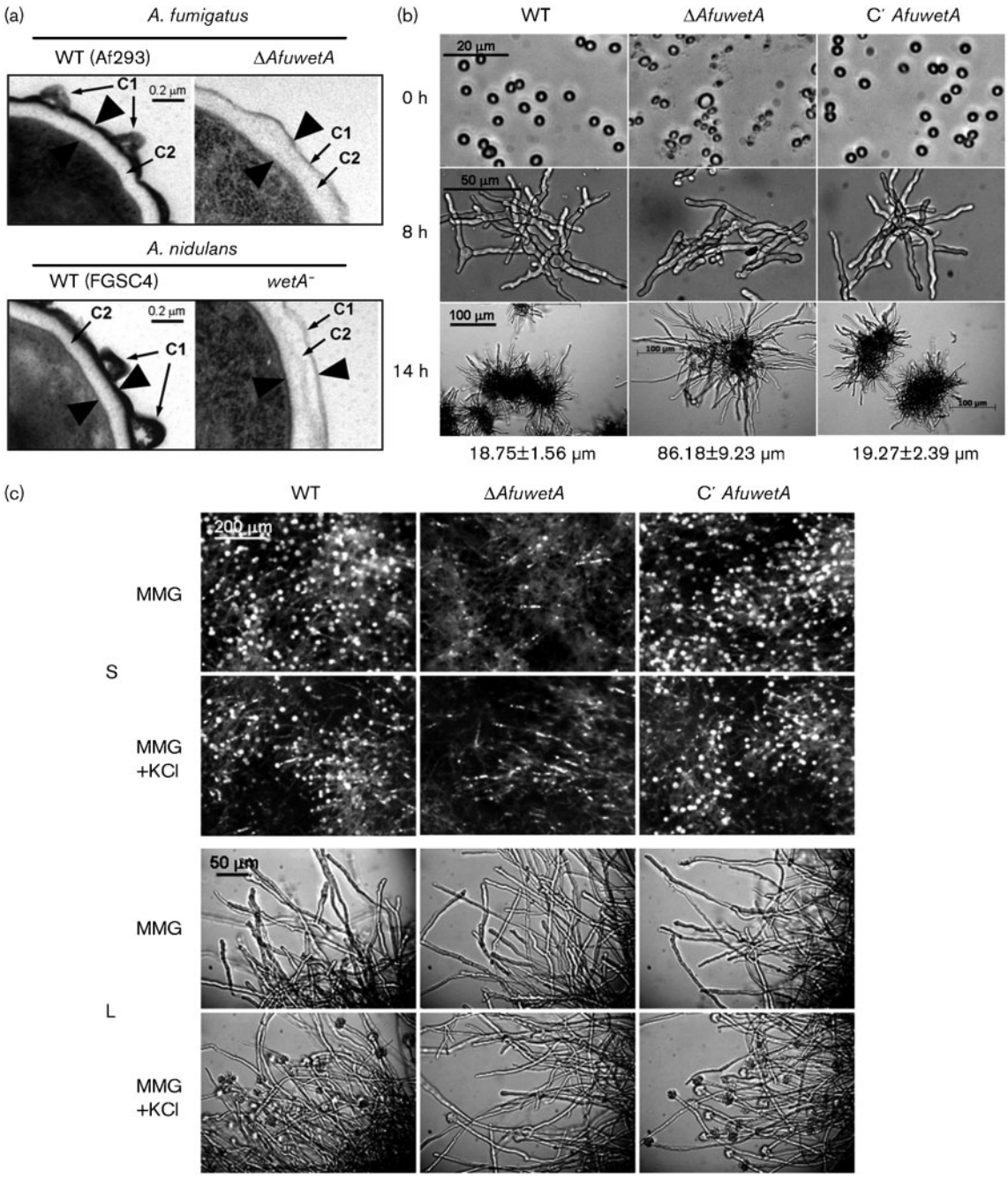


Fig. 6. Additional phenotypes resulting from Δ*AfuwetA*. (a) TEM images of 2-day-old conidia wall structures of *A. fumigatus* WT (Af293) and the Δ*AfuwetA* mutant (TSGw4), and *A. nidulans* WT (FGSC4) and the *wetA*⁻ mutant (AJC472.1). Arrowheads indicate the wall thickness in four conidia. C1 (outer) and C2 (inner) indicate the wall layers of *A. nidulans* conidia as reported by Sewall *et al.* (1990b). (b) Conidia of WT, Δ*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, Δ*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 *AfuwetA*.

central regulatory pathway (*AfubrlA*→*AfuabaA*→*AfuwetA*) of conidiation. Subsequently, *AfuvosA* (Ni & Yu, 2007) is activated by *Afu*AbaA with the assistance of *Afu*WetA. *Afu*WetA and *Afu*VosA coordinately complete spore maturation, including trehalose biogenesis. As found in *A. nidulans*, both *Afu*BrlA and *Afu*AbaA are required for

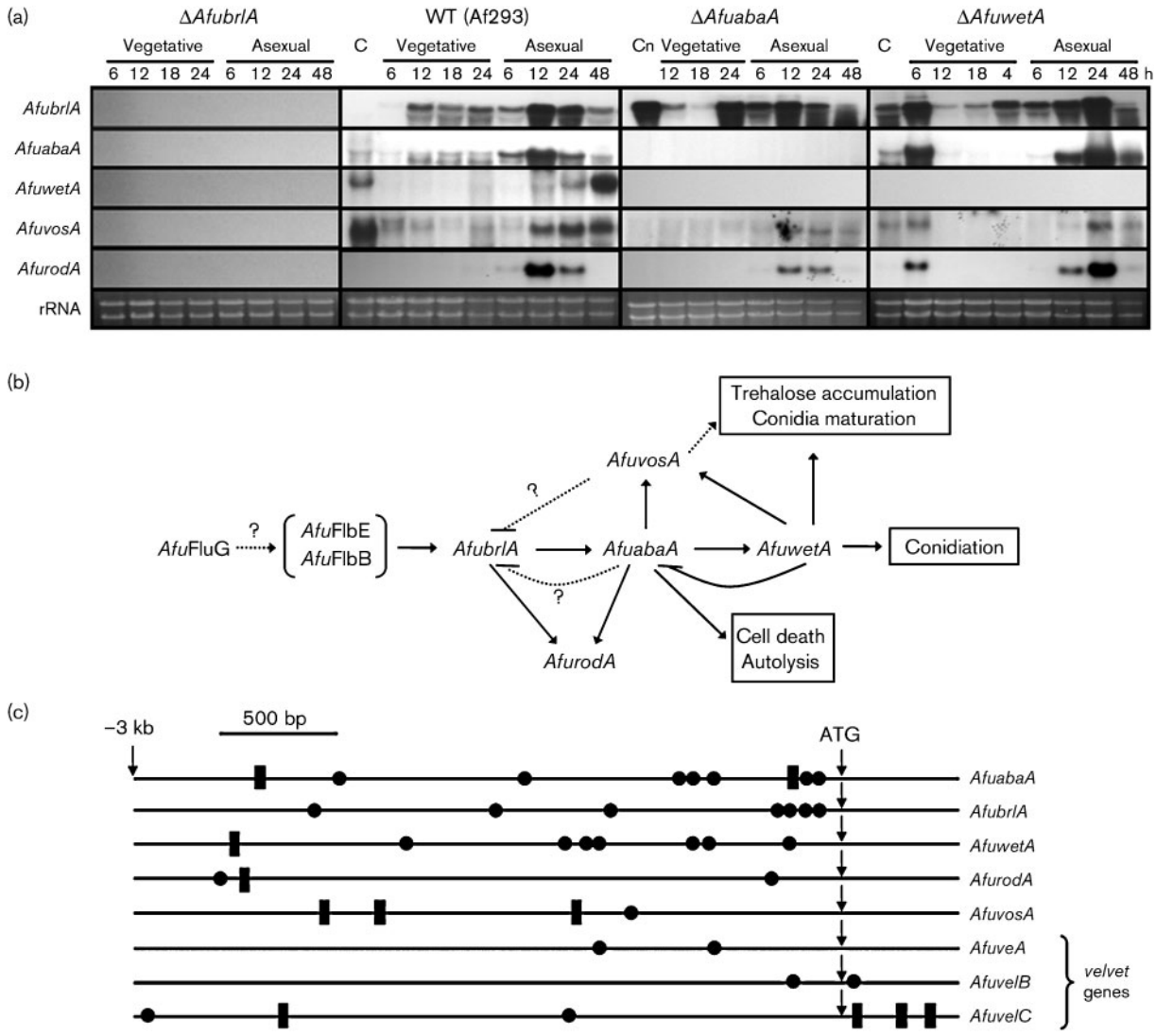


Fig. 7. Further expression studies and a model for developmental regulation in *A. fumigatus*. (a) Levels of various developmental genes (*AfubrlA*, *AfuabaA*, *AfuwetA*, *AfuvosA* and *AfurodA*) in four strains Δ*AfubrlA* (A1176), WT (Af293), Δ*AfuabaA* (TSGa17) and Δ*AfuwetA* (TSGw4). C, conidia; Cn, conidiophore. (b) Model for regulation of *A. fumigatus* conidiation (see Discussion). (c) Positions of BREs (■, 5′-(C/A)(G/A)AGGG(G/A)-3′) and AREs (●, 5′-CATTCY-3′) in selected regulatory genes in *A. fumigatus*.

proper expression of *AfurodA* (see Adams *et al.*, 1998). The repressive roles of *Afu*AbaA and *Afu*WetA on expression of *AfubrlA* may be attributed to *Afu*VosA (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 *Afu*WetA may negatively regulate *AfuabaA* and that *Afu*AbaA may directly repress *AfubrlA* are indicated. *Afu*AbaA 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′-CATTCY-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 *Afu*AbaA might bind to these genes and affect their expression. Among those tested, *Afu*VeA, *Afu*VelB and *Afu*VelC 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). *Afu*AbaA may also influence secondary metabolism by regulating expression of *AfuveA*,

AfuvelB and *AfuvelC*. As one, two and three BREs are found in the *AfuwetA*, *AfuabaA* and *AfuvosA* genes, respectively, and one is found in *AfurodA*, *Afu*BrlA may control *AfuwetA*, *AfuabaA*, *AfuvosA* and *AfurodA* expression. Further studies to identify *Afu*AbaA and *Afu*WetA target genes in *A. fumigatus* are in progress.

ACKNOWLEDGEMENTS

The authors express special thanks to Shilpa Gadwal, Na Gao and Hee-Soo Park for technical assistance and Ellin Doyle for critically reviewing the manuscript. L. T. was supported by the Graduate Scholarship Program sponsored by China Scholarship Council (CSC) and the Ministry of Education of China. This work was supported by National Science Foundation (IOS-0640067, IOS-0950850) and UW-Madison Food Research Institute grants to J.-H. Y.

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Edited by: A. H. Herrera-Estrella