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Characterization of the *velvet* regulators in *Aspergillus fumigatus*

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Summary

Fungal development and secondary metabolism is intimately associated via activities of the fungi-specific *velvet* family proteins. Here we characterize the four *velvet* regulators in the opportunistic human pathogen *Aspergillus fumigatus*. The deletion of *AfuvosA*, *AfuveA* and *AfuvelB* causes hyperactive asexual development (conidiation) and precocious and elevated accumulation of *AfubrlA* during developmental progression. Moreover, the absence of *AfuvosA*, *AfuveA* or *AfuvelB* results in the abundant formation of conidiophores and highly increased *AfubrlA* mRNA accumulation in liquid submerged culture, suggesting that they act as repressors of conidiation. The deletion of *AfuvosA* or *AfuvelB* causes a reduction in conidial trehalose amount, long-term spore viability, conidial tolerance to oxidative and UV stresses, and accelerated and elevated conidial germination regardless of the presence or absence of an external carbon source, suggesting an interdependent role of them in many aspects of fungal biology. Genetic studies suggest that *Afu*AbaA activates *AfuvosA* and *AfuvelB* expression during the mid to late phase of conidiation. Finally, the *AfuveA* null mutation can be fully complemented by *Aspergillus nidulans* VeA, which can physically interact with *Afu*VelB and *Afu*LaeA *in vivo*. A model depicting the similar yet different roles of the *velvet* regulators governing conidiation and sporogenesis in *A. fumigatus* is presented.

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Introduction

Aspergillus fumigatus is a saprophytic ascomycete fungus that plays a crucial role in nutrient recycling. It is also the most prevalent airborne fungal pathogen (Latge, 1999; 2001). This airborne fungus propagates in the environment by producing a massive number of asexually derived spores called conidia. Inhaled conidia are normally removed by the innate immune system including alveolar macrophages. However, inhalation of conidia by immune-compromised individuals causes serious invasive pulmonary aspergillosis with a human mortality rate of at least 50% (Latge, 1999; Dagenais and Keller, 2009; McCormick *et al*., 2010; Cramer *et al*., 2011). Moreover, the airborne fungal spores contain potent allergens to which certain people respond with exaggerated hypersensitive reaction causing allergic bronchopulmonary aspergillosis (Stevens *et al*., 2003; Tillie-Leblond and Tonnel, 2005).

Asexual development (conidiation) in *Aspergillus* involves the formation of elaborate multicellular conidia-bearing structures called conidiophores (Adams *et al*., 1998; Yu, 2010). The asexual reproductive cycle of *A. fumigatus* can be divided into vegetative growth and developmental phases. Vegetative growth begins with the spore germination leading to the formation of a network of undifferentiated interconnected hyphae known as the mycelium. After a certain period of hyphal proliferation, in response to appropriate stimuli (e.g. exposure to air or nutrient deficiency), some of the hyphal cells cease normal growth and initiate conidiation, which can be subdivided into conidiophore formation and spore maturation. Conidiophores consist of a thick-walled foot cell, stalk, vesicle, phialides (no metulae) and (up to 50 000) conidia (Adams *et al*., 1998; Rhodes and Askew, 2010; Yu, 2010). The process of conidiation is a precisely timed and genetically programmed event responding to internal and external signals (Mirabito *et al*., 1989; Adams *et al*., 1998). Our previous studies demonstrated that BrlA, AbaA and WetA control spatial and temporal expression of conidiation-specific genes during conidiophore development and

spore maturation in *A. fumigatus* (Mah and Yu, 2006; Tao and Yu, 2011). The roles of these central regulatory components are conserved in two distantly related fungi: *Aspergillus nidulans* (*Ani*; model) and *A. fumigatus* (*Afu*; pathogen) (Mah and Yu, 2006; Yu *et al*., 2006; Yu, 2010; Tao and Yu, 2011).

Fungal conidia contain high levels (up to 15% of the dry mass) of trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), which plays a crucial role in long-term spore viability and tolerance against environmental stresses including heat, oxidative and osmotic stresses (Sussman and Lingappa, 1959; Winkler *et al*., 1991; Elbein *et al*., 2003; Paul *et al*., 2008). Importantly, trehalose biosynthesis is involved in the virulence of several pathogenic fungi including *A. fumigatus*, *Candida albicans* and *Cryptococcus neoformans* (Alvarez-Peral *et al*., 2002; Petzold *et al*., 2006; Martinez-Esparza *et al*., 2007; Al-Bader *et al*., 2010; Puttikamonkul *et al*., 2010). Due to the lack of orthologues involved in trehalose biosynthesis in humans and the importance of trehalose biosynthesis in fungal pathogenesis, proteins associated with trehalose biosynthesis have been considered as potential antifungal drug targets. Recent study demonstrated that *Afu*WetA plays an essential role in trehalose biogenesis in conidia of *A. fumigatus* (Tao and Yu, 2011).

Our previous studies have demonstrated that *An*VosA plays a key role in trehalose biogenesis in conidia, negative feedback regulation of conidiation and conidial maturation in *A. nidulans* (Ni and Yu, 2007). *An*VosA and three other proteins (*An*VeA, *An*VelB and *An*VelC) share high levels of similarity (Ni and Yu, 2007), and together they define the *velvet* family regulators. The *velvet* regulators are highly conserved, are only found in filamentous ascomycetes and basidiomycetes and all contain the *velvet* domain (Ni and Yu, 2007; Bayram and Braus, 2012). They play differential roles in governing development, sporogenesis and secondary metabolism by forming the multiple *velvet* complexes such as VelB–VeA–LaeA, VelB–VosA and VelB–VelB in *A. nidulans* (Bayram *et al*., 2008a; Sarikaya Bayram *et al*., 2010; Park *et al*., 2012). The *velvet* regulators, particularly VeA and VelB homologues, have been characterized in various fungi and shown to be involved in controlling development, secondary metabolism and pathogenicity (reviewed in Calvo, 2008; Bayram and Braus, 2012). However, these crucial regulators have not been characterized in *A. fumigatus*.

In this study, we characterize the four *velvet* genes by genetic and biochemical approaches in *A. fumigatus*, and present evidence that they play pivotal roles in governing development, trehalose biosynthesis and conidial germination. Somewhat distinct from *A. nidulans*, the deletion of *AfuveA* or *AfuvelB* results in hyperactivation of conidiation, leading to abundant formation of conidiophores in liquid submerged culture and near constitutive accumulation of high levels of *AfubrlA* mRNA in vegetative growth, suggesting that *Afu*VeA and *Afu*VelB are the primary negative regulators of conidiation during vegetative growth. It appears that the feedback control of development upon completion of conidiophore is conferred by *Afu*VelB and *Afu*VosA, which are activated by *Afu*AbaA. In addition, both *Afu*VosA and *Afu*VelB participate in coupling trehalose biogenesis in conidia and sporogenesis, and *Afu*VosA, *Afu*VeA and *Afu*VelB play a differential role in controlling conidial germination. Finally, we show that the *AfuveA* null mutation is complemented by *AniveA* and *An*VeA physically interacts with *Afu*VelB and *Afu*LaeA in *A. fumigatus*. We propose a genetic model depicting the *velvet*-mediated developmental regulation in *A. fumigatus*.

Results

Summary of A. fumigatus velvets

The *AfuvosA* gene (EF544392, AFUG_4G10860) was previously identified by a genome search (Ni and Yu, 2007). The ORF of *AfuvosA* consists of 2012 bp nucleotides (nt) with 10 exons, predicted to encode a 435 aa length protein (Fig. 1A). As shown in Fig. 1B, levels of *AfuvosA* transcript are particularly high in conidia (C) and during the early vegetative growth. Then, the levels drop quickly during late vegetative growth (vegetative 9–24 h) and increase during late phase of conidiation (asexual 12, 24, 48 and 72). The *Afu*VosA protein contains the *velvet* domain in the N-terminal half and a putative NLS-pat7 (239 PVKRQRT 245; http://psort.hgc.jp/form2.html). Searching the Pfam database revealed that the *Afu*VosA C-terminal portion is similar to the PAT1 protein, a topoisomerase II-associated deadenylation-dependent mRNA-decapping factor, necessary for accurate chromosome transmission and mRNA turnover in *Saccharomyces cerevisiae* (Fig. 1C) (Wang *et al*., 1996; Marnef and Standart, 2010).

The *AfuveA* gene (CAE47975; AFUA_1G12490) was previously reported by Krappmann *et al*. (2005): the ORF of *AfuveA* consists of 1713 bp, interrupted by one intron (70 nt), encoding a 570-amino-acid length protein (63.0 kDa). Northern blot analysis revealed that *AfuveA* mRNA is detectable at 3 h of vegetative growth, maintained at high levels during vegetative growth and the early phase (6–24 h) of conidiation, then reduced (Fig. 1B). Protein database searches revealed that *Afu*VeA contains the *velvet* domain and a putative classical bipartite nuclear localization signal (NLS) in the N-terminal region (Ni and Yu, 2007; Stinnett *et al*., 2007). Whereas *An*VeA contains a putative export signal (NES) (Bayram *et al*., 2008b), no canonical NES motif is found in *Afu*VeA (http://www.cbs.dtu.dk/services/NetNES/). The Web-based program ePESTfind (http://

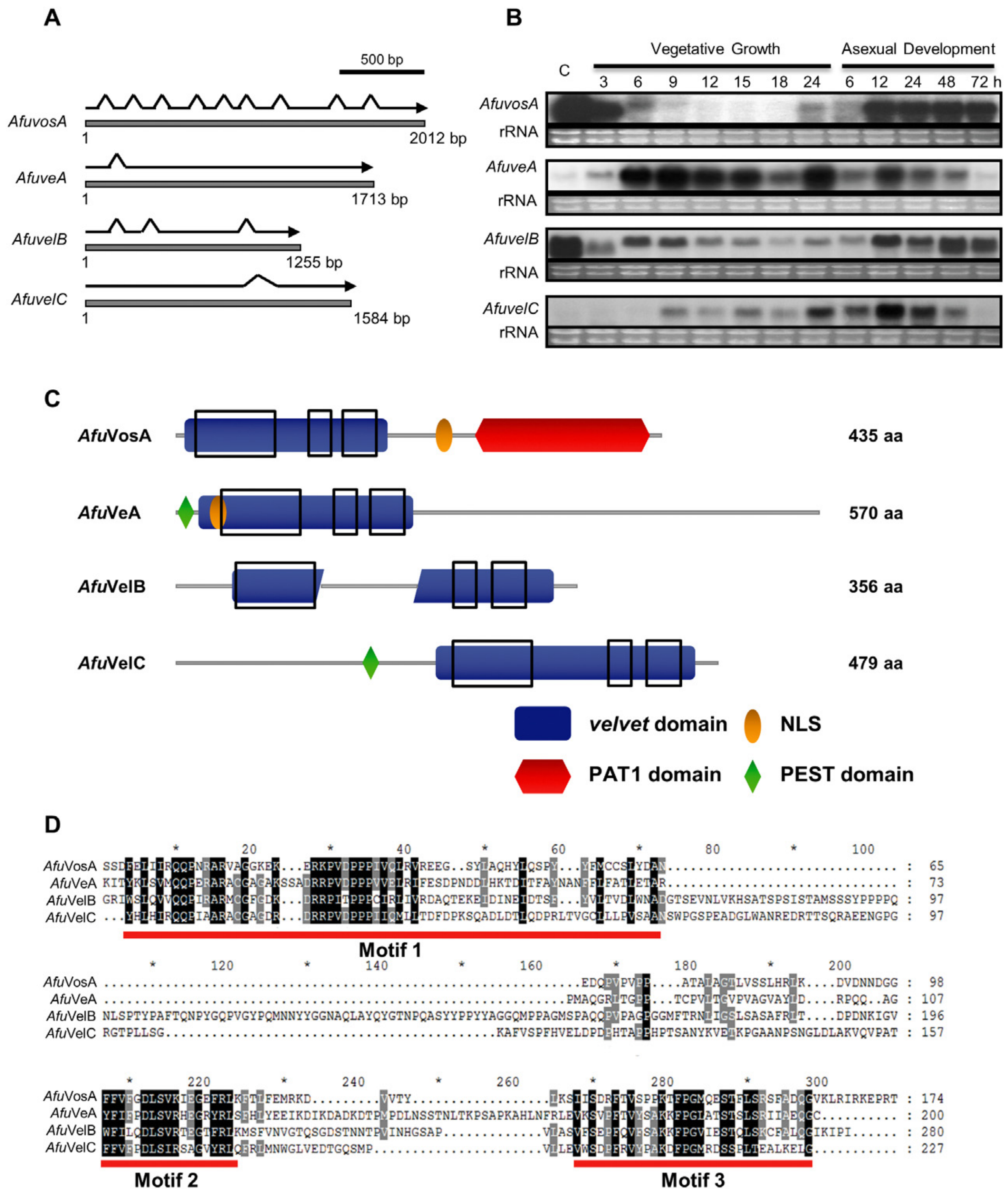

Fig. 1. Summary of *Afuvelvet*.

A. Schematic presentation of the *Afuvelvet* ORF (shaded box), transcripts (arrows) and introns (shown by discontinuity in the arrow). Gene structures were verified by sequence analyses of cDNA of individual *Afuvelvets*. Start codon is assigned as 1.

B. Northern blot showing levels of *Afuvelet* mRNA during the lifecycle of *A. fumigatus* WT (AF293). Conidia (asexual spores) were indicated as 'C'. The time (hours) of incubation in liquid submerged culture and post asexual developmental induction is shown. Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA.

C. Domain architecture of the *Afuvelvet* regulators in *A. fumigatus*. Three conserved motifs were indicated by boxes.

D. Alignment of the velvet domain in *Afu*VosA, *Afu*VeA, *Afu*VelB and *Afu*VelC. The conserved motifs are marked by lines. ClustalW (http://align.genome.jp/) was used for the alignment.

emboss.bioinformatics.nl/cgi-bin/emboss/epestfind) predicted a putative PEST sequence for rapid degradation at the N terminus of *Afu*VeA.

The *AfuvelB* gene (EAL87909; AFUA_1G01970) is composed of a 1255 bp ORF with three (64, 58 and 62 nt) introns (Fig. 1A). As shown in Fig. 1B, levels of the *AfuvelB* transcript(s) are high in conidia (C) and during the late phase of conidiation (asexual 24, 48 and 72 h) when conidia differentiate and become mature. Levels of the *AfuvelB* transcript(s) quickly drop during vegetative growth and early asexual developmental induction. The *AfuvelB* gene appears to encode two overlapping transcripts that are detectable during the late phase of conidiation and sporogenesis. Previously, Bayram and Braus proposed that VelB contains two *velvet* domains in *A. nidulans* (Bayram and Braus, 2012). Our further analyses of the *velvet* domain using ClustalW and MEME (Bailey *et al.*, 1994) revealed that it contains three conserved motifs (boxes in Fig. 1C). The N-terminal and C-terminal parts of *Afu*VelB include motif 1 and motifs 2&3, respectively, separated by 90 aa residues (Fig. 1D), suggesting that *Afu*VelB contains one *velvet* domain.

The ORF of *AfuvelC* (EAL89811; AFUA_4G09770) consists of 1584 bp, interrupted by one intron (144 nt), and is predicted to encode a 479-amino-acid length protein (52.0 kDa) containing the *velvet* domain in the C-terminal region (Fig. 1A and C). The *Afu*VelC protein contains one putative PEST sequence (ePESTfind) in the N-terminal region (Fig. 1C). We examined mRNA levels of *AfuvelC* and found that levels of *AfuvelC* mRNA are low during vegetative growth, increase at 6 and 12 h of conidiation and are undetectable in conidia (Fig. 1B).

Differential roles of velvets *in asexual development*

To investigate the roles of the *Afuvelvet* genes in *A. fumigatus*, we generated *Afuvelvet* null mutants (Δ*Afuvelvet*) by replacing the entire coding region of each *Afuvelvet* gene with the *A. nidulans pyrG+* or *argB+* marker. We also generated complemented strains for each *Afuvelvet* deletion by introducing the wild-type (WT) allele (Fig. 2A). Multiple Δ*Afuvelvet* and complemented strains displaying identical phenotypes were isolated and further examined. The first noticeable changes were that, when point-inoculated on solid medium, the deletion of *AfuveA* and *AfuvelB* resulted in a significant reduction ($P < 0.05$) of radial colony growth (~ 80% of WT) and enhanced accumulation of green pigment(s), implying hyperactive conidiation (Fig. 2A and B).

When we further examined the effects of *Afuvelvet* during asexual developmental induction, we found that, while WT and Δ*AfuvelC* strains began to show formation of conidiophores at 5 h, the Δ*AfuvosA*, Δ*AfuveA* and Δ*AfuvelB* mutants started to produce conidiophores at 3–4 h after induction of development (data not shown). Further examination of mRNA levels of *AfubrlA* and *AfuabaA* in WT and Δ*Afuvelvet* strains revealed precocious and elevated accumulation of these two key developmental activators by the absence of *AfuveA*, *AfuvelB* or *AfuvosA*. As shown in Fig. 2C, in WT accumulation of *AfubrlA* mRNA increased from 6 h, peaked at 12 h, decreased after 24 h and became almost undetectable at 48 h post developmental induction. *AfubrlA* mRNA in the Δ*AfuvosA* mutant started to accumulate at 0 h, increased from 6 h, peaked at 24 h and decreased after 48 h. The deletion of *AfuveA* resulted in near constitutive accumulation of high levels of *AfubrlA* mRNA at 0–48 h post developmental induction. *AfubrlA* mRNA levels in Δ*AfuvelB* strains were high during early (0 h) asexual development and somewhat declined during late conidiation. In the Δ*AfuvelC* mutant it appears that levels of *AfubrlA* mRNA decreased compared to WT. *AfuabaA* mRNA levels were high in the Δ*AfuveA*, Δ*AfuvelB* and Δ*AfuvosA* mutants, but low in the Δ*AfuvelC* mutant (Fig. 2C). These results indicate that *Afuvelvet* regulators play differential roles in (negatively) controlling conidiation and expression of *AfubrlA* during the progression of development.

Negative regulation of conidiation by Afu*VosA,* Afu*VeA and* Afu*VelB*

To further investigate the developmental regulatory roles of *Afuvelvet*, conidia of WT, Δ*Afuvelvet* and complemented strains were inoculated into liquid minimal medium with glucose (MMG) with 0.1% yeast extract (YE), and examined for the conidiophore formation. As shown in Fig. 3A, whereas WT, Δ*AfuvelC* and complemented strains did not elaborate conidiophores, the absence of *AfuvosA* caused swelling of the hyphal tips and production of some conidiophores. Furthermore, the deletion of *AfuveA* and *AfuvelB* resulted in the formation of a high number of conidiophores and also green conidia in liquid submerged culture, suggesting that *Afu*VeA and *Afu*VelB play a primary role in proper repression of conidiation during vegetative proliferation (Fig. 3A). We further examined whether the deletion of *Afuvelvet* affected *AfubrlA* mRNA levels via Northern blot analyses. As shown in Fig. 3B, *AfubrlA* mRNA in the Δ*AfuvosA* mutant was detectable at 24 h of liquid submerged culture conditions, and accumulated even higher levels in the Δ*AfuveA* and Δ*AfuvelB* vegetative cells (see V24 and V48 in Fig. 3B).

To test a genetic relationship between *Afu*VelB and *Afu*VeA or *Afu*VelB and *Afu*VosA, we generated the Δ*AfuveA* Δ*AfuvelB* and Δ*AfuvelB* Δ*AfuvosA* double mutants and examined conidiophore formation and *AfubrlA* mRNA levels. Similar to the *AfuveA* and *AfuvelB* single deletion mutants, both Δ*AfuveA* Δ*AfuvelB* and

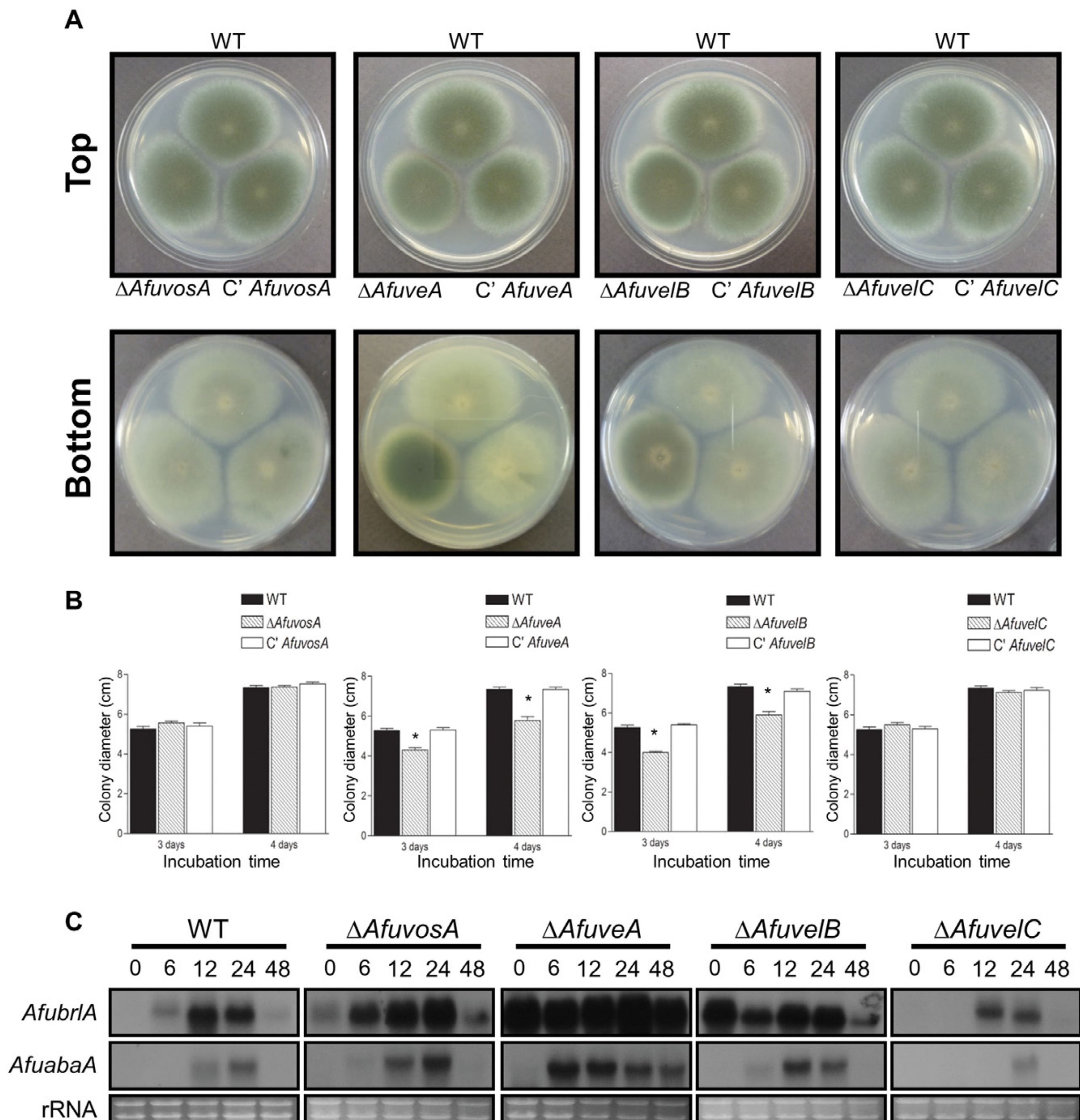

Fig. 2. The role of *Afuvelvet* regulating asexual development.
A. Colony photographs of WT (AF293), Δ*AfuvosA* (THSF11.1), Δ*AfuveA* (THSF1.1), Δ*AfuvelB* (THSF2.1), Δ*AfuvelC* (THSF3.1) and complemented strains THSF12.1 (C′ *AfuvosA*), THSF4.1 (C′ *AfuveA*), THSF5.1 (C′ *AfuvelB*) and THSF6.1 (C′ *AfuvelC*) point-inoculated on solid MMG with 0.1% YE and grown for 3 days (top and bottom panels). The bottom panel shows the underside of the plates.
B. Colony diameters of the designated strains point-inoculated on solid MMG with 0.1% YE and grown for 3 and 4 days. $^{*}P < 0.05$.
C. Northern blot for *AfubrlA* and *AfuabaA* mRNA in WT (AF293), Δ*AfuvosA* (THSF11.1), Δ*AfuveA* (THSF1.1), Δ*AfuvelB* (THSF2.1) and Δ*AfuvelC* (THSF3.1) strains post asexual developmental induction (Asex). Numbers indicate the time (hours) post induction of asexual development. Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA.

Δ*AfuvelB* Δ*AfuvosA* double mutants exhibited abundant formation of conidiophores and green conidia, and enhanced accumulation of *AfubrlA* in liquid submerged culture (Fig. 3B). These corroborate the idea that *Afu*VeA and *Afu*VelB are the principal negative regulators of conidiation during vegetative growth in *A. fumigatus*, and they may play an interdependent role in repression of development, most likely by forming a heterocomplex(es).

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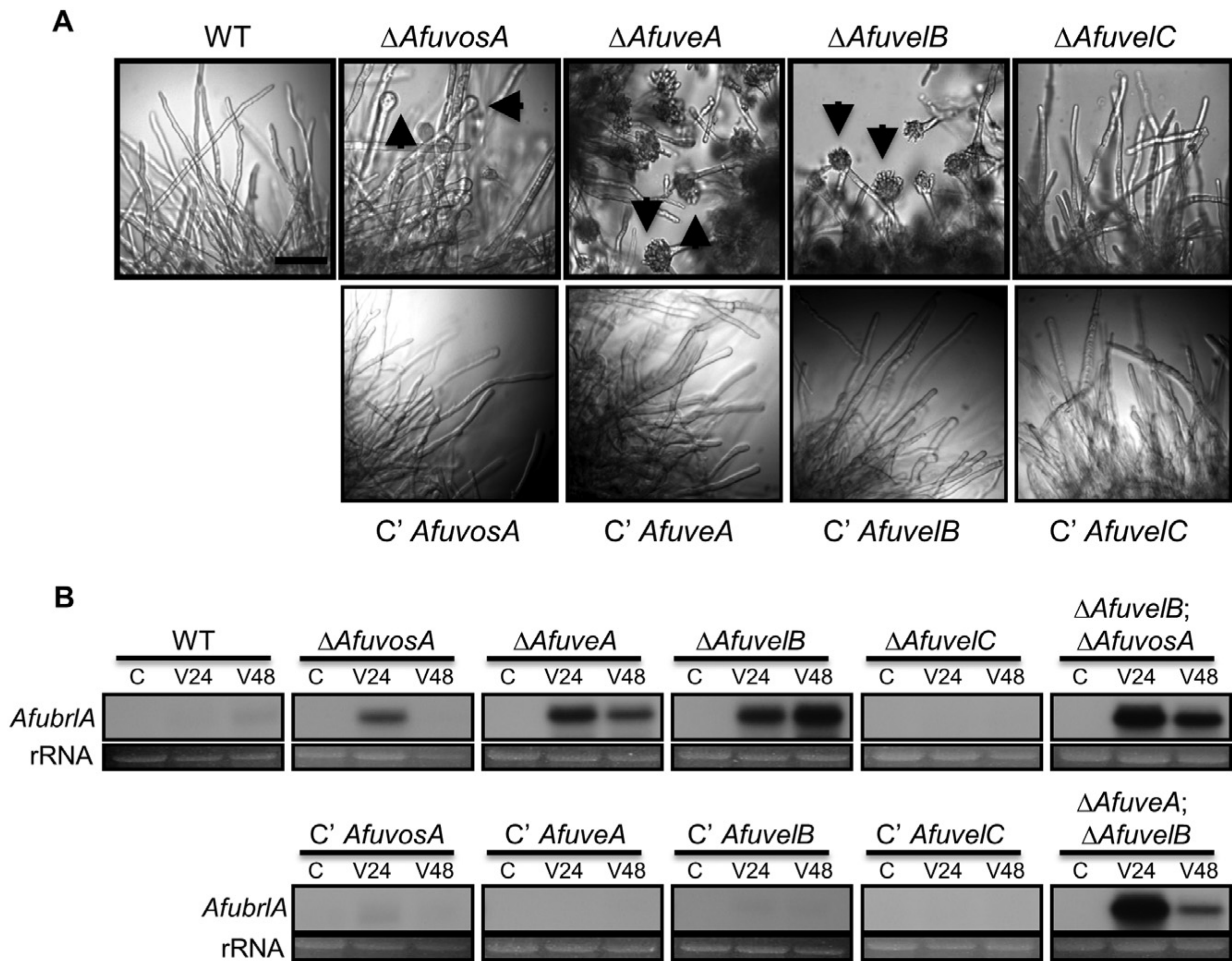

Fig. 3. Effects of Δ*Afuvelvet* in liquid submerged culture.
A. Photomicrographs of the mycelium of WT (AF293), Δ*AfuvosA* (THSF11.1), Δ*AfuveA* (THSF1.1), Δ*AfuvelB* (THSF2.1), Δ*AfuvelC* (THSF3.1) and complemented strains THSF12.1 (C′ *AfuvosA*), THSF4.1 (C′ *AfuveA*), THSF5.1 (C′ *AfuvelB*), THSF6.1 (C′ *AfuvelC*) grown in liquid MMG with 0.1% YE for 24 h at 37°C. The arrows indicate conidiophores.
B. mRNA levels of *AfubrlA* in above-mentioned strains (A), and double deletion mutant strains Δ*AfuveA* Δ*AfuvelB* (THSF13.1) and Δ*AfuvelB* Δ*AfuvosA* (THSF14.1). Conidia are indicated as C. The time (hours) of incubation in liquid submerged culture (V) is shown.

We further propose that *Afu*VosA and *Afu*VelB play a primary role in negative feedback regulation of conidiation and *brlA* upon completion of conidiogenesis in *A. fumigatus* (see *Discussion*).

VosA *and* VelB *are required for proper trehalose biogenesis in* Afu *conidia*

Previous studies demonstrated that both VelB and VosA play an essential role in trehalose biogenesis in conidia and sporogenesis in *A. nidulans* (Ni and Yu, 2007; Sarikaya Bayram *et al*., 2010). To test a potential role of *Afu*-Velvet regulators in spore maturation and trehalose biosynthesis, viability of conidia, trehalose amount and tolerance of conidia to oxidative and UV stresses were examined and compared. For the viability, the ability of 30 day-old WT and Δ*Afuvelvet* conidia to generate colonies was examined. The results revealed that the Δ*AfuvosA* and Δ*AfuvelB* conidia exhibited ~ 40% reduced viability compared to conidia of WT and other strains (data not shown). To test whether the *Afu*Velvet regulators are needed for the proper accumulation of trehalose in spore, trehalose concentrations in 2 day-old fresh conidia of WT, Δ*Afuvelvet* and complemented strains were measured. In the Δ*AfuvosA* and Δ*AfuvelB* mutants conidia, trehalose content was decreased compared to that of WT, whereas amounts of trehalose of conidia of Δ*AfuveA* and Δ*AfuvelC* strains were comparable to that of conidia of WT, suggesting that *Afu*VelB and *Afu*VosA are required for the proper biogenesis of trehalose in conidia (Fig. 4A).

Because trehalose serves as a vital protectant against various environmental stresses, we further questioned

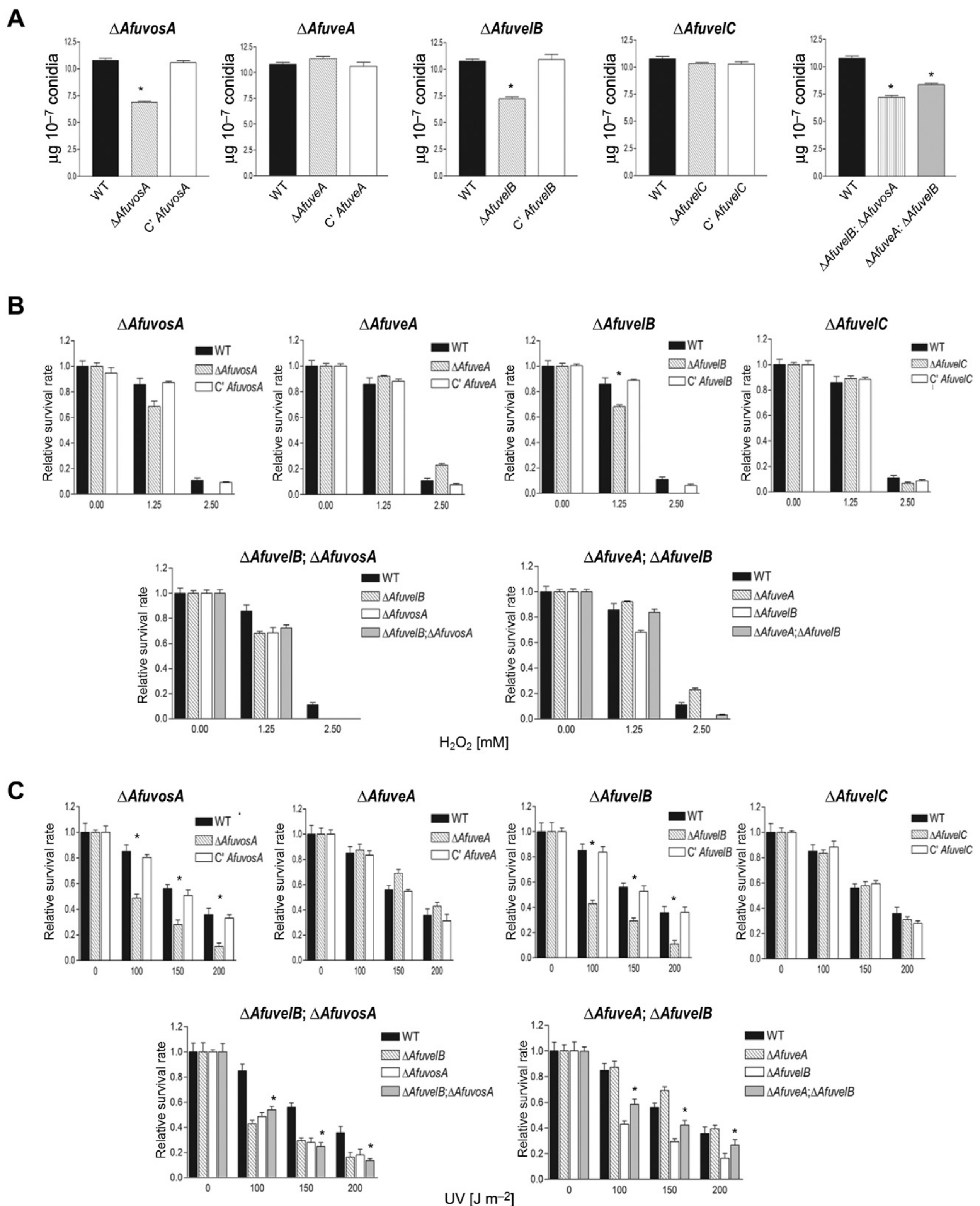

Fig. 4. Requirement of *Afuvelvet* for trehalose biosynthesis and stress tolerance of conidia.
A. Amount of trehalose per 10^7 conidia in the 2 day-old conidia of WT, Δ*Afuvelvet* single and double mutant and complemented strains. Differences between WT and mutant marked by an asterisk (*) are statistically significant according to the *t*-test with $P < 0.05$.
B. Tolerance of the conidia of WT, Δ*Afuvelvet* single and double mutant and complemented strains against oxidative stress. $^*P < 0.05$.
C. Tolerance of conidia of WT, Δ*Afuvelvet* single and double mutant and complemented strains against ultraviolet (UV) irradiation. $^*P < 0.05$.

whether the absence of *AfuvosA* and *AfuvelB* resulted in increased sensitivity of the conidia against oxidative and UV stresses. We found that the Δ*AfuvosA* and Δ*AfuvelB* mutant conidia exhibited reduced tolerance to oxidative and UV stresses (Fig. 4B and C).

Our previous studies suggested that *Ani*VelB primarily interacts with *Ani*VosA in asexual and sexual spores, and *Ani*VelB and *Ani*VosA play an interdependent role in trehalose biosynthesis in conidia (Sarikaya Bayram *et al.*, 2010; Park *et al.*, 2012). To examine the relationship between *Afu*VosA and *Afu*VelB, we checked trehalose amount and tolerance of spores against oxidative and UV stresses, and found that there were no differences between single and double deletion mutants (Fig. 4). Taken together, these results suggest that both *Afu*VosA and *Afu*VelB are required for proper trehalose biosynthesis and stress tolerance of spores, and they play an interdependent (not additive) role in spore maturation.

The roles of Afuvelvet *controlling conidial germination*

In our previous study, we demonstrated that the absence of *AnivelB* or *AnivosA* resulted in elevated conidial germination in *A. nidulans*, regardless of the presence or absence of an external carbon source (Park *et al.*, 2012). To examine the roles of *Afuvelvet* in spore germination, we first inoculated conidia of WT, Δ*Afuvelvet* and complemented strains in liquid MMG with 0.1% YE and examined germ tube formation. As shown in Fig. 5A, WT, Δ*AfuvelC* and all complement strains exhibited about 30% conidial germination at 8 h and near 100% germination at 13 h in liquid submerged culture. On the other hand, the Δ*AfuvosA*, Δ*AfuveA* and Δ*AfuvelB* mutants showed 60–70% conidial germination at 8 h and near 100% germination at 11 h in liquid medium. Moreover, the Δ*AfuveA* Δ*AfuvelB* and Δ*AfuvelB* Δ*AfuvosA* double mutants exhibited increased conidial germination rates similar to individual single deletion strains. To test further, conidia of WT, Δ*Afuvelvet* and complemented strains were inoculated in liquid medium without an external carbon source and observed for germination. We found that no conidia of all strains showed any sign (swelling) of germination until 48 h (data not shown).

We then examined germination rates of conidia of WT, Δ*Afuvelvet* and complemented strains on solid MMG or MMG with 0.1% YE. In both media, similar to liquid medium, Δ*AfuveA*, Δ*AfuvelB* and Δ*AfuvosA* single mutants exhibited enhanced conidial germination rates compared to WT (data not shown). Finally, we examined germination rates in the absence of external carbon source by inoculating conidia of WT, Δ*Afuvelvet* and complemented strains on solid MM with 1.6% agarose as a solidifying agent. As shown in Fig. 5B, at 24 h after inoculation, whereas only 10% of WT, Δ*AfuveA*, Δ*AfuvelC* and complemented strain conidia showed germling formation, 80% of the Δ*AfuvosA* or Δ*AfuvelB* single mutant conidia and the Δ*AfuveA* Δ*AfuvelB* and Δ*AfuvelB* Δ*AfuvosA* double mutant conidia germinated ($P < 0.05$). These indicate that *AfuvosA*, *AfuveA* and *AfuvelB* are associated with controlling conidial germination, and that *AfuvosA* and *AfuvelB* play an interdependent role in proper regulation of spore germination in the absence of external carbon source.

Afu*AbaA is necessary for expression of AfuvelB and AfuvosA*

Previously, we showed that *Ani*AbaA binds to the promoter regions of *AnivelB* and *AnivosA*, and activates *AnivelB* and *AnivosA* expression during the mid-late phase of conidiation in *A. nidulans* (Park *et al.*, 2012). We analysed the promoter regions of the *AfuvosA*, *AfuveA* and *AfuvelB* genes for the presence of the predicted AbaA response elements (AREs, 5′-CATTCY-3′, where Y is a pyrimidine; Andrianopoulos and Timberlake, 1994) and TEC1p, the *Saccharomyces cerevisiae* homologue of *Ani*AbaA, binding sequence (Gavrias *et al.*, 1996; Heise *et al.*, 2010) using the JASPAR program (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl?rm=browse&db=core&tax_group=fungi; Vlieghe *et al.*, 2006). As shown in Fig. 6A, one to three predicted AREs are present in the promoter regions of the *AfuvosA*, *AfuveA* and *AfuvelB* genes. We further hypothesized that *Afu*AbaA participates in the regulation of *AfuvosA*, *AfuveA* and *AfuvelB* expression during conidiation and examined mRNA levels of these genes in WT and Δ*AfuabaA* strains. The absence of *AfuabaA* abolished and reduced accumulation of *AfuvosA* and *AfuvelB* transcripts, respectively, but did not affect *AfuveA* expression (Fig. 6B). However, when *Afu*AbaA was overexpressed under the control of the *alcA* promoter, levels of *AfuveA* and *AfuvelB*, but not *AfuvosA*, mRNA increased at 6 and 12 h in liquid MMT submerged culture, indicating that *Afu*AbaA is sufficient to induce the activation of *AfuveA* and *AfuvelB* (Fig. 6C). Taken together, these results suggest that *Afu*AbaA differentially functions in proper expression of *AfuvosA*, *AfuveA* and *AfuvelB*.

Function and interaction of VeA *is conserved in two aspergilli*

The *Ani*VeA protein regulates sexual development and sterigmatocystin production by interacting with *Ani*VelB and *Ani*LaeA (Bayram *et al.*, 2008a). Many VeA homologues have been shown to control fungal development and secondary metabolism in other filamentous fungi (Calvo, 2008; Bayram and Braus, 2012), and they are functionally conserved among different ascomycetes (Bayram *et al.*, 2008b; Wiemann *et al.*, 2010). To check

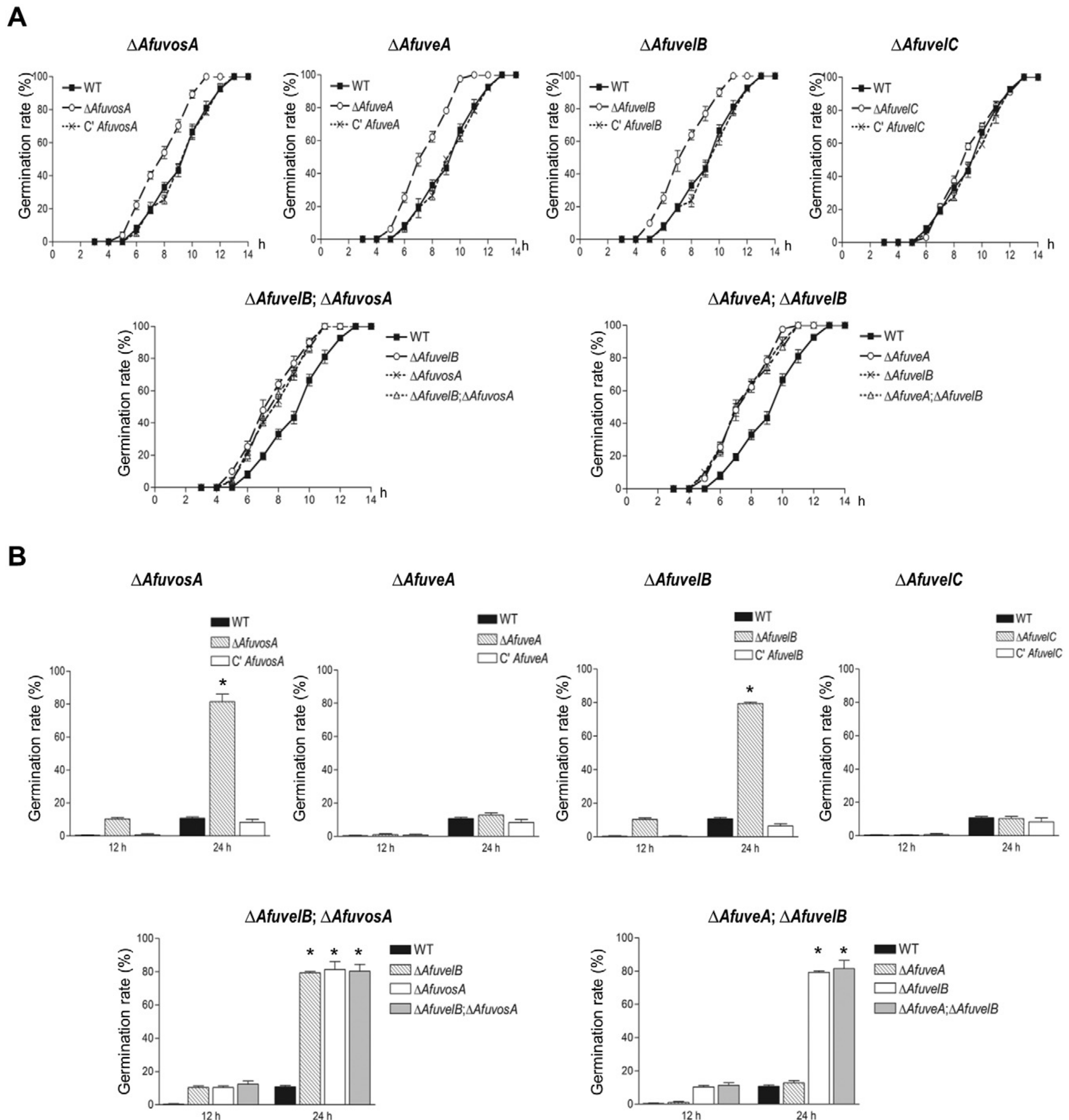

Fig. 5. Negative roles of *Afuvelvet* in controlling germination of conidia.
A. Kinetics of conidial germ tube formation of WT, Δ*Afuvelvet* single/double mutants and complemented strains inoculated in liquid MMG with 0.1% YE at 37°C, 220 r.p.m. Number of conidia showing a germ tube protrusion was recorded at indicated times and is presented as a percentage of the total number of conidia in these fields.
B. Conidia of the designated strains were inoculated on solid MM (using agarose as a solidifying agent) without an external carbon source and incubated at 37°C for 6–24 h. Number of conidia showing a germ tube protrusion was recorded at 12 or 24 h, and is presented as a percentage of the total number of conidia in these fields. $^*P < 0.05$.

the functional conservancy of VeA in two aspergilli, we generated strains expressing the *Ani*VeA protein fused with a tap C-terminal tag in the Δ*AfuveA* background in *A. fumigatus*, and examined the phenotypes and interacting proteins *in vivo*. We found that the introduction of *AniveA* fully restored growth and development in the Δ*AfuveA* mutant, suggesting the functional conservancy of VeA (Fig. 7A). To further test whether *Ani*VeA also inter-

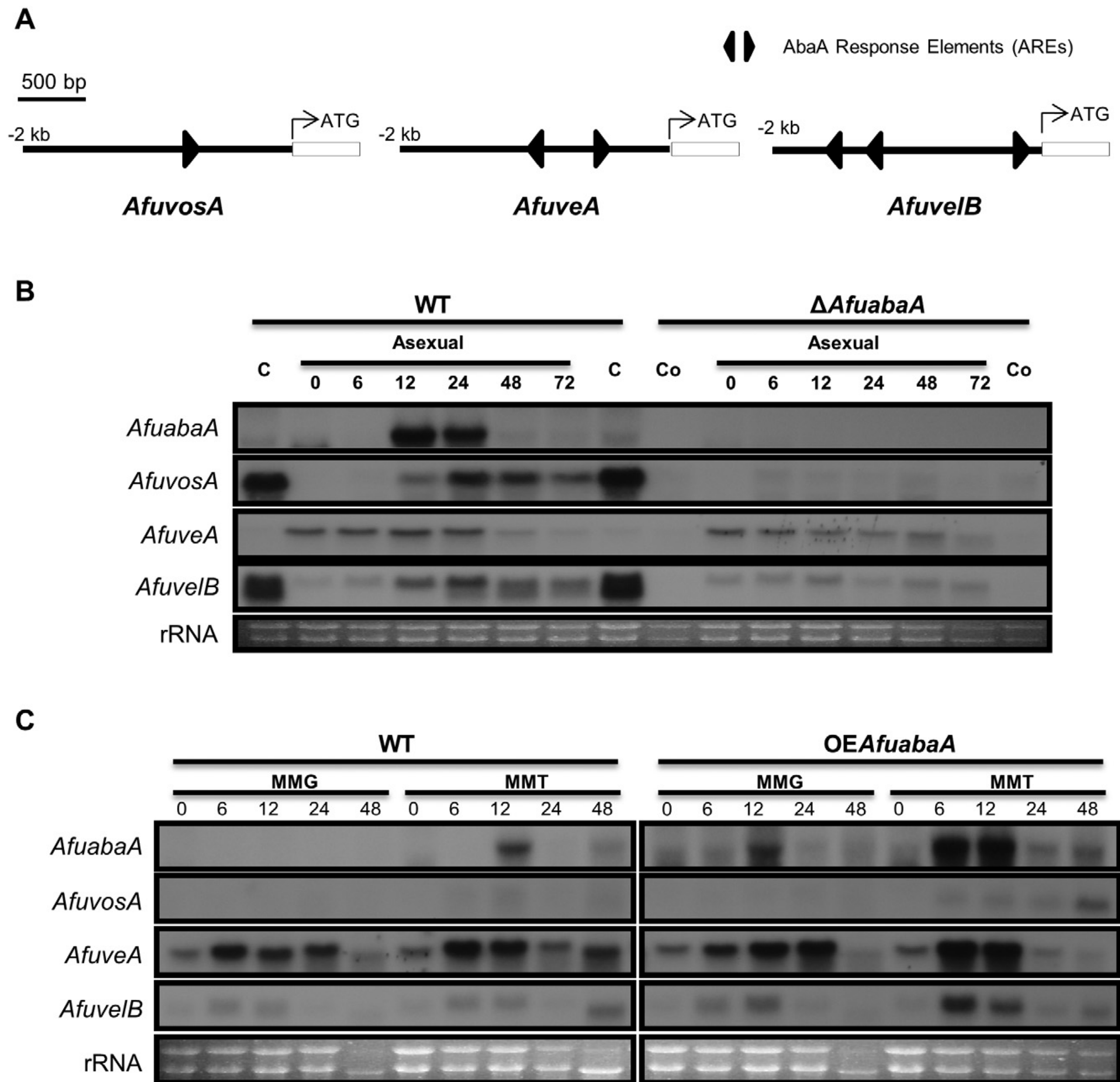

Fig. 6. The roles of *Afu*AbaA for *Afuvelvet* expression.
A. Positions of putative AREs (5′-CATTCY-3′, indicated by the black arrowhead) in the promoter regions of the *AfuvosA*, *AfuveA* and *AfuvelB* genes.
B. Northern blot analyses for the levels of *AfuabaA*, *AfuvosA*, *AfuveA* and *AfuvelB* transcripts in WT (AF293) and Δ*AfuabaA* (TSGa17) mutant strains at 0, 6, 12, 24, 48, 72 h post asexual developmental induction. C, conidia; Co, conidiophore.
C. Northern blot analyses for the levels of *AfuabaA*, *AfuvosA*, *AfuveA* and *AfuvelB* mRNAs in WT (AF293) and *AfuabaA* overexpression mutant (TLI9) strains. Strains were grown in liquid MMG at 37°C for 14 h (designated as time point '0') and transferred into liquid MMG (non-inducing) or liquid MMT (inducing). Samples were collected at designated time points after the transfer.

acts with *Afu*VelB or *Afu*LaeA, we used tandem affinity purification (TAP) (Bayram *et al*., 2008a) and found that the tagged *An*VeA was co-purified with *Afu*VelB and *Afu*LaeA in vegetative cells (Fig. 7B and C). These data suggest that the function and interaction of VeA is conserved in two *Aspergillus* species.

Discussion

In *A. nidulans*, the *velvet* regulators play differential roles controlling asexual and sexual development (Kim *et al*., 2002; Ni and Yu, 2007; Bayram *et al*., 2008a; Park *et al*., 2012). *An*VelB and *An*VeA are absolutely required for

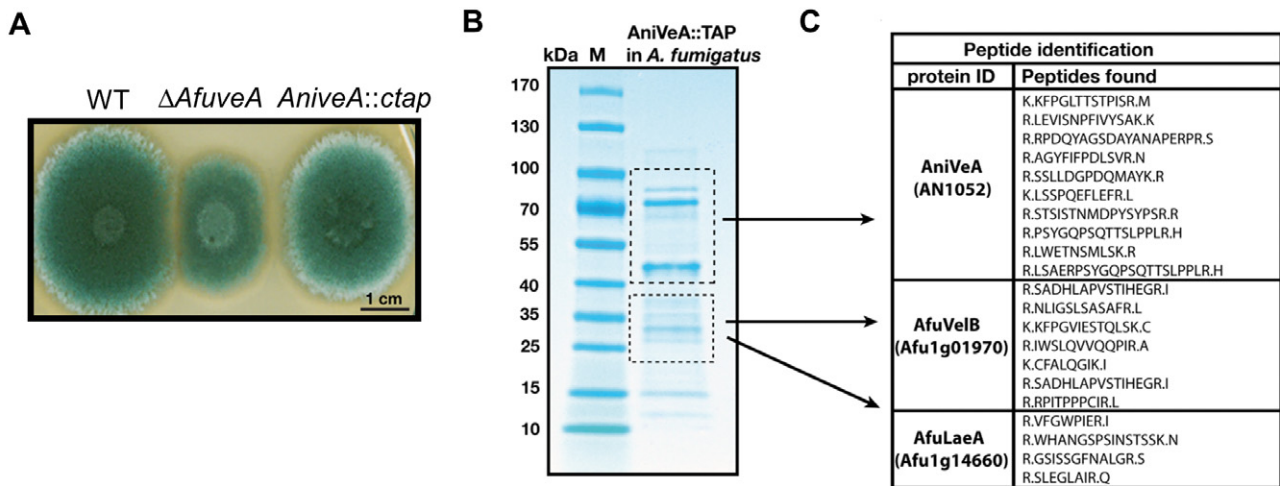

Fig. 7. Conservation of function and interaction of *An*VeA in *A. fumigatus*.
A. Phenotypes WT (D141), Δ*AfuveA* (AfS13) and *AniveA*::*ctap* strains. Conidia (10^4) of individual strains were point-inoculated on MMG and further incubated at 37°C for 3 days.
B. Colloidal Coomassie blue stained 4–15% gradient SDS polyacrylamide gel of *An*VeA–TAP purification from vegetative cells of *A. fumigatus*.
C. Peptide identifications from the indicated sectors in the SDS polyacrylamide gel. *An*VeA::cTAP recruits the *Afu*VelB (Afu1g01970) and *Afu*LaeA (Afu1g14660). This figure is available in colour online at wileyonlinelibrary.com.

sexual development and *An*VosA is necessary for proper ascosporogenesis (Ni and Yu, 2007; Bayram *et al.*, 2008a). Our recent studies reveal that *An*VelC is a positive regulator of sexual development (H-S. Park *et al.*, unpublished). Additional studies demonstrated that *An*VelB acts as a positive regulator of conidiation and *An*VosA (either as homodimer or heterodimer with *An*VelB) confers feedback regulation of *AnibrlA* and conidiation (Ni and Yu, 2007; Park *et al.*, 2012). In *A. fumigatus*, as shown in Fig. 2C, the Δ*AfuvosA*, Δ*AfuveA* and Δ*AfuvelB* mutants exhibited considerably increased accumulation of *AfubrlA* and *AfuabaA* mRNAs under the conidiation-inducing conditions. In liquid submerged culture conditions, the Δ*AfuvosA*, Δ*AfuveA* and Δ*AfuvelB* mutants exhibited abundant formation of conidiophores at 24 h, whereas WT, Δ*AfuvelC* and complemented strains did not develop (Fig. 3A). The Δ*AfuvosA*, Δ*AfuveA* and Δ*AfuvelB* mutants accumulated *AfubrlA* mRNA at 24 h of vegetative growth, whereas no *AfubrlA* expression was detected in the WT, Δ*AfuvelC* and complemented strains (Fig. 3B). These imply that *Afu*VosA, *Afu*VeA and *Afu*VelB are necessary for normal progression of growth and proper-down regulation development in *A. fumigatus*.

We previously showed that the *An*VosA and *An*VelB proteins play a major role in trehalose biogenesis of conidia and completion of sporogenesis in *A. nidulans* (Ni and Yu, 2007; Sarikaya Bayram *et al.*, 2010; Park *et al.*, 2012). Similar to accumulation patterns of *AnivosA* and *AnivelB* mRNAs, *AfuvosA* and *AfuvelB* transcripts highly accumulate in the late phase of conidiation and mature conidia (Fig. 1B). The phenotypes of conidia caused by mutational inactivation of *AnivosA* or *AnivelB* are also similar to those of the Δ*AfuvosA* and Δ*AfuvelB* mutants (Figs 4 and 5). These results suggest that the roles of VelB and VosA in coupling trehalose biogenesis and conidiogenesis are conserved in two aspergilli. However, it is important to note that while the trehalose in the Δ*AnivelB* and Δ*AnivosA* conidia was absent (Ni and Yu, 2007; Sarikaya Bayram *et al.*, 2010), the conidia of Δ*AfuvelB* and Δ*AfuvosA* strains contain a reduced (~ 60% of WT) amount of trehalose. Also unlike in *A. nidulans* no significant differences in mRNA levels of *AfutpsA*, *AfutpsB*, *AfuorlA* between WT and Δ*AfuvelB* or Δ*AfuvosA* mutant strains were observed (data not shown), indicating that *Afu*VosA and *Afu*VelB are not required for proper activation of genes necessary for the accumulation of trehalose in conidia. Moreover, whereas the deletion of *AnivosA* resulted in high-level accumulation of *AnibrlA* mRNA in conidia (Ni and Yu, 2007), the deletion of *AfuvosA*, *AfuvelB* and/or *AfuveA* did not cause accumulation of *AfubrlA* in conidia (Fig. 3B), indicating that feedback regulation of conidiation in *A. fumigatus* may not be conferred by *Afu*Velvet regulators alone. Importantly, *Afu*WetA is required for trehalose biogenesis, conidial maturation and negative feedback regulation of *AfubrlA* (Tao and Yu, 2011). Tao and Yu showed that the lack of *AfuwetA* caused the total absence of trehalose in conidia, high-level accumulation of *AfubrlA* mRNA in conidia and reduced accumulation of *AfuvosA* mRNA. We propose that *Afu*WetA together with *Afu*Velvets confers trehalose biogenesis, the completion of conidiogenesis and feedback regulation of conidiation in *A. fumigatus*.

Our studies have revealed that the *velvet* proteins interact with each other and form complexes, including the

VelB–VeA–LaeA, VelB–VelB and VosA–VelB complexes, which play differential roles in regulating development, sporogenesis and secondary metabolism in *A. nidulans* (Bayram *et al*., 2008a; Sarikaya Bayram *et al*., 2010). Results of our double mutant analyses are generally in agreement that *Afu*VelB and *Afu*VosA, similar to *Ani*VelB and *Ani*VosA, play an interdependent role in trehalose biosynthesis and conidial germination (Figs 4 and 5), indicating that *Afu*VosA–*Afu*VelB complex may also be a functional unit in *A. fumigatus*. Furthermore, our cross-complementation results demonstrate that the *AfuveA* null mutation is fully complemented by *AniveA*, and *Ani*VeA physically interacts with *Afu*VelB and *Afu*LaeA and may form a heterotrimeric complex (Fig. 7). These results indicate that function and interaction of VeA is conserved in both aspergilli. The components of the *Ani*VelB–*Ani*VeA–*Ani*LaeA complex are required for secondary metabolite production in various filamentous fungi (Kato *et al*., 2003; Bok and Keller, 2004; Calvo *et al*., 2004; Bayram *et al*., 2008a; Kale *et al*., 2008; Wiemann *et al*., 2010; Lee *et al*., 2012; Park *et al*., 2012). In addition, Bok and Keller previously demonstrated that gliotoxin production was reduced in the Δ*AfulaeA* mutants in *A. fumigatus* (Bok and Keller, 2004). Based on these data, we thought that the absence of *AfuveA* and *AfuvelB* may affect gliotoxin production and checked the gliotoxin accumulation in WT and mutants. Thin layer chromatography (TLC) analyses, however, indicated that WT, Δ*AfuveA* and Δ*AfuvelB* strains showed equal levels of gliotoxin under liquid submerged culture conditions (data not shown). We speculate two possible explanations for these results. First, this heterotrimeric complex is not associated with gliotoxin production and may be involved in other secondary metabolites. Second, a large accumulation of *AfubrlA* mRNA caused by the absence of *AfuveA* and *AfuvelB* may be sufficient to activate gliotoxin production. Several studies support the idea that *Afu*BrlA may play a vital role in co-ordinating gliotoxin production and conidiation in *A. fumigatus* (Gardiner and Howlett, 2005; Kwon-Chung and Sugui, 2009; Xiao *et al*., 2010). Additional molecular studies of the *velvet* complexes in *A. fumigatus* need to be carried out.

Multiple AREs are present in the promoter regions of the *velvet* genes in both aspergilli (Tao and Yu, 2011; Park *et al*., 2012). In our previous study, we demonstrated that AbaA positively regulates *velB* and *vosA* expression during the late phase of asexual development in *A. nidulans*, and that AbaA binds to the promoters of *velB* and *vosA in vitro* (Park *et al*., 2012). Likewise, *Afu*AbaA is necessary for proper expression of *AfuvelB* and *AfuvosA* during conidiation and in conidia (Fig. 6). Recently, we proposed a model that *AfuvosA* is primarily activated by *Afu*AbaA with the assistance of *Afu*WetA during late phase of conidiation (Tao and Yu, 2011). Collectively, we present working models for developmental regulation in *A. nidulans* and *A. fumigatus* (Fig. 8). In both aspergilli, the proposed *brlA* → *abaA* → *wetA* central regulatory pathway in conidiation is fully conserved (Adams *et al*., 1998; Mah and Yu, 2006; Yu, 2010; Tao and Yu, 2011). VosA and VelB are required proper regulation of conidial germination in both aspergilli and *Afu*VeA, together with *Afu*VosA and *Afu*VelB, plays a negative role in regulating conidial germination. The upstream regulators play similar yet slightly different roles, and are required for proper expression of *brlA* in both aspergilli (Mah and Yu, 2006; Xiao *et al*., 2010; Yu, 2010). During early asexual development *Ani*VelB (activator) and *Ani*VelC (repressor) indirectly affect the *AnibrlA* expression. While *Ani*VelB acts as a positive regulator of the formation of conidiophores and *AnibrlA* expression in early phase of conidiation, *Afu*VelB together with *Afu*VeA acts as a key negative regulator of conidiation and *AfubrlA* expression during vegetative growth and conidiogenesis. For spore maturation, the two aspergilli share key regulators and the VelB–VosA complex may be a functional unit for controlling trehalose biosynthesis in conidia, tolerance of spores to environmental stresses and feedback regulation of conidiation. Regulation of *vosA* and *velB* mRNA expression in the late phase of conidiation is exerted by AbaA in both species. It is important to note that, while *Ani*VosA and *Ani*VelB play an essential role in trehalose accumulation in conidia, it appears that *Afu*WetA is the principal regulator of trehalose biogenesis and conidial wall maturation. WetA and VosA–VelB co-ordinately regulate the completion of spore maturation and trehalose biosynthesis in both aspergilli (Boylan *et al*., 1987; Marshall and Timberlake, 1991; Ni and Yu, 2007; Tao and Yu, 2011; Park *et al*., 2012). Finally, while both *Ani*VelB and *Ani*VeA are required for mycotoxin sterigmatocystin biosynthesis by forming the key heterotrimeric complex *Ani*VelB–*Ani*VeA–*Ani*LaeA, *Afu*VelB and *Afu*VeA are not necessary for gliotoxin biosynthesis. The molecular mechanisms governing *velvet*-mediated regulation are being investigated and the outcomes will expand our knowledge in fungal biology.

Experimental procedures

Strains, media and culture conditions

Aspergillus fumigatus strains used in this study are listed in Table 1. The fungal strains were grown on solid or liquid MMG and 0.1% YE at 37°C, as previously described (Kafer, 1977; Mah and Yu, 2006). For auxotrophic mutants (strains AF293.1 and AF293.6) the medium was supplemented with 5 mM uridine + 10 mM uracil, and 0.1% arginine (Xue *et al*., 2004). For liquid submerged culture, about 5×10^5 conidia ml^{-1} were inoculated into liquid MMG with 0.1% YE and incubated at 37°C.

For Northern blot analysis, samples were collected as described (Seo *et al*., 2003; Ni and Yu, 2007). Briefly, for vegetative growth phases, conidia (5×10^5 conidia ml^{-1}) of WT and mutant strains were inoculated in 100 ml of liquid

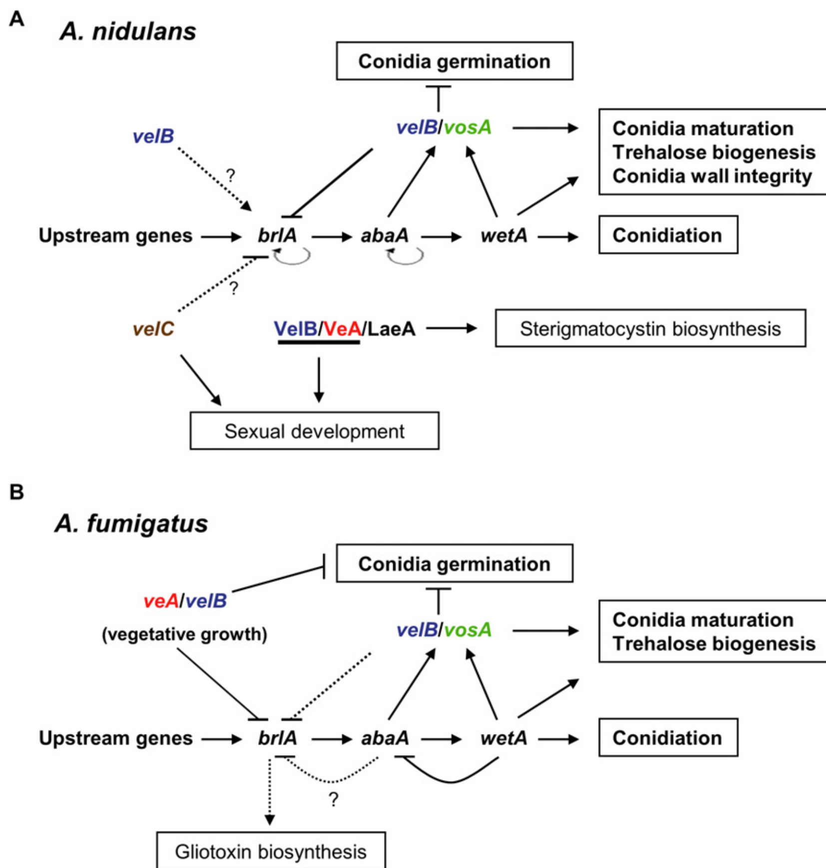

Fig. 8. Model for regulation of conidiation.
A. A simplified genetic model for the *velvet*-mediated developmental regulation in *A. nidulans* (see *Discussion*).
B. A comprehensive model for regulation of *A. fumigatus* asexual development (see *Discussion*).

Table 1. *Aspergillus* strains used in this study.

Strain name	Relevant genotype	References
AF293	Wild type	Brookman and Denning (2000)
AF293.1	*AfupyrG1*	Xue *et al.* (2004)
AF293.6	*AfupyrG1*; *AfuargB1*	Xue *et al.* (2004)
TNI17.1	*AfupyrG1*; *ΔAfuvosA*::*AnipyrG*$^+$	Ni and Yu (2007)
TSGa17	*AfupyrG1*; *ΔAfuabaA*::*AnipyrG*$^+$	Tao and Yu (2011)
TLI9	*AfupyrG1*, *alcA(p)*::*AfuabaA*::*AfupyrG*$^{+a}$	Tao and Yu (2011)
D141	Wild type, clinical isolate	Reichard *et al.* (1990)
AfS13	*ΔAfuveA*::*loxP-phleo/tk*	Krappmann *et al.* (2005)
AfGB11	*AniveA(p)*::*AniveA*::*ctap* tag*, *ptrA*; *ΔAfuveA*::*loxP-phleo/tk*	This study
THSF1.1	*ΔAfuveA*::*AnipyrG*$^+$; *AfupyrG1*	This study
THSF2.1	*ΔAfuvelB*::*AnipyrG*$^+$; *AfupyrG1*	This study
THSF3.1	*AfupyrG1*; *ΔAfuvelC*::*AnipyrG*$^+$	This study
THSF4.1	*ΔAfuveA*::*AnipyrG*$^+$; *AfupyrG1*; *AfuveA*$^+$::*hygB*$^+$	This study
THSF5.1	*ΔAfuvelB*::*AnipyrG*$^+$; *AfupyrG1*; *AfuvelB*$^+$::*hygB*$^+$	This study
THSF6.1	*AfupyrG1*; *ΔAfuvelC*::*AnipyrG*$^+$; *AfuvelC*$^+$::*hygB*$^+$	This study
THSF8.1	*ΔAfuvelB*::*AniargB*$^+$; *AfupyrG1*; *AfuargB1*	This study
THSF10.1	*AfupyrG1*; *AfuargB1*; *ΔAfuvosA*::*AniargB*$^+$	This study
THSF11.1	*AfupyrG1*; *AfuargB1*; *ΔAfuvosA*::*AniargB*$^+$; *AnipyrG*$^+$	This study
THSF12.1	*AfupyrG1*; *AfuargB1*; *ΔAfuvosA*::*AniargB*$^+$; *AfuvosA*$^+$; *AnipyrG*$^+$	This study
THSF13.1	*ΔAfuvelB*::*AniargB*$^+$; *ΔAfuveA*::*AnipyrG*$^+$; *AfupyrG1*; *AfuargB1*	This study
THSF14.1	*ΔAfuvelB*::*AnipyrG*$^+$; *AfupyrG1*; *AfuargB1*; *ΔAfuvosA*::*AniargB*$^+$	This study

a. The 3/4 *AfupyrG* marker in pNJ25 causes targeted integration at the *AfupyrG*1 locus.

MMG with 0.1% YE in 250 ml flasks and incubated at 37°C, 220 r.p.m. Individual mycelial samples were collected at designated time points from liquid submerged cultures. For synchronized asexual developmental induction, about 5×10^5 conidia ml^{-1} WT and relevant mutant strains were inoculated in 500 ml of liquid MMG with 0.1% YE and incubated at 37°C and 250 r.p.m. for 16 h (0 h for developmental induction). The mycelium was harvested by filtering through Miracloth (Calbiochem, CA), transferred to solid MMG with 0.1% YE and incubated at 37°C for air-exposed asexual developmental induction. Samples were collected at various time points post asexual developmental induction. To induce *AfuabaA* overexpression, MM with 100 mM threonine as sole carbon source (MMT) and 0.1% YE was used. Briefly, to overexpress *AfuabaA* under the control of the *alcA* promoter, strains were inoculated in liquid MMG, incubated at 37°C, 220 r.p.m. for 14 h, and the mycelial aggregates were collected, rinsed with liquid MMT, transferred into liquid MMG or MMT with 0.1% YE, and further incubated at 37°C, 220 r.p.m. All samples were collected, squeeze-dried, quick-frozen in liquid N_2 and stored at −80°C subject to RNA isolation.

Generation of A. fumigatus *mutants*

The oligonucleotides used in the present study are listed in Table S1. The *Afuvelvet* genes were deleted in *A. fumigatus* AF293.1 (*pyrG1*) and AF293.6 (*pyrG1*; *argB1*) strains (Xue *et al*., 2004) employing double-joint PCR (DJ-PCR) (Yu *et al*., 2004). The deletion constructs containing the *A. nidulans* selective markers with the 5′ and 3′ franking regions of the *Afuvelvet* genes were introduced into the recipient strains AF293.1 or AF293.6 respectively (Szewczyk *et al*., 2006). The selective markers were amplified from FGSC4 genomic DNA with the primer pairs oBS8/oBS9 (*AnipyrG*+) and oKH60/oNK105 (*AniargB*+). The *Afuvelvet* mutants were isolated and confirmed by PCR, followed by restricted enzyme digestion (Yu *et al*., 2004). At least three deletion strains for each case were isolated.

To complement Δ*AfuveA*, Δ*AfuvelB* and Δ*AfuvelC*, single-joint PCR (SJ-PCR) method was used (Yu *et al*., 2004). The *AfuveA*, *AfuvelB* and *AfuvelC* genes' ORF with presumed promoter and terminator were amplified with specific primer pairs where the 3′ reverse primer carries overlapping sequences with the *hygB* gene's 5′ end. Amplification of the *hygB* gene was carried out with primer pairs where the 5′ forward primer carries overlapping sequences with each *Afuvelvet* gene's 3′ end. The final amplicons were amplified with nested primer pairs OHS335;OHS333 (*AfuveA*), OHS336;OHS333 (*AfuvelB*) and OHS336;OHS333 (*AfuvelC*) and introduced into strains THSF1.1, THSF2.1 and THSF3.1 respectively. For the complementation of the *AfuvosA* null mutation, two PCR amplicons, the *AfuvosA* gene region including its presumed promoter and terminator (amplified by OHS203;OHS204) and the marker for *AnipyrG* (OBS8;OBS9) were co-introduced into a Δ*AfuvosA* strain (THSF10.1). Multiple complemented strains were isolated and confirmed by PCR and Northern blot analyses. To test the ability of *AniveA* to complement Δ*AfuveA*, the plasmid pME3157 (Bayram *et al*., 2008a) containing *AniveA*::*ctap* tag fusion was introduced into the *AfuveA* deletion strain AfS13 (Krappmann *et al*., 2005) to yield AfGB11.

Nucleic acid isolation and manipulation

To isolate genomic DNA from *A. fumigatus*, about 10^6 conidia were inoculated in 2 ml of liquid MMG + 0.5% YE, and stationary cultured at 37°C for 24 h. The mycelial mat was collected, squeeze-dried and genomic DNA was isolated as described (Yu *et al*., 2004). All deletion mutants were confirmed by PCR amplification of the coding region of individual genes followed by restriction enzyme digestion amplicons.

Total RNA isolation and Northern blot analyses were carried out as previously described (Han *et al*., 2004; Mah and Yu, 2006). For total RNA isolation, the prepared sample was homogenized using a Mini Bead beater in the presence of 1 ml of TRIzol® reagent (Invitrogen) and 0.3 ml of silica/zirconium beads (Biospec). RNA extraction was performed according to the manufacturer's instruction (Invitrogen). For Northern blot analyses, total approximately 10 g (per lane) of total RNA isolated from individual samples was separated by electrophoresis using an 1% (v/w) agarose gel containing 3% (v/v) formaldehyde and blotted onto a Hybond-N membrane (Amersham). RNA blots were hybridized with individual probes using modified Church buffer as described (Yu and Leonard, 1995; Xiao *et al*., 2010). The DNA probes were prepared by PCR amplification of the coding regions of individual genes with appropriate oligonucleotide pairs using AF293 genomic DNA as template (Table S1). Probes were labelled with [32P]dCTP (PerkinElmer) using Random Primer DNA Labeling Kit (Takara) and purified by illustra MicroSpin G-25 columns (GE Healthcare).

Trehalose assay

Trehalose was extracted from conidia and analysed as described (d'Enfert and Fontaine, 1997; Ni and Yu, 2007). Two day-old conidia (2×10^8) were collected and washed with ddH_2O. Conidia were resuspended in 200 μl of ddH_2O and incubated 95°C for 20 min, and then the supernatant was separated by centrifugation and collected. The supernatant was then mixed with equal volume of 0.2 M sodium citrate (pH 5.5) and further incubated at 37°C for 8 h with or without 3 mU of trehalase from porcine kidney, which hydrolyses trehalose to glucose. The amount of glucose generated by trehalase was assayed with a glucose assay kit (Sigma) following manufacturer's instruction, and converted into the trehalose amount per 10^7 conidia (triplicates). Each sample not treated with trehalase served as a negative control.

Oxidative stress tolerance test

Oxidative stress tolerance test was carried out as described (Han *et al*., 2004; Sarikaya Bayram *et al*., 2010) with some modifications. Hydrogen peroxide sensitivity of conidia was tested by incubating 1 ml of conidial suspensions containing 10^5 conidia with varying concentrations (0, 1.25 or 2.50 mM) of H_2O_2 and incubated for 30 min at room temperature. Each conidia suspension was then diluted with ddH_2O, and conidia were inoculated into solid MMG with 0.1% YE. After incubation at 37°C for 48 h, colony numbers were counted and calculated as a survival ratio of the untreated control.

UV light stress tolerance test

UV light tolerance test was carried out as described previously (Lima *et al*., 2005; Sarikaya Bayram *et al*., 2010) with some modifications. Two day-old fresh conidia were collected in ddH_2O and plated out on solid MMG with 0.1% YE plates (100 conidia per plate). The plates were then irradiated immediately with UV using a UV cross-linker and incubated at 37°C for 48 h. The colony numbers were counted and calculated as a ratio of the untreated control.

Germination of conidia

Germination rates were measured as previously described with a slight modification (Ni *et al*., 2005). To examine germination levels in liquid media, conidia of WT and mutants were inoculated in 5 ml of liquid MMG with 0.1% YE, or liquid medium lacking a carbon source, and incubated at 37°C, 220 r.p.m. Levels of germination (isotropic growth and germ tube formation) were examined every 1 h after inoculation under a microscope. To examine germination levels on solid media without any carbon sources, conidia of WT and mutants were spread onto solid MM with 1.6% agarose and incubated at 37°C. Levels of germination were examined every 6 h after inoculation under a microscope.

Microscopy

The colony photographs were taken by using a Sony digital camera (DSC-F828). Photomicrographs were taken using a Zeiss M^2 BIO microscope equipped with AxioCam and AxioVision digital imaging software (Zeiss).

Tandem affinity purification and LC-MS/MS protein identification

Tandem affinity purification method and peptide identifications were carried out as given in detail (Bayram *et al*., 2008a).

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