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WetA and VosA are distinct regulators of conidiation capacity, conidial quality, and biological control potential of a fungal insect pathogen

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Abstract Many filamentous fungi produce only conidia for dispersal and survival in vitro or in vivo. Here, we show that the developmental regulator WetA and the velvet protein VosA are not only required for conidial maturation but indispensable for conidiation in *Beauveria bassiana*, a filamentous entomopathogen. Deletion of *wetA* or *vosA* resulted in more than 90 % transcriptional depression of *brlA* and *abaA*, two activator genes in the central developmental pathway, during the critical period of conidiophore development and conidiation. Consequently, Δ*wetA* and Δ*vosA* strains lost 98 % in and 88 % of their conidiation capacities under optimal culture conditions, respectively. The conidia of Δ*wetA* showed more defective features than those of Δ*vosA*, including smaller size, lesser density, lower hydrophobicity, and impaired cell walls although intracellular trehalose content decreased more in the aging culture of Δ*vosA* than of Δ*wetA*. As a result, conidial sensitivity to cell wall perturbation was elevated in Δ*wetA* but unaffected in Δ*vosA*, which produced conidia more sensitive to the oxidant menadione and the wet-heat stress at 45 °C. Both deletion mutants showed similar defects in conidial tolerance to high osmolarity or UV-B irradiation but no change in conidial sensitivity to the other oxidant H_2O_2 or the fungicide carbendazim. Moreover, Δ*wetA* lost more virulence to *Galleria mellonella* larvae than Δ*vosA*. All these phenotypical changes were restored by either *wetA* or *vosA* complementation. Taken together, WetA and VosA are indispensable for asexual development and contribute differentially to conidial quality and hence the biological control potential of *B. bassiana* against insect pests.

Keywords Entomopathogenic fungi · Central development activator WetA · Velvet protein VosA · Asexual development · Conidial maturation and quality · Biological control potential

Introduction

The entomopathogenic fungus *Beauveria bassiana* produces aerial conidia to infect the broadest spectrum of insects as far as known to date (Vega et al. 2012; Wang and Feng 2014). Such conidia can be massively produced as active ingredients of fungal insecticides on inexpensive solid substrates, such as small grains (Ye et al. 2006). Thus, both conidiation capacity and conidial quality are crucial for the biological control potential of the filamentous fungal pathogen against arthropod pests.

Fungal conidiation is an event that is precisely timed and genetically programmed by a central developmental pathway in response to internal and external cues. In *Aspergillus*, the central pathway comprises the developmental regulators BrlA, AbaA, and WetA that activate the expression of downstream conidiation-specific genes in a hierarchical manner during conidiophore development, conidiation, and conidial maturation (Mirabito et al. 1989; Yu et al. 2006). Of those, WetA functions in the late phase of condiophore development to activate the expression of proteins/enzymes involved in the synthesis of spore wall components (Sewall et al. 1990a; Marshall and Timberlake 1991; Son et al. 2014) and hence is essential for conidiation and conidial maturation (Park and Yu 2012). This hints to a close link of WetA to the conidial

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quality that largely determines the biological control potential of a filamentous fungal insect pathogen. The maturation process also requires the activation of the velvet protein VosA to depress the *brlA* expression for termination of its control cycle and to facilitate trehalose biogenesis and accumulation in newly formed conidia (Ni and Yu 2007). The family of velvet proteins share a velvet domain that is highly conserved in filamentous fungi (Ni and Yu 2007; Bayram and Braus 2012). The velvet domain has been shown to be a novel DNA-binding motif that specifically recognizes an 11-nucleotide consensus sequence consisting of two motifs in the promoters of key developmental regulatory genes (Ahmed et al. 2013). Therefore, VosA is not only required for conidial maturation but likely involved in a transcriptional relationship with the regulators in the central pathway due to the DNA-binding motif existing in its velvet domain.

The roles of these developmental regulators have been intensively studied in *Aspergillus* (Adams et al. 1988; Sewall et al. 1990a, b; Marshall and Timberlake 1991; Ogawa et al. 2010; Tao and Yu 2011) and *Penicillium* (Borneman et al. 2000; Andrianopoulos 2002; Sigl et al. 2011; Wang et al. 2015) and summarized in several reviews (Adams et al. 1998; Etxebeste et al. 2010; Park and Yu 2012; Alkhayyat et al. 2015). However, none of these regulators has been functionally explored in filamentous entomopathogens, such as *B. bassiana* and *Metarhizium anisopliae* sensu lato, which are widely applied for the biological control of global arthropod pests (Wang and Feng 2014). During asexual development, *B. bassiana* lacking sexual stage develops very short, zigzag, or denticulate rachises (conidiophores) from hyphal cells and produces several subspherical conidia of 1–2 μm on each denticula, forming spore balls with all conidia surrounding the rachises. This asexual developmental pattern is largely different from that seen in *Aspergillus* species, which develop dozens of phialides (conidiophores) from a single foot cell and form a chain of conidia on each phialide (Park and Yu 2012; Alkhayyat et al. 2015). For *B. bassiana*, cultivation of conidia on a standard medium requires a period of less than 24 h for full germination. After 1 or 2 days of hyphal growth, conidiophore development and low level, but measurable, conidiation usually occur on day 3, followed by increasing conidiation until a peak of conidial yield is achieved approximately on day 7. The sequenced genome of *B. bassiana* (Xiao et al. 2012) harbors the orthologs of all the developmental activators well characterized in *Aspergillus*. This study seeks to elucidate how WetA and VosA regulate conidiation compacity and conidial quality in *B. bassiana* and hence contribute to the fungal potential against arthropod pests. Our phenotypic and transcriptional analyses of single-gene deletion mutants show that both WetA and VosA are indispensable for conidiation as well as conidial maturation in *B. bassiana* due to their interactions with the central activators BrlA and AbaA at transcriptional level.

Material and methods

Microbial strains and culturing conditions

The wild-type strain *B. bassiana* ARSEF 2860 (BbWT herein) and its mutants were grown in rich SDAY [Sabouraud dextrose agar (4 % glucose, 1 % peptone, and 1.5 % agar) plus 1 % yeast extract] at 25 °C and 12:12 h (light/dark cycle) for standard cultures. Their stress responses were assayed in 1/4 SDAY (amended with 1/4 of each SDAY nutrient) or minimal CZA (Czapek agar: 3 % sucrose, 0.3 % $NaNO_3$, 0.1 % K_2HPO_4, 0.05 % KCl, 0.05 % $MgSO_4$, and 0.001 % $FeSO_4$). *Escherichia coli* DH5α and *E. coli* Top10 from Invitrogen (Shanghai, China) were cultured in Luria-Bertani medium plus kanamycin (100 μg/ml) or ampicillin (100 μg/ml) at 37 °C for plasmid propagation. *Agrobacterium tumefaciens* AGL-1 used as a T-DNA donor for fungal transformation was cultivated at 28 °C in YEB medium (Fang et al. 2004).

Cloning and analysis of *wetA* and *vosA* in *B. bassiana*

The *B. bassiana* genome under the NCBI Accession NZ_ADAH00000000 (Xiao et al. 2012) was searched via online BLASTP analysis (http://blast.ncbi.nlm.nih.gov/blast.cgi) using the queries of *wetA* and *vosA* homologs in *Aspergillus nidulans* and *Aspergillus fumigatus*. The coding sequences of the identified *wetA* and *vosA* (tag loci: BBA_06126 and BBA_01023) were amplified from BbWT via PCR with paired primers (Table S1 in the Supplementary Material) and verified by sequencing at Invitrogen. The sequences of the deduced proteins were compared with the counterparts of other filamentous fungi in the NCBI database via online analyses of BLASTP, SMART (http://smart.embl-heidelberg.de/), and PANTHER (http://www.pantherdb.org/), followed by phylogenetic analysis with MEGA5 software (Tamura et al. 2011).

Generation and identification of *wetA* and *vosA* mutants

Our backbone plasmids p0380-bar and p0380-sur-gateway (Xie et al. 2012; Wang et al. 2012) were used for targeted gene manipulation via *Agrobacterium*-mediated transformation (Fang et al. 2004). Either *wetA* or *vosA* was deleted from BbWT via homogenous replacement of its partial coding (5′ and 3′ fragments) sequence with a *bar*-inclusive cassette and rescued by ectopic integration into Δ*wetA* or Δ*vosA* of a cassette consisting of full-length coding sequence (with flank regions) and a *sur* marker. The 5′ and 3′ fragments and the full-length sequences of *wetA* or *vosA* were cloned from BbWT with paired primers, digested with appropriate restriction enzyme sites, and inserted into the backbone plasmids with paired primers (Table S1 in the

Supplementary Material). Putative mutants were screened in terms of the *bar* resistance to phosphinothricin (200 μg/ml) or the *sur* resistance to chlorimuron ethyl ammonium (10 μg/ml) and then identified via PCR and Southern blot analyses with paired primers and amplified probes (Table S1 in the Supplementary Material). Positive deletion mutants were analyzed in conjunction with parental BbWT and rescued mutants (control strains) in triplicate experiments as follows.

Assessing growth rates on different media

Hyphal mass plugs (5 mm diameter) were bored from 3-day-old cultures grown at 25 °C on cellophane-overlaid SDAY (CO-SDAY) plates, which were spread with 100 μl aliquots of 10^6 conidia/ml suspension to initiate uniform cultures, and attached centrally to the plates of SDAY, CZA, and 18 CZA-derived media with altered carbon/nitrogen sources and availability. The CZA-derived media were prepared by removing 3 % sucrose, 0.3 % $NaNO_3$ or both from CZA, replacing the sole carbon with 3 % of glycerol, ethanol, acetate (NaAc), glucose, trehalose, maltose, fructose, lactose, mannitol, sorbitol, oleic acid, or olive oil, and replacing the sole nitrogen with 0.3 % of NH_4Cl, NH_4NO_3, or $NaNO_2$, respectively. After 7 days of incubation at 25 °C and 12:12 h, each colony diameter was cross-measured as an index of radial growth on a given medium.

Assessing conidiation capacity and conidial viability

Aliquots of 100 μl conidial suspension were evenly spread on CO-SDAY plates and incubated for 7 days at 25 °C and 12:12 h. From day 3 onwards, three plugs (5 mm diameter) were bored daily from each plate culture using a cork borer. Conidia on each plug were released into 1 ml of 0.02 % Tween 80 by ~5 min vibration. The conidial concentration in the suspension was determined with a hemocytometer and converted to the number of conidia per cm^2 plate culture. During cultivation, fungal mass samples were stained with calcofluor white (a dye specific to cell wall) and examined under a confocal microscope to reveal possible changes in the abundance of hyphae, conidiophores, and conidia of each deletion mutant versus BbWT.

To assess the viability of the conidia from the 7-day-old cultures, 100 μl aliquots of conidial suspension were evenly spread onto the plates of a germination medium (GM; 2 % sucrose, 0.5 % peptone, and 1.5 % agar), followed by 24 h incubation at 25 °C. Germination percentage was determined at 2 h intervals using three microscopic counts per plate. Time (GT_{50}) required for 50 % germination was estimated by fitting the germination trends to the time of incubation. In addition, viability changes of the conidia in the 10- to 50-day-old cultures were also assessed based on germination percentage within 24 h at the same regime.

Examination and assessment of conidial quality

Several methods were used to examine newly formed and mature conidia, collected from the 7- and 15-day-old cultures, respectively, for their quality difference. First, ultrastructural changes on the surfaces of conidia were observed via scanning electron microscopy (SEM). Second, size and density of conidia were determined with the respective readings of forward scatter (FSc) and side scatter (SSc) detectors from the flow cytometry of 2×10^4 conidia per sample (three samples per strain) as described previously (Puttikamonkul et al. 2010; Zhang et al. 2013). Third, carbohydrate epitopes on the surfaces of conidia were determined in different lectin-binding assays (Wanchoo et al. 2009). Briefly, washed conidia were suspended in the binding buffers of the Alexa fluor 488-labeled lectins ConA [i.e., concanavalin A specific to α-N-acetylglucosamine (α-GlcNAc) and α-glucose], WGA (wheat germ agglutinin specific to β-GlcNAc and sialic acids) and GNL (*Galanthus nivalis* lectin specific to mannose residues) from Molecular Probes-Invitrogen (Eugene, OR, USA) and Vector Laboratories (Burlingame, CA, USA) following the user's guide. After 1 h labeling in darkness, the unbound lectin was removed by washing in the buffer. The fluorescence intensity for the amount of each lectin bound to conidial surfaces was read through the flow cytometry of 2×10^4 labeled conidia with an argon laser at excitation/emission wavelengths of 488/530 nm. Finally, an index of conidial hydrophobicity, which is important for conidial adhesion to host integument, was assessed using a diphase method as described previously (Holder et al. 2007; Wang et al. 2014).

Assaying cellular responses to chemical and environmental stresses

Conidial responses to different types of chemical stressors were assayed by spreading 100 μl aliquots of conidial suspension onto GM plates alone (control) or supplemented with a sensitive concentration of menadione (0.2 mM), H_2O_2 (2 mM), SDS (0.2 mg/ml), Congo red (0.8 mg/ml), NaCl (1.2 M), or carbendazim fungicide (1 μg/ml). After 24 h incubation at 25 °C, germination percentage was determined with three microscopic counts per plate.

Conidial thermotolerance and UV-B resistance of each strain were quantified by exposing conidial samples to a hot water bath at 45 °C for 0–120 min and to the irradiation of the weighted UV-B wavelength of 312 nm at the gradient doses of 0–0.6 J/cm^2 as described previously (Wang et al. 2012; Xie et al. 2012). Median lethal time (LT_{50}) and median lethal dose (LD_{50}) were estimated as indices of the two parameters by

fitting conidial survival (relative germination) trend to the intensities of each stress.

Assessing intracellular trehalose content

Aliquots of 1 g fungal mass (mixtures of mycelia and conidia) from 3-, 5-, 7-, 10- and 15-day-old SDAY cultures were ground in liquid nitrogen and resuspended in 1 ml dd-H_2O. Each suspension was boiled in a water bath for 6 h, followed by 30-min centrifugation at 16,000×*g*. The content of trehalose in the supernatant was determined in an HPLC system as described previously (Liu et al. 2009; Wang et al. 2012) and expressed as mg/g dry mass.

Bioassays for fungal virulence

Conidia from the 7-day-old SDAY cultures of all the strains were bioassayed for virulence to *Galleria mellonella* larvae (~300 mg each) from a vendor (Da Mai Chong Insectaries, Wuxi, Jiangsu, China). For each strain, briefly, three cohorts of ~35 larvae were inoculated by immersing them for ~10 s in 30-ml aliquots of 1×10^7 conidia/ml suspension for normal cuticular infection or injecting 5 μl of 1×10^5 conidia/ml suspension into the hemocoel of each larva (i.e., 500 conidia per larva) for cuticle-bypassing infection. The same volume of 0.02 % Tween 80 was used as a control for the immersion or injection treatment. All treated cohorts were maintained in large Petri dishes (15 cm diameter) for 8 days at 25 °C and 12:12 h and monitored every 12 h for mortality records. LT_{50} (no. days) was generated as an index of the fungal kill action by probit analysis of each time-mortality trend.

Transcriptional profiling of conidiation-required genes

The CO-SDAY cultures of all deletion mutants and BbWT were initiated by spreading 100 μl of 10^7 conidia/ml suspension per plate, followed by 7 days of incubation at 25 °C and 12:12 h. In addition, Δ*brlA* and Δ*abaA* mutants, created with the same protocol as described previously, were grown for 7 days in CO-SDAY by spreading 100 μl of hyphal suspension per plate because none of them produced any conidium. Total RNAs were extracted daily from the cultures with an RNAiso™ Plus Reagent kit (TakaRa, Dalian, China) and reversely transcribed into complementary DNAs (cDNAs) with a PrimeScript® RT reagent kit (TaKaRa). Each cDNA (10-fold dilution) was used as a template to determine transcript levels of *brlA*, *abaA*, *wetA*, and *vosA* via quantitative real-time PCR (qRT-PCR) with paired primers (Table S1 in the Supplementary Material) using the fungal 18S rRNA as an internal standard. All the qRT-PCR experiments of four cDNA samples per strain were performed under the action of SYBR® Premix Ex Taq™ (TaKaRa). Relative transcript level (RTL) of each gene was computed as the ratio of its transcript in each deletion mutant over that in BbWT on a given day using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

The same protocol was used to determine temporal transcription patterns of the four genes in BbWT during 7 days of cultivation at the same regime. The RTL of each gene was estimated as the ratio of its transcript on a given day over that on day 1.

Statistical analysis

All phenotypic observations, measurements, and fitted estimates from the experiments of three replicates were differentiated by one-factor (strain) analysis of variance, followed by Tukey's honestly significant difference (HSD) test of the means from each experiment.

Results

Features of WetA and VosA in *B. bassiana*

An online search through the *B. bassiana* genome with the queries of homologous sequences of *A. nidulans* and *A. fumigatus* in the NCBI database resulted in identified *wetA* (1920 bp) and *vosA* (926 bp). The identified genes amplified from BbWT encode 639 and 273 amino acids (molecular sizes: 66.96 and 31.17 kDa), respectively. The deduced WetA and VosA are featured with a Esc1/WetA-related protein domain at the C-terminal end (Mi et al. 2010; Son et al. 2014) and a conserved Velvet domain at the C-terminal side (Bayram and Braus 2012) (Fig. S1A in the Supplementary Material) and share 30–74 and 31–91 % sequence identities (Fig. S1B, C) with the counterparts of other 13 fungi in the NCBI database, respectively.

During 7 days of standard cultivation, *wetA* was transcribed more abundantly in BbWT on days 3–7, a period for the initiation of conidiophore development through full conidiation, than the first 2 days for conidial germination and hyphal growth (Fig. 1a). The *vosA* transcript was largely increased only on days 5–7, a period for the conidiation after conidiophore development. Interestingly, transcriptional expressions of *brlA* and *abaA* began from days 3 and 4, respectively, and maintained at high levels for the following days. Notably, *brlA* and *abaA* showed similar maxima of transcript levels 10- to 25-fold higher than those of *wetA* on days 4 and 5, a critical period for the formation of most conidiophores and a rapid increase of conidiation in *B. bassiana*. These transcription profiles implicated sequential activation of *brlA* and *abaA* during the asexual development of *B. bassiana*, as documented in *Aspergillus* (Alkhayyat et al. 2015), but a somewhat difference in *wetA*, which was activated as early as *brlA* in *B. bassiana* rather than after the *abaA* activation in *Aspergillus*.

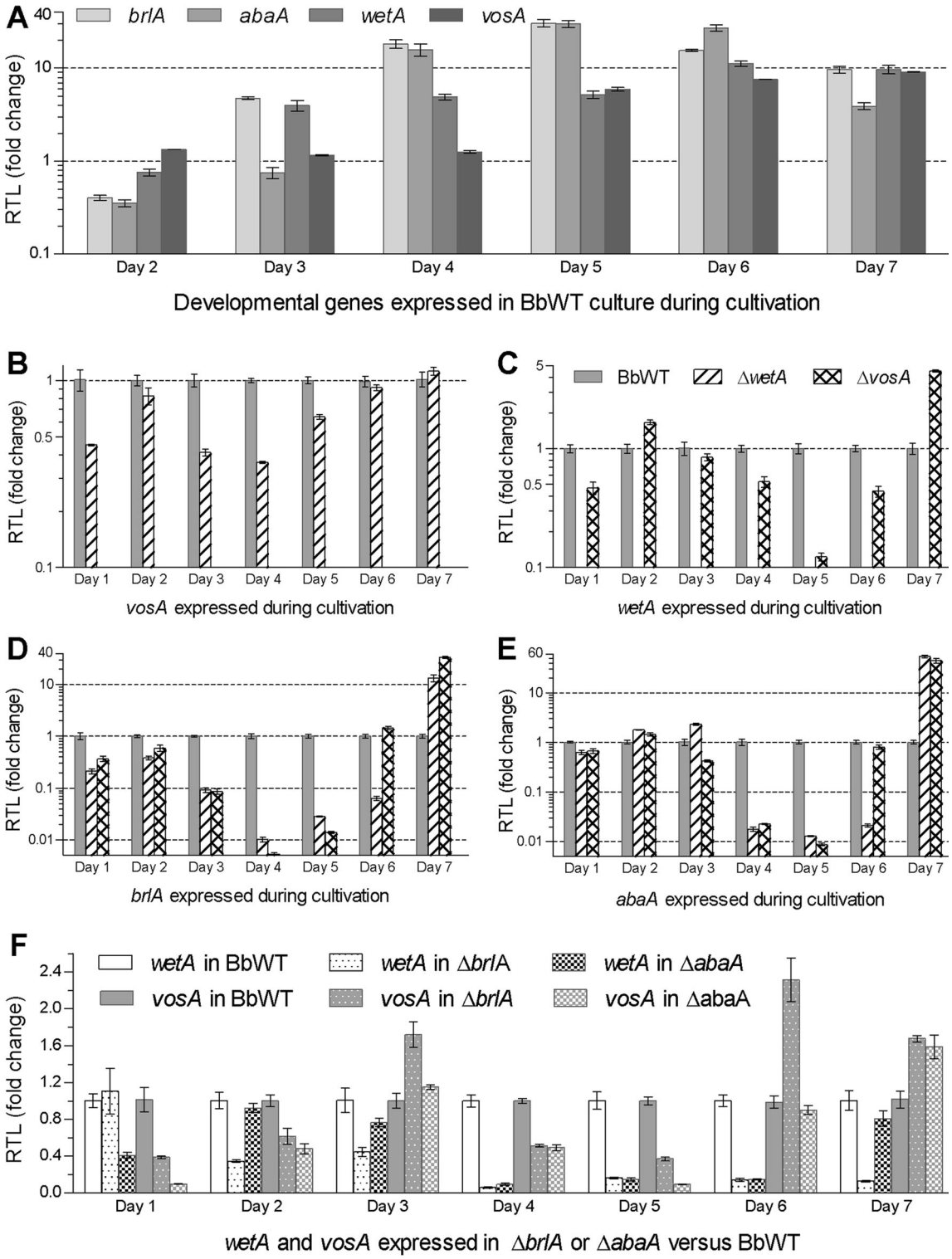


Fig. 1 Deleted *wetA* or *vosA* affects profoundly the central developmental pathway of *B. bassiana*. **a** Relative transcript levels (RTL) of *wetA* and *vosA* in wild-type culture grown for 2–7 days in SDAY at 25 °C. The first-day transcript of each gene at the end of conidial germination was used a standard. **b**–**e** RTLs of *wetA*, *vosA*, *brlA*, and *abaA* in Δ*wetA* and Δ*vosA* mutants versus wild-type (BbWT) over the days of cultivation in SDAY, respectively. **f** RTLs of *wetA* and *vosA* in Δ*brlA* and Δ*abaA* mutants versus BbWT during cultivation in SDAY. *Error bars*: standard deviations (SD) from four cDNA samples

Either *wetA* or *vosA* deletion alters transcriptional profiles of other developmental activators

The expected recombination events in Δ*wetA*, Δ*vosA*, and complementary mutants were confirmed by PCR and Southern blot analyses (Fig. S2 in the Supplementary Material). Consequently, either *wetA* or *vosA* was undetectable at transcription level during the 7-day cultivation. Compared with that in BbWT, the *vosA* transcript in Δ*wetA* decreased by ~50 % on days 1, 4, and 6 and 88 % on day 5 but increased by 4-fold on day 7 and slightly changed on days 2 and 3 (Fig. 1b). In Δ*vosA*, the *wetA* transcript was repressed by 55, 59, 64, and 37 % on days 1 and 3–5, respectively, despite a minor change on other days (Fig. 1c). These suggested a strong interaction between *wetA* and *vosA* at transcriptional level. Intriguingly, the *brlA* transcript in Δ*wetA* was drastically depressed by 79 % on day 1, 62 % on day 2, and 91–99 % on days 3–6 but upregulated by 12-fold on day 7 (Fig. 1d). The *abaA* transcript in Δ*wetA* was also depressed by 37 % on day 1 and 98–99 % on days 4–6 but upregulated by 53-fold on day 7, accompanied with a minor upregulation on days 2 and 3 (Fig. 1e). The *vosA* deletion resulted in more drastic depression of *brlA* on days 3–5 (92–99 %) than on days 1 (64 %) and 2 (41 %), and of *abaA* on days 4 (98 %) and 5 (99 %) than on days 1 (35 %) and 3 (58 %), followed by their sharp upregulation by 33- and 44-fold on day 7. In Δ*brlA*, more interestingly, the *wetA* transcript was reduced by 65 % on day 2, 56 % on day 3, and 84–94 % on days 4–7, and the *vosA* transcript was reduced by 60 % on day 1 and 85–90 % on days 4–6 (Fig. 1f). The deletion of *abaA* from BbWT also led to a remarkable down-regulation of *wetA* by 50–63 % on days 1, 4, and 5 and of *vosA* by 51–90 % on days 1, 2, 4, and 5 despite minor effects on other days. The temporal transcription patterns of *brlA* and *abaA* in Δ*wetA* or Δ*vosA* and of *wetA* and *vosA* in Δ*brlA* or Δ*abaA* indicated a tight link of each to three others in the central developmental pathway of *B. bassiana*.

Contributions of WetA and VosA to growth, asexual cycle, and virulence

Compared with BbWT, all the deletion mutants showed different degrees of growth defects in rich SDAY or 1/4 SDAY and minimal CZA or 18 CZA-derived media, which were initiated with small hyphal plugs to initiate fungal colonies. After 7 days of standard incubation, colony sizes were significantly smaller in Δ*vosA* than the control strains grown on all the rich and minimal media (Tukey's HSD, $P < 0.05$), as summarized in Table S2 in the Supplementary Material. The Δ*wetA* mutant exhibited milder growth defects in 1/4 SDAY and most minimal media.

Apart from smaller sizes, the colonies of Δ*wetA* and Δ*vosA* were more cottony and thicker than those of the control strains (Fig. 2a) on SDAY, a standard medium for cultivation of fungal entomopathogens (Feng et al. 1994; Roberts and St Leger 2004). Additionally, deeper folds appeared in the colonies of Δ*vosA* than of Δ*wetA*. In microscopic examination of fungal masses taken from 5-day-old SDAY cultures, BbWT showed many spore balls on clustered zigzag rachises and hence far more abundant conidia than Δ*wetA* and Δ*vosA* (Fig. 2b). However, neither rachis nor conidium was visible in the cultures of Δ*brlA* and of Δ*abaA* at the same time (Fig. S3 in the Supplementary Material). Thus, the latter two mutants were excluded in the following phenotypic experiments.

Quantification of conidiation over 7 days of standard culivation demonstrated a drastic reduction of conidial yield in each deletion mutant (Fig. 3a). Compared with BbWT, Δ*wetA* and Δ*vosA* suffered conidial yield losses of 88 and 56 % on day 3, 98–99 % and 95–98 % on days 4–6, and 98 and 88 % on day 7, respectively. Conidia from the 7-day-old culture of BbWT germinated significantly faster than those from the cultures of Δ*wetA* and Δ*vosA* (Tukey's HSD, $P < 0.01$), whose GT_{50}s were prolonged by 4.4 and 1.2 h under optimal culture conditions, respectively (Fig. 3b).

In the bioassay of *G. mellonella* larvae, hemocoel injection and topical application of conidial suspension resulted in the LT_{50}s of Δ*wetA* 3 and 0.8 days longer than those of BbWT, respectively (Fig. 3c). The Δ*vosA* mutant also suffered a significant, but minor, LT_{50} delay irrespective of topical application (Tukey's HSD, $P = 0.0117$) or injection (Tukey's HSD, $P = 0.0035$).

All the changes were restored by either *wetA* or *vosA* complementation. Apparently, Δ*wetA* suffered more severe defects in conidiation than Δ*vosA* under normal culture conditions. In addition, the conidia produced by Δ*wetA* not only germinated slower but were less virulent than those produced by Δ*vosA*.

Different effects of WetA and VosA on conidial maturation and quality

Since full conidiation in BbWT was achieved at the end of 7-day cultivation in SDAY, newly formed and mature conidia from the 7- and 15-day-old cultures were examined to reveal the effects of *wetA* and *vosA* on conidial maturation and quality, which are indicated by morphology, viability, and surface features. As demonstrated by SEM, newly formed conidia produced by BbWT showed an ultrastructural coat of well-defined fascicles or bundles (Fig. 4a, upper row), presumably composed of hydrophobin rodlets (Holder et al. 2007). Such bundles became larger and less compact in Δ*vosA* while most of them were poorly defined in Δ*wetA*. The bundles of the mature conidia humped more in BbWT than in Δ*vosA* (Fig. 4a, lower row). In contrast, the mature conidia of Δ*wetA* lacked conspicuously humping

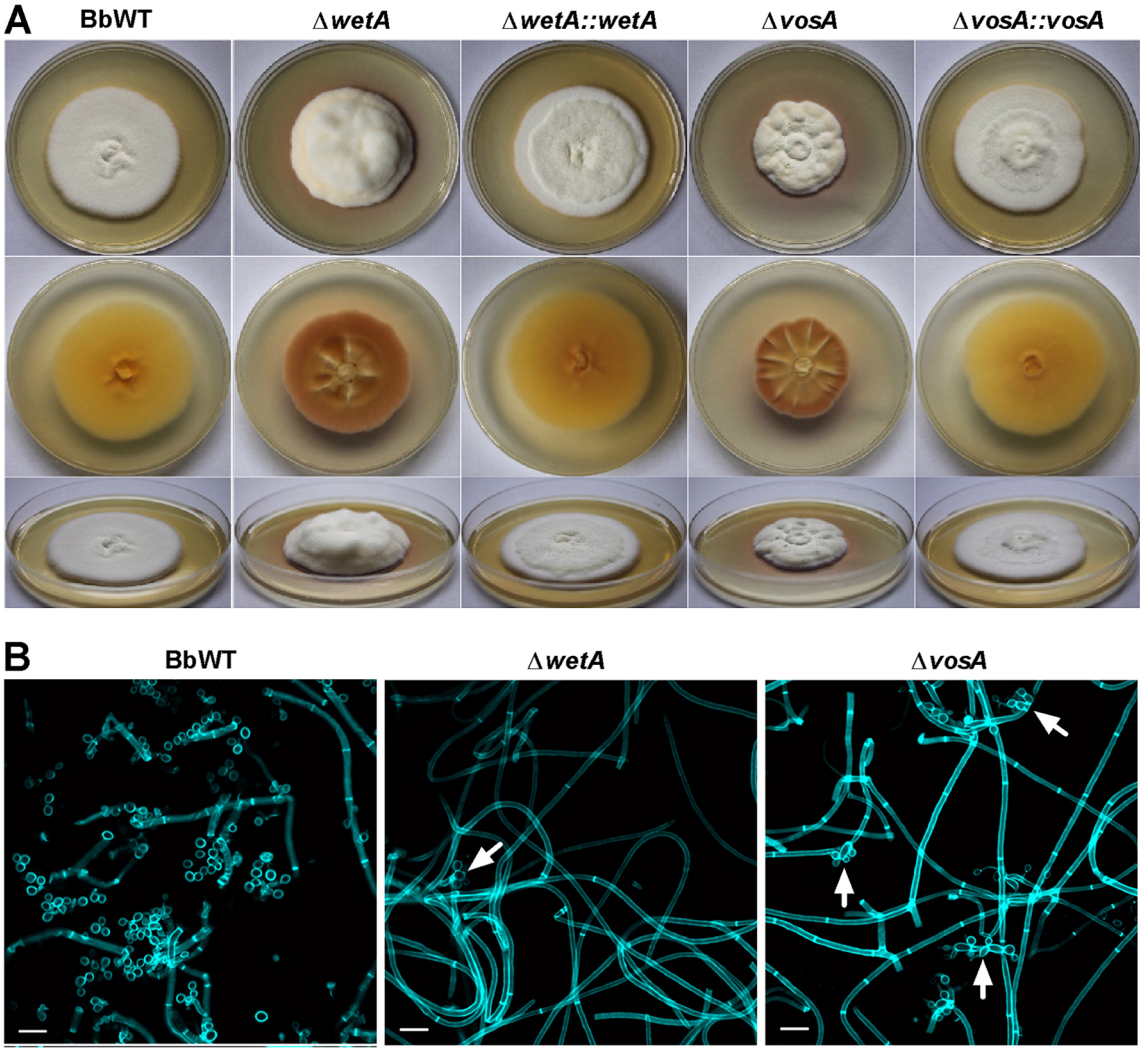


Fig. 2 Deleted *wetA* or *vosA* affects vegetative growth and conidiation of *B. bassiana*. **a** *Top* (row 1), *bottom* (row 2), and *side* (row 3) views of 10-day-old SDAY colonies initiated with hyphal mass plugs. **b** Microscopic views of fungal mass samples taken from 5-day-old SDAY cultures and stained with calcofluor white. *Arrows* indicate only a very few conidia visible in Δ*wetA* and Δ*vosA*, contrasting to abundant conidia in BbWT. *Scale bars*: 5 μm

bundles and were readily deformed in the process of pre-treatment for SEM.

Moreover, size and density of newly formed conidia were reduced by 58 and 40 % in Δ*wetA* compared with BbWT but not significantly affected in Δ*vosA*, as indicated by the respective FSc and SSc readings from the flow cytometry of large conidial samples (Fig. 4b). The FSc and SSc readings from the mature conidia (Fig. 4c) were 48 and 4 % smaller in Δ*wetA* than in BbWT, respectively. In Δ*vosA*, mature conidia suffered a significant, but minor, decrease (9.3 %) in density and little change in size.

In fluorescent lectin-binding assays through flow cytometry, Δ*wetA* and Δ*vosA* displayed significant alterations of carbohydrate epitopes (Tukey's HSD, $P < 0.05$) on the surfaces of newly formed and mature conidia labeled with the Alexa fluor 488-labeled lectins ConA, WGA, and GNL. The amount of ConA bound to the surfaces of newly formed conidia was reduced by 57 % in Δ*wetA* and 18 % in Δ*vosA* compared with BbWT (Fig. 4d); the relative reduction of ConA bound to the surfaces of mature conidia changed to 54 % in Δ*wetA* and 24 % in Δ*vosA* (Fig. 4e). In contrast, both WGA and GNL were much more bound to conidial surfaces of both Δ*wetA* and Δ*vosA*. The amounts of bound WGA and GNL increased from 92 % and 1.4-fold in newly formed conidia to 7.7- and 8-fold in the mature conidia of Δ*wetA*, respectively. The same estimates increased from 7 and 78 to 98 % and 1.5-fold in Δ*vosA*. Additionally, newly formed and mature conidia produced by Δ*wetA* were significantly less (6.4 and 7.4 %) hydrophobic than the BbWT counterparts (Fig. 4f) whereas Δ*vosA* showed an insignificant change in conidial hydrophobicity (Tukey's HSD, $P > 0.05$). All these changes were restored in the complementary rescued mutants.

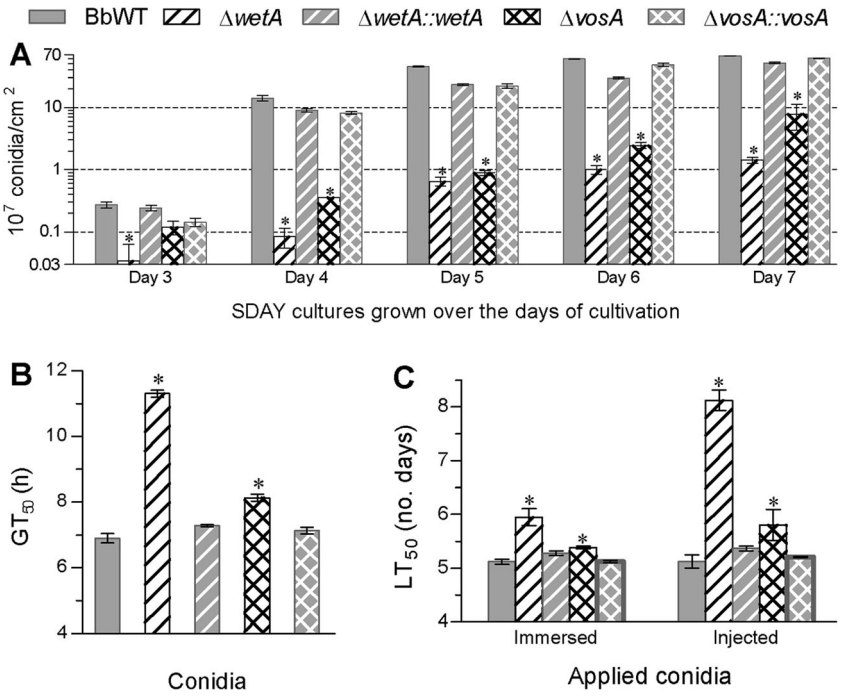


Fig. 3 Deleted *wetA* or *vosA* affects asexual development, conidial germination, and virulence of *B. bassiana*. **a** Conidial yields quantified from SDAY cultures initiated with 100 μl of conidial suspension per plate. **b** GT_{50} (h) for the time required to achieve 50 % germination of conidia. **c** LT_{50} (no. days) for conidial virulence to *G. mellonella* larvae inoculated via topical application (immersed) and hemocoel injection. *Asterisked bars* in each group differ significantly from those unmarked (Tukey's HSD, $P < 0.05$). *Error bars*: SD from three replicates

Contributions of WetA and VosA to intracellular trehalose accumulation, conidial viability, and multi-stress responses

The level of intracellular trehalose accumulation important for fungal stress response (Elbein et al. 2003) fluctuated greatly in each strain during 15 days of standard cultivation in SDAY. Compared with that in BbWT, trehalose content in Δ*wetA* increased by 2.5-fold on day 3 and 32 % on day 5 but decreased to 53, 56, and 67 % of those in BbWT on days 7, 10, and 15 (Fig. 5a), respectively. In Δ*vosA*, trehalose content increased by 5-fold on day 3, 93 % on day 5, and 61 % on day 10 but rapidly decreased to only 61 and 31 % of those in BbWT on days 10 and 15, respectively.

We examined more phenotypes indicative of conidial quality from different strains. Germination percentages of the conidia from less than 20-day-old SDAY cultures did not differ significantly between each deletion mutant and the control strains at the end of 24 h incubation (data not shown). However, Δ*wetA* and Δ*vosA* lost conidial viability much more rapidly with the aging of cultures than the control strains (Fig. 5b). Compared with those in BbWT, germination percentages of the conidia from the 30-, 40-, and 50-day-old cultures were decreased by 75, 85, and 82 % in Δ*vosA*, and by 10, 49, and 61 % in Δ*wetA*, respectively.

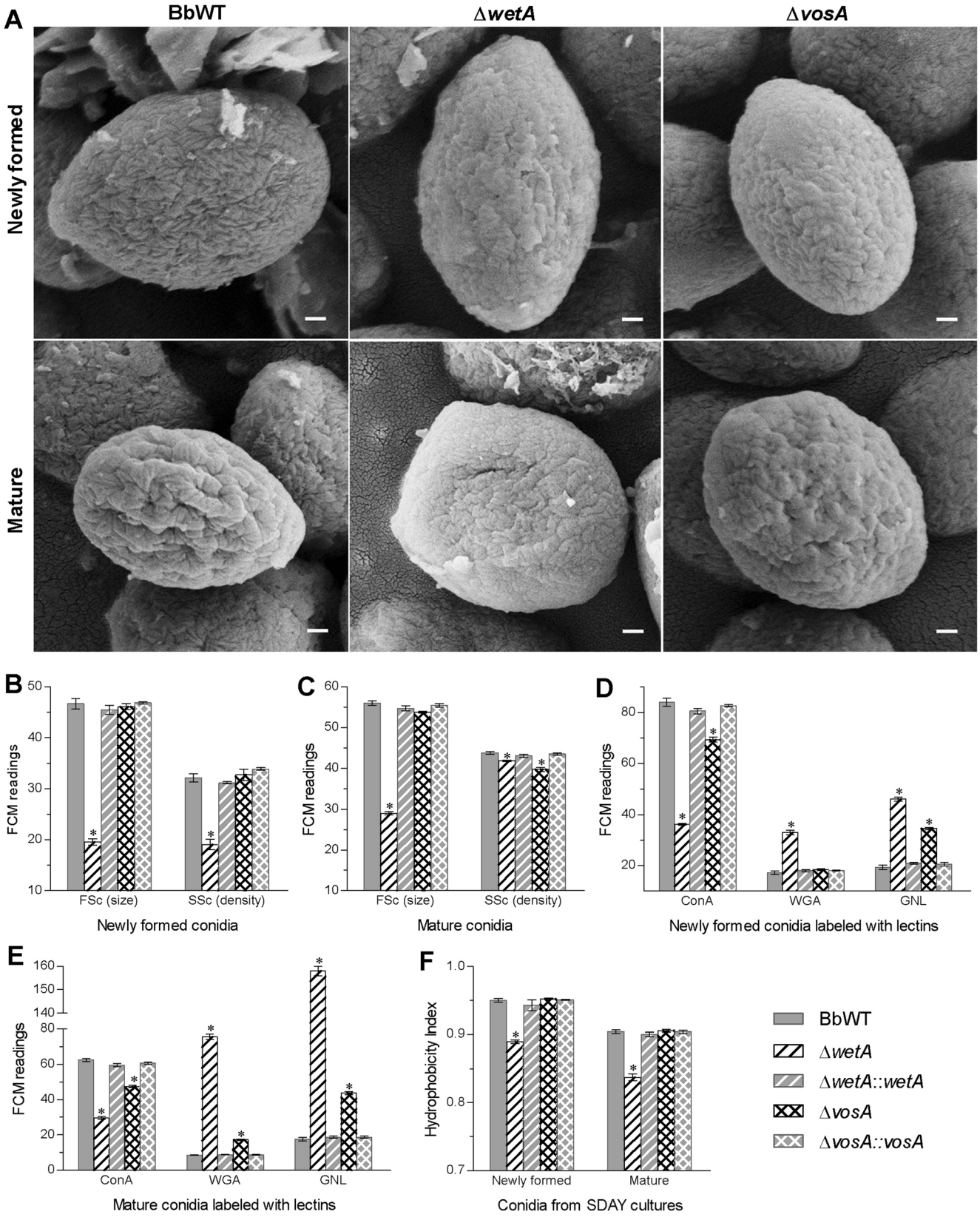
In stress assays, the conidia of Δ*wetA* and Δ*vosA* were equally more sensitive to a high osmolarity of NaCl (1.2 M) than those of BbWT (Fig. 5c). Conidial sensitivity to the oxidant menadione increased only in Δ*vosA*. Only did the Δ*wetA* conidia become more sensitive to cell wall perturbation by Congo red or SDS. However, neither Δ*wetA* nor Δ*vosA* showed a significant change in sensitivity to H_2O_2 or carbendazim (Tukey's HSD, $P > 0.05$).

Conidial thermotolerance and UV-B resistance were quantified as LT_{50}s after exposure to wet-heat stress at 45 °C and LD_{50}s after exposure to UV-B irradiation (Fig. 5d). Compared with BbWT, Δ*wetA* and Δ*vosA* lost 25 and 19 % of thermotolerance, and 13 and 10 % of UV-B resistance, respectively.

Discussion

As presented above, WetA and VosA are not only indispensable for conidiation of *B. bassiana* but responsible for maturation and quality of aerial conidia. The indispensability is largely dependent upon transcriptional interactions of either WetA or VosA with the crucial regulators BrlA and AbaA in the central development pathway, as discussed below.

Fig. 4 Deleted *wetA* or *vosA* affects conidial features indicative of ▶ maturation and quality in *B. bassiana*. **a** SEM images of newly formed and mature conidia from the 7- and 15-day-old SDAY cultures. Note a difference between BbWT and Δ*wetA* or Δ*vosA* in the bundles of conidial hydrophobin rodlets (*scale bars*: 200 nm). **b**, **c** Size and density of newly formed and mature conidia indicated by the respective FSc and SSc readings from the flow cytometry (FCM) of 2×10^4 conidia per sample. **d**, **e** Relative fluorescence intensities (FCM readings) in 2×10^4 newly formed and mature conidia (per sample) labeled with the fluorescent lectins ConA, WGA, and GNL, respectively. **f** Hydrophobicity indices of newly formed and mature conidia. *Asterisked bars* in each group differ significantly from those unmarked (Tukey's HSD, $P < 0.05$). *Error bars*: SD from three independent samples


A
BbWT
ΔwetA
ΔvosA
Newly formed
Mature
B
FCM readings
FSc (size)
SSc (density)
Newly formed conidia
C
FCM readings
FSc (size)
SSc (density)
Mature conidia
D
FCM readings
ConA
WGA
GNL
Newly formed conidia labeled with lectins
E
FCM readings
ConA
WGA
GNL
Mature conidia labeled with lectins
F
Hydrophobicity Index
Newly formed
Mature
Conidia from SDAY cultures
BbWT
ΔwetA
ΔwetA::wetA
ΔvosA
ΔvosA::vosA

First of all, WetA and VosA are required for conidiation due to their contributing to 98 and 88 % of conidiation capacity in *B. bassiana*. The drastic severity of conidiation defects attributed to either *wetA* or *vosA* deletion in *B. bassiana* has not been reported from other filamentous fungi. We also tried to create a double deletion mutant of *wetA* and *vosA* but failed in repeated attempts perhaps due to a lethality of the double deletion, which has not been disclosed in reported studies. Previously, deletion of *wetA* resulted in only 35 % reduction of conidial yield in *Fusarium graminearum* (Son et al. 2014) but unaffected conidiation accompanied with immature or colorless conidia in *Aspergillus* (Sewall et al. 1990a; Tao and Yu 2011) and *Penicillium* (Wang et al. 2015). However, a previous study on the Δ*vosA* mutant of *A. nidulans* (Ni and Yu 2007) focused on conidial maturation instead on conidiation level, not showing an effect of the *vosA* deletion on the fungal conidiation. The severe conidiation defects observed in this study are apparently attributable to the central pathway blocked by either *wetA* or *vosA* deletion, which led to almost all transcriptional depression of *brlA* on days 3–5 or *abaA* on days 4 and 5. This is because BrlA and AbaA are highly conserved activators of asexual development in filamentous fungi (Etxebeste et al. 2010; Park and Yu 2012). The absence of *wetA* in *A. fumigatus* has been shown to increase *brlA* and

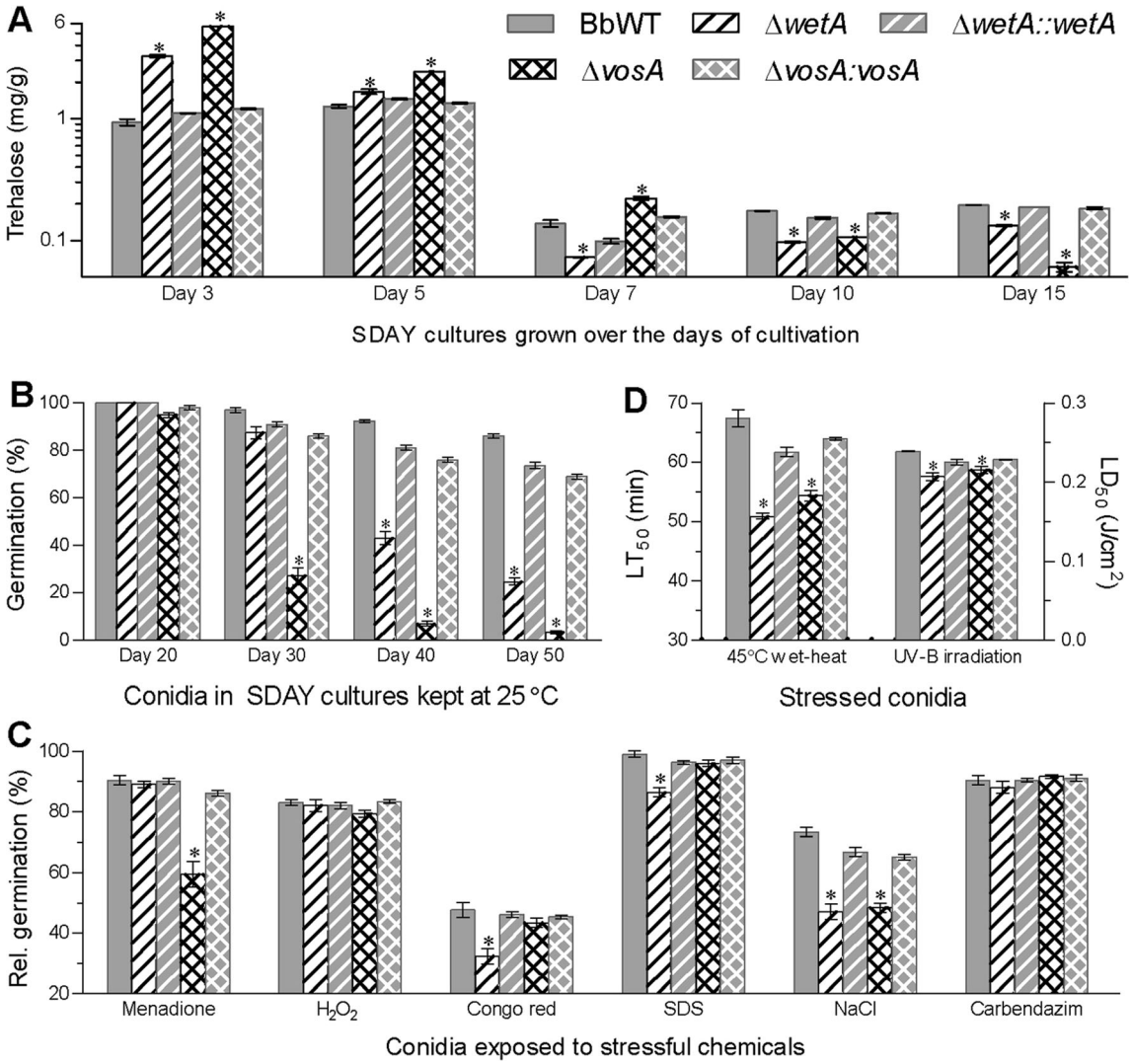


Fig. 5 Effects of deleted *wetA* or *vosA* on intracellular trehalose accumulation, conidial viability, and multi-stress tolerance in *B. bassiana*. **a** Trehalose contents in SDAY cultures grown for 3–15 days under normal conditions. **b** Germination percentages of the conidia collected from 20-, 30-, 40-, and 50-day-old SDAY cultures after 24 h of normatin incubation. **c** Relative germination (%) of the conidia collected from 7-day-old cultures and incubated for 24 h at 25 °C on a germination medium supplemented with menadione (0.2 mM), H_2O_2 (2 mM), Congo red (0.8 mg/ml), SDS (0.2 mg/ml), NaCl (1.2 M), and carbendazim (1 μg/ml), respectively. An unstressed control was used as a standard for the estimates of each strain. **d** LT_{50} (min) and LD_{50} (J/cm^2) estimated as indices of conidial tolerance to 45 °C wet-heat stress and UV-B irradiation. *Asterisked bars* in each group differ significantly from those unmarked (Tukey's HSD, $P < 0.05$). *Error bars*: SD from three replicates

abaA mRNA accumulation, suggesting a negative feedback control of conidiation involving *wetA* (Tao and Yu 2011). In another study, the deletion of *vosA* in *A. nidulans* caused uncontrolled activation of asexual development, whereas the enhanced expression of *vosA* led to blocked sporulation, suggesting that VosA may also function in a negative feedback regulation of sporogenesis (Ni and Yu 2007). In our study, the *wetA* or *vosA* deletion did not elevate transcript levels of *brlA* and *abaA* during the standard cultivation until full conidiation was achieved on the last day. Instead, the two gene transcripts were largely reduced in our Δ*wetA* and Δ*vosA* mutants during the critical period of conidiophore development and conidiation. In *B. bassiana*, therefore, both *wetA* and *vosA* could interact with *brlA* and *abaA* at transcriptional level in a way very different from the negative feedback control revealed in *Aspergillus*. This inference is also evident with the inhibitory effect of either *brlA* or *abaA* deletion on the *wetA* and *vosA* transcripts.

Apart from the most severe defects in conidiation, our Δ*wetA* and Δ*vosA* mutants exhibited different degrees of phenotypic defects tightly associated with conidial maturity and quality. These defects include smaller size, lesser density, slower germination, more impaired cell walls, and lower hydrophobicity in the conidia of Δ*wetA* than of Δ*vosA*. These results confirm that WetA plays more important role than VosA in the conidial maturation of *B. bassiana* perhaps by activating sets of possible proteins or enzymes involved in the synthesis and assembly of conidial wall components, as revealed in other fungi (Sewall et al. 1990a; Marshall and Timberlake 1991; Tao and Yu 2011; Son et al. 2014). A more significant role of VosA in the conidial maturation of *A. nidulans* has been shown to contribute to trehalose accumulation in conidia (Ni and Yu 2007). In this study, the level of the multi-functional trehalose (Elbein et al. 2003) largely increased in the Δ*wetA* and Δ*vosA* cultures grown for 3, 5, and 7 days except for a significant reduction in Δ*wetA* on day 7, when full conidiation was achieved in the BbWT culture with a very low level of hyphal debris. From then on, intracellular trehalose accumulation was decreased more rapidly in Δ*vosA* than in Δ*wetA*. We also tried to quantify conidial trehalose contents but failed in several attempts because the conidiation of either Δ*wetA* or Δ*vosA* was too poor to produce a sufficient amount of conidia for the purpose. Thus, our data simply indicate greater contribution of VosA than WetA to intracellular trehalose content in the aging culture of *B. bassiana*. More rapid loss of conidial viability during the culture aging hints to lower maturity of conidia in the aging culture of Δ*vosA* than of Δ*wetA*.

Moreover, the *wetA* or *vosA* deletion exerted profound effect on the biological control potential of *B. bassiana*. The two deletion mutants produced the conidia impaired in quality aside from a drastic loss of their conidiation capacity discussed above. The impaired quality was featured not only with cell wall damage, slower germination, and rapid viability loss but with attenuated virulence and reduced stress tolerance. These changes implicate that the mutant conidia are less capable of infecting insect pests and tolerating environmental stresses, which are often encountered in the field where a fungal insecticide containing the active ingredients of normal conidia is applied (Wang and Feng 2014). Some of the changes indicative of the impaired quality have also been shown in other fungal Δ*wetA* mutants with their conidia suffering reduced viability and survival rates (Tao and Yu 2011; Son et al. 2014). The increased sensitivity of our Δ*wetA* conidia to cell wall perturbation and wet-heat stress coincides well with their cell walls more impaired than those of the Δ*vosA* conidia, which were not sensitive to the cell wall stress. Interestingly, both deletion mutants showed a similar reduction in conidial osmotolerance or UV-B resistance and an insignificant change in conidial sensitivity to H_2O_2 or carbendazim. These phenotypic changes are largely, if not all, consistent with increased conidial sensitivity to thermal and/or osmotic stress in *A. fumigatus* Δ*wetA* (Tao and Yu 2011) and *A. nidulans* Δ*vosA* (Ni and Yu 2007). However, either the Δ*wetA* or Δ*vosA* mutant was highly sensitive to the oxidant H_2O_2 in *Aspergillus* but not in *B. bassiana*, in which only Δ*vosA* showed increased conidial sensitivity to the other oxidant menadione. In *Penicillium digitatum*, the Δ*wetA* conidia were extremely sensitive to menadione but showed increased tolerance to H_2O_2 (Wang et al. 2015). Thus, antioxidant responses attributed to WetA may vary with fungal species. We consider that the differential change of each stress tolerance in the *B. bassiana* Δ*wetA* and Δ*vosA* was attributable to not only impaired cell walls but likely reduced trehalose content, which has been shown in the previous studies (Ni and Yu 2007; Tao and Yu 2011) but failed to be quantified in this study due to their too poor conidiation. In addition, Δ*wetA* was much less virulent to the susceptible insect than Δ*vosA* irrespective of cuticular or cuticle-bypassing infection while Δ*vosA* was more sensitive to nutritional stress and less capable of utilizing environmental carbon and nitrogen sources for growth. All the defects in conidial virulence and responses to nutritional and abiotic stresses indicate that either WetA or VosA plays an important role in the fungal adaptation to host insect and environment.

Taken together, WetA and VosA are crucial regulators of both conidiation capacity and conidial quality in *B. bassiana* despite their differential roles in conidial maturation. WetA is more involved in the synthesis and assembly of conidial wall components to ensure cell wall integrity while VosA is more responsible for the synthesis and accumulation of intracellular trehalose. Therefore, both of them are required for conidiation capacity and conidial quality, thereby contributing to the biological control potential of *B. bassiana*. This also implicates that both WetA and VosA could likely be exploited to improve the fungal

potential against arthropod pests via overexpression, warranting a further study.

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