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rtfA, a putative RNA-Pol II transcription elongation factor gene, is necessary for normal morphological and chemical development in *Aspergillus flavus*

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Abstract The filamentous fungus *Aspergillus flavus* is an agriculturally important opportunistic plant pathogen that produces potent carcinogenic compounds called aflatoxins. We identified the *A. flavus rtfA* gene, the ortholog of *rtf1* in *Saccharomyces cerevisiae* and *rtfA* in *Aspergillus nidulans*. Interestingly, *rtfA* has multiple cellular roles in this mycotoxin-producing fungus. In this study, we show that *rtfA* regulates conidiation. The *rtfA* deletion mutant presented smaller conidiophores with significantly reduced conidial production compared to the wild-type strain. The absence of *rtfA* also resulted in a significant decrease or lack of sclerotial production under conditions that allowed abundant production of these resistance structures in the wild type. Importantly, the deletion of *rtfA* notably reduced the production of aflatoxin B_1, indicating that *rtfA* is a regulator of mycotoxin biosynthesis in *A. flavus*. In addition, the deletion *rtfA* also altered the production of several unknown secondary metabolites indicating a broader regulatory scope. Furthermore, our study revealed that *rtfA* controls the expression of the global regulators *veA* and *laeA*, which further influence morphogenesis and secondary metabolism in *A. flavus*.

Keywords *rtfA* · Aflatoxin · Conidiation · Sclerotium · *Aspergillus flavus* · Secondary metabolism

Introduction

Fungal secondary metabolites are bioactive low-molecular-weight compounds. Some of these metabolites present benefits as pharmaceutical drugs, such as the antibiotics or cholesterol-lowering drugs (Paláez 2004; Kennedy et al. 1999; Unkles et al. 2014) while others, such as mycotoxins, are detrimental to human health, agriculture, and industry. The most well-known mycotoxins are aflatoxins (AFs), particularly the acutely carcinogenic AFB_1 (Bhatnager et al. 2002; Cary et al. 2000; Payne and Brown 1998; Sweeny and Dobson 1999; Trail et al. 1995), produced by various *Aspergillus* species including the opportunistic plant pathogen *Aspergillus flavus*. This species disseminates efficiently by producing air-borne asexual conidia on specialized structures called conidiophores (Adams et al. 1998). In addition, *A. flavus* forms structures known as sclerotia. These structures, composed of condensed masses of mycelium, are able to resist extreme environmental conditions (Coley-Smith and Cooke 1971; Malloch and Cain 1972; Wicklow 1987), contributing to the survival of *A. flavus* in the field. This fungus is often found infecting economically important oil-seed crops, such as peanuts, corn, cottonseed, and tree nuts, contaminating them with mycotoxins, including AFs.

The genes required for AF biosynthesis are clustered and activated by the transcription factor gene, *aflR*, located in the AF gene cluster (Woloshuk et al. 1995). *aflR* has been shown to be under the regulation of several global regulators, among them the *velvet* protein VeA (Duran et al. 2007, 2009; Calvo et al. 2004). A similar role was observed in the model fungus *Aspergillus nidulans*, where VeA also controls the production

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of an AF-related mycotoxin sterigmatocystin (ST), the penultimate precursor in the AF biosynthetic pathway (Kato et al. 2003). In this model, organism great progress has been made in elucidating the mode of action of VeA. The VeA protein forms, together with LaeA and VelB, a complex designated Velvet (Bayram et al. 2008). VeA also interacts with the red phytochrome-like protein FphA, which interacts with blue sensing proteins LreA and LreB (Purschwitz et al. 2008). FphA and other VeA-interacting proteins, such as LlmF and the VipC-VapB heterodimer also modulate VeA mechanism of action by posttranslational modifications or altering its subcellular localization (Stinnett et al. 2007; Araújo-Bazán et al. 2009; Palmer et al. 2013; Sarikaya-Bayram et al. 2014; Purschwitz et al. 2008). These protein interactions and protein modifications affect multiple cellular processes (Calvo et al. 2016) in response to environmental stimuli, including morphogenesis and secondary metabolism. VeA is conserved in many fungal species (Calvo et al. 2004; Duran et al. 2007; Dhingra et al. 2012; Hoff et al. 2010; Kato et al. 2003; Laskowski-Peak et al. 2012; Li et al. 2006; Myung et al. 2012), where its function and the composition of VeA protein complexes could present some variation depending on species (Calvo et al. 2016).

In order to further dissect the VeA mechanism of action, we previously searched for new *veA*-dependent genetic elements utilizing a mutagenesis screening method in the model fungus *A. nidulans* (Ramamoorthy et al. 2012). Several *veA*-dependent genes were identified, among them *rtfA*, encoding a putative RNA-polymerase II transcription elongation factor-like protein conserved in fungal species and other eukaryotes (Jaehning 2010; Ramamoorthy et al. 2012; Tenney et al. 2006). In the model yeast *Saccharomyces cerevisiae*, the *rtfA* homolog *rtf1* is a subunit of the Paf1 complex, that physically interacts with the RNA-polymerase II and promotes optimal gene expression by controlling histone modifications (Jaehning 2010; Krogan et al. 2002; Mueller and Jaehning 2002; Rosonina and Manley 2005; Squazzo et al. 2002; Warner et al. 2007). In yeast *rtf1* is required for ubiquitination of histone H2B and di-methylation and tri-methylation of histone H3 (Briggs et al. 2002; Ng et al. 2003; Sun and Allis 2002; Warner et al. 2007), as well as TATA site selection by TATA box binding proteins (TBP) (Stolinski et al. 1997), interactions with active open reading frames (ORFs), proper attachment of components from RNA-pol II, and binding chromatin remodeling proteins such as the ATP-dependent protein Chd1 (Jaehning 2010; Warner et al. 2007). In *A. nidulans*, it has been shown that *rtfA* is important for the activation of the ST gene cluster and concomitant ST biosynthesis as well as for proper asexual and sexual development (Ramamoorthy et al. 2012).

We found the *rtfA* homolog present in the *A. flavus* genome. Our current study revealed that *rtfA* regulates development, affecting conidiophore structure and conidial production, as well as sclerotial production. Furthermore, *rtfA* controls the biosynthesis of AF and other secondary metabolites in this agriculturally and medically important fungus.

Materials and methods

Fungal strains and culture conditions

A. flavus CA14 (*pyrG*−, *niaD*-, Δ*ku70*) (SRRC collection # 1709), CA14 pyrG-1 (*pyrG*+, *niaD*-, Δ*ku70*), TJML1.1 (Δ*rtfA*, *pyrG*+, *niaD*-, Δ*ku70*), and TJML2.1 (Δ*rtfA*, *niaD*-, Δ*ku70*, *rtfA*+) strains were used in this study. All strains were grown on YGT medium (5 g yeast extract, 20 g glucose, 1 mL of trace elements per liter (Käfer 1977)) at 30 °C in the dark unless otherwise specified. Agar (15 g/L) was added to obtain solid medium. Strains were maintained as 30 % glycerol stocks at −80 °C.

Construction of the *rtfA* deletion (Δ*rtfA*) strain

The deletion vector to eliminate *rtfA* (AFL2G_05224.2) by gene replacement was generated as follows: first, a 975 bp 5′ and a 1 kb 3′ UTRs fragments were PCR amplified using ExTaq HS polymerase (Takara, Mountain View, CA, USA) and primers 5′RtfAEcoRI and 5′RtfABamHI and 3′RtfASalI and 3′RtfAHindIII, respectively (Table 1) utilizing *A. flavus* CA14 genomic DNA as template. PCR products were subcloned into the TOPO pCR2.1 (Invitrogen, Carlsbad, CA, USA) vector. The correct inserts were further confirmed by DNA sequencing. The 5′ and 3′ UTR PCR products were then released from the TOPO pCR2.1 vector using *Eco*RI and *Bam*HI, and *Sal*I and *Hin*dIII, respectively. The released fragments were then ligated to pPG15–5 previously digested with the corresponding restriction enzymes. pPG15–5 contains the *Aspergillus parasiticus pyrG* selectable marker gene for fungal transformation (Chang 2003). The resulting deletion vector was designated as pRtfA-pyrG. Fungal transformation was performed as described in Cary et al. (2006) using *A. flavus* CA14 as host strain. The transformants were grown on Czapek agar (CZ, Difco, Franklin Lakes, New Jersey, USA) supplemented with 10 mM ammonium sulfate (CZ-AS) and sucrose as osmotic stabilizer. The *rtfA* deletion strain was confirmed by Southern blot analysis.

Construction of the complementation (Δ*rtfA*-com) strain

The *rtfA* complementation strain was generated by first obtaining a 4.8-kb PCR product containing the complete *rtfA* wild-type locus using the primers 1169 and 1170 (Table 1) and *A. flavus* CA14 genomic DNA as template. The PCR product was digested with *Bam*HI and ligated to the *Bgl*II-digested pJet + ptrA vector (containing the *ptrA* selection marker at the *Pst*I site). This resulted in the final vector pSD42.1 that was

Table 1 Primers used in this study

Primer name	Sequence
5′ RtfA EcoRI	5′-GAATTCCAAGTTCAGTCAAAGTCAACTTCTTC-3′
5′ RtfA BamHI	5′-GGATCCTCCAGGTTTGCCATGCTGGCTTTCC-3′
3′ RtfA SalI	5′-GTCGACCTCTTCTAAACCTCGTGCAGACAAG-3′
3′ RtfA HindIII	5′-AAGCTTGGTCATAGGAGGTGGGAAAATGTACAG-3′
1169	5′-AAAAAGGATCCCGTTTCCAAGGATCGTCAATGTTCATGG-3′
1170	5′-AAAAAGGATCCTGCTCCGAATCTTCCGCTCCATCCT-3′
1246(P1)	5′-CGCGCTTGCGGGTGACGCCTC-3′
1247(P2)	5′-CCATCGTCCCACTCAGCATCGC-3’
18S F	5′- TTCCTAGCGAGCCCAACC T-3’
18S R	5′- CCCGCCGAAGCAACTAAG-3’
RtfA F	5′- GTTCCCTTTCGTTGCTTGTTCAGACTC-3’
RtfA R	5′ CAGTCGACTTGGTGTCCAGTGATCC-3’
brlA F	5′- CCGCTTATGATGACAACGTG-3’
brlA R	5′- GGCTGTGTGTTCCAGTCTCA-3’
abaA F	5′- GAGTGGCAGACCGAATGTATGTTG-3’
abaA R	5′ TAGTGGTAGGCATTGGGTGAGTTG-3’
nsdC F	5′- GGAAGTTACGCTCCTGAAGATG-3’
nsdC R	5′- CGTTCGTCCTCTTCATCCATAC-3’
aflR F	5′- CCGGGATAGCTGTACGAGTTG-3’
aflR R	5′- GAGCACAGGCCGGTTTCTC-3’
ver1 F	5′- ACTTTCACCGATGAGCAGGTAGA-3’
ver1 R	5′- AGGCCCACCCGGTTCA-3’
omtA F	5′- CGGTTCCCTGGCTCCTAAG-3’
omtA R	5′- TGGGTGGCAGCAGCTAGAC-3’
veA F	5′- GTATGAAACGCCCGGTTACT-3’
veA R	5′- CTCGCCGGATGGCATATTTA-3’
laeA F	5′- TTTCTAGACTTGGGCTGCGG-3’
laeA R	5′- AGCAAGGTCAACCCCAACAA-3’

transformed into *A. flavus* Δ*rtfA*. Positive transformants were identified on Czapek agar (CZ, Difco, Franklin Lakes, New Jersey, USA) supplemented with 0.4 M ammonium tartrate, pyrithiamine (0.1 μg/mL), and sucrose as osmotic stabilizer. The transformants were confirmed by Southern blot analysis.

Fungal growth analysis

Conidia of the *A. flavus* wild-type, Δ*rtfA* and Δ*rtfA*-com strains were point inoculated on solid YGT medium. The strains were incubated for 3, 4, and 5 days at 30 °C in the dark. Fungal growth was then measured as colony diameter (in millimeter). The experiment contained three replicates. The plates were photographed after incubation with a Sony Cybershot DSC-W120 camera (Sony, New York, NY, USA).

Conidiophore structural analysis

The *A. flavus* strains were point inoculated on YGT solid medium and allowed to incubate at 30 °C in the dark for 3 days. Cores (16 mm^2) were taken 1 cm from the center and vortexed in sterile ddH_20 to remove conidia. Micrographs were taken with a Nikon E-600 bright-field microscope (Nikon Inc., Melville, NY, USA) attached to a Nikon DXM 1200 digital camera (Nikon Inc., Melville, NY, USA). Conidiophore vesicle diameters were measured in 30 conidiophores of each strain.

Quantification of conidial production

The *A. flavus* strains were point inoculated on YGT solid medium and incubated in the dark at 30 °C for 3, 4, and 5 days. After incubation, a 7-mm core was taken from each plate and homogenized. Conidia were quantified with a hemocytometer (Hausser Scientific, Horsham, PA) under a Nikon Eclipse E-400 bright-field microscope (Nikon Inc., Melville, NY, USA). The experiment was performed in triplicate.

An additional experiment was carried out by point inoculating the *A. flavus* strains onto YGT medium and incubated for 7 and 11 days. Then, a 1/8th radial sector of the fungal

colony was collected and homogenized in a 15-mL Falcon tube that contained 10 mL of ddH_2O. Spores were also quantified with a hemacytometer under the microscope. The experiment was carried out in triplicate.

Sclerotial analysis

The *A. flavus* strains were point inoculated on 35 mL solid YGT medium. The strains were grown for 7, 11, and 17 days at 30 °C in the dark. Cultures were visualized using a Leica MZ75 dissecting microscope attached to a Leica DC50LP camera (Leica Microsystems Inc., Buffalo Grove, IL, USA). Micrographs were taken from the cultures after an ethanol (70 %) wash to remove conidiophores. The experiment was performed with three replicates.

A similar analysis was also carried out using Wickerham agar medium (2 g yeast extract, 3 g peptone, 5 g corn steep solids, 2 g dextrose, 30 g sucrose, 2 g $NaNO_3$, 1 g $K_2HPO_4{\cdot}3H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, 0.1 g $FeSO_4{\cdot}7H_2O$, 15 g agar per liter [pH 5.5] (Chang et al. 2012)). The strains were incubated for 13 and 20 days at 30 °C in the dark.

Aflatoxin B_1 analysis

Cores from top-agar and point inoculated cultures were extracted with chloroform. The solvent was allowed to dry overnight. The samples were resuspended in 300 μL of chloroform. Extracts were analyzed by thin layer chromatography (TLC) as previously described (Cleveland et al. 1987). Briefly, 25 μL of each extracts were spotted on a TLC silica plate and separated using a chloroform: acetone (85:15 *v*/*v*) solvent system. The TLC plate was air dried, sprayed with a 12.5 % $AlCl_3$ solution in 95 % ethanol and baked at 80 °C for 10 min. The TLC plate was then exposed to UV light at 365-nm for visualization of compounds. The experiment was carried out with three replicates. The AFB_1 standard was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

Effect of *rtfA* in resistance to temperature and light stresses

The *A. flavus* strains were point inoculated on 25 mL of solid YGT medium. Fungal cultures were incubated under light or dark conditions at 30, 37, 42, or 44 °C for 5 days. Colony growth and conidiation patterns were observed. Cultures were photographed with a Sony Cybershot DCS-W120 camera (Sony, New York, NY, USA). Micrographs were taken at ×50 magnification and visualized using a Leica MZ75 dissecting microscope attached to a Leica DC50LP camera (Leica Microsystems Inc., Buffalo Grove, IL, USA).

Gene expression analysis

The wild-type Δ*rtfA* and Δ*rtfA*-com strains were top-agar inoculated on YGT medium (10^6 conidia/plate) and allowed to incubate at 30 °C under dark. Mycelia were harvested after 3, 4, and 5 days of incubation and immediately frozen in liquid nitrogen. RNA was extracted using Zymo Research ZR Fungal/Bacterial RNA Mini Prep Kit (Zymo, Irvine, CA, USA) and DNAse I digestion was carried out according to manufacture's protocol. cDNA was obtained by using Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qRT-PCR was carried out using iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA) and a iCycler iQ5 Multicolorreal-time PCR detection system (Bio-Rad, Hercules, CA, USA) as previously described in Cary et al. (2007). The primers used in the qRT-PCR analysis are listed in Table 1. Gene expression levels obtained were normalized to the expression of the 18S rRNA gene by the $2^{-\Delta\Delta CT}$ Method previously described by Livak and Schmittgen (2001).

Statistical analysis

Statistical analysis was applied to analyze all of the quantitative data in this study utilizing ANOVA (analysis of variance), in conjunction with a Tukey's multiple comparison test using a p value of $p < 0.05$ for samples that are determine to be significantly different.

Results

rtfA is necessary for normal colony growth and asexual development

To assess the role of *rtfA* in *A. flavus* growth and asexual development, an *A. flavus rtfA* deletion strain (Δ*rtfA*) was constructed (Fig. 1a). Southern blot analysis confirmed the genotype of the selected transformants. Genomic DNA from the wild-type control, Δ*rtfA* strains, and the Δ*rtfA*-com was digested with *Sal*I (Fig. 1a-c). A fragment corresponding to the 5′ UTR of *rtfA* was used as probe template. A 2.1-kb band shows the presence of the *rtfA* wild-type allele, while a 3.7-kb band indicates gene replacement of *rtfA* with the *A. parasiticus pyrG*. Absence of *rtfA* expression in the Δ*rtfA* strain was verified by qRT-PCR (Fig. 1d). The *A. flavus* Δ*rtfA* strain demonstrated a slight but statistically significant reduction in colony growth when compared to the isogenic control strains at each time point analyzed (Fig. 2).

Additionally, the *A. flavus* Δ*rtfA* strain demonstrated a statistically significant reduction in the production of conidia at 3, 4, and 5 days of incubation when compared to the control strains (Fig. 3a). However, after 7 days of incubation, the number of asexual conidiophores were similar to those of

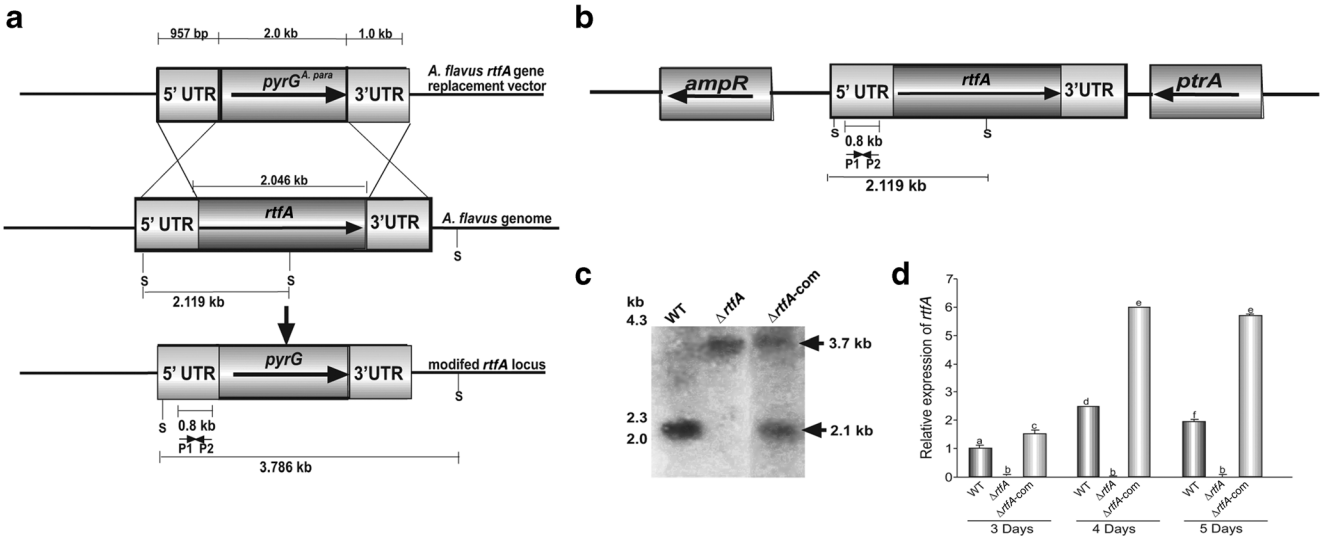

Fig. 1 Southern blot analysis confirming the deletion of the *rtfA* gene as well as the complementation of this deletion mutant with the *rtfA* wild-type allele. **a** The 800 bp probe template shown was obtained by PCR using primers 1246 (P1) and 1247 (P2) (Table 1) and genomic DNA from a CA14 strain as template. Genomic DNA from wild-type CA14 pyrG-1 (WT), Δ*rtfA* and complementation (Δ*rtfA*-com) strains was digested with *Sal*I (S). A 2.1-kb band indicates an *rtfA* wild-type locus, while a 3.7-kb band indicates a gene replacement of *rtfA* with the selection marker *A. parasiticus pyrG*. **b** Linear representation of the complementation vector. **c** Southern blot results confirming the correct integration of the deletion cassette in the Δ*rtfA* mutant and successful complementation with the *rtfA* wild-type allele in the Δ*rtfA*-com strain. **d** Relative expression of *rtfA*. *Error bars* represent standard error. *Different letters on the columns* indicate values that are statistically different ($p < 0.05$)

the isogenic controls, and at 11 days of incubation the *A. flavus* Δ*rtfA* strain presented statistically greater conidiation levels than those observed in the isogenic wild-type and Δ*rtfA*-com strains (Fig. 3b). Gene expression analysis of *brlA* and *abaA* showed that both genes were abnormally upregulated in the *A. flavus* Δ*rtfA* strain compared to the controls after 3 days of incubation. However, after 4 and 5 days, the expression levels of these genes were significantly reduced with respect to those in the control strains (Fig. 3c–d). Partial recovery of *brlA* and *abaA* expression is observed in the complementation strain, leading to normal conidiation levels. Microscopic analysis of Δ*rtfA* conidiophores showed a statistically significant reduction in conidiophore vesicle diameter when compared to the isogenic control strains (Fig. 4).

rtfA is necessary for normal sclerotial production

We examined whether *A. flavus rtfA* plays a role in sclerotial production. To test this hypothesis, the *A. flavus* wild-type, *rtfA* deletion mutant and complementation strains were point inoculated on YGT medium, which allows formation of *A. flavus* sclerotia in the wild type (Fig. 5a). Under these experimental conditions Δ*rtfA* produced a very limited number of immature sclerotia, at a time that the wild-type and Δ*rtfA*-com strains produced large numbers of mature resistant bodies.

A similar effect was also observed on Wickerham medium [Fig. S1 in the Electronic supplementary material (ESM)].

In order to provide further insight on how *rtfA* regulates sclerotial production in *A. flavus*, we examined the expression

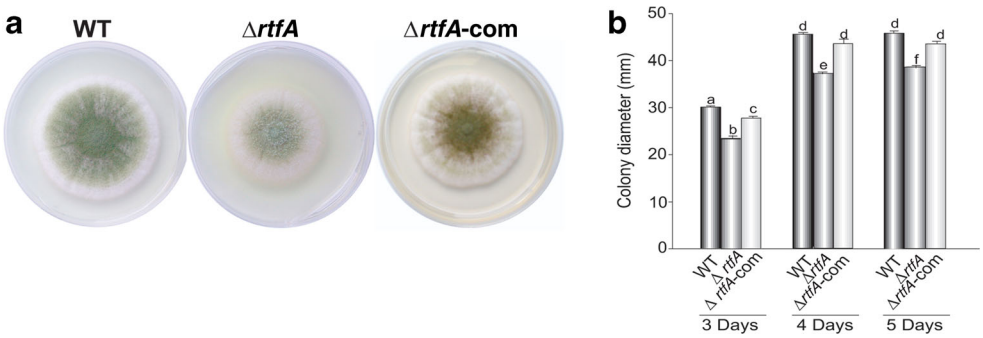

Fig. 2 Effects of *rtfA* on *A. flavus* colony growth. **a** Photographs showing point inoculated cultures of *A. flavus* wild-type (WT), Δ*rtfA* and complementation (Δ*rtfA*-com) strains growing on YGT medium at 30 °C in the dark for 5 days. **b** Quantification of colony growth, as colony diameter (in millimeter). *A. flavus* strains were point inoculated on YGT medium and incubated at 30 °C in the dark. Measurements were taken at 3, 4, and 5 days after inoculation. *Error bars* represent standard error. *Different letters on the columns* indicate values that are statistically different ($p < 0.05$)

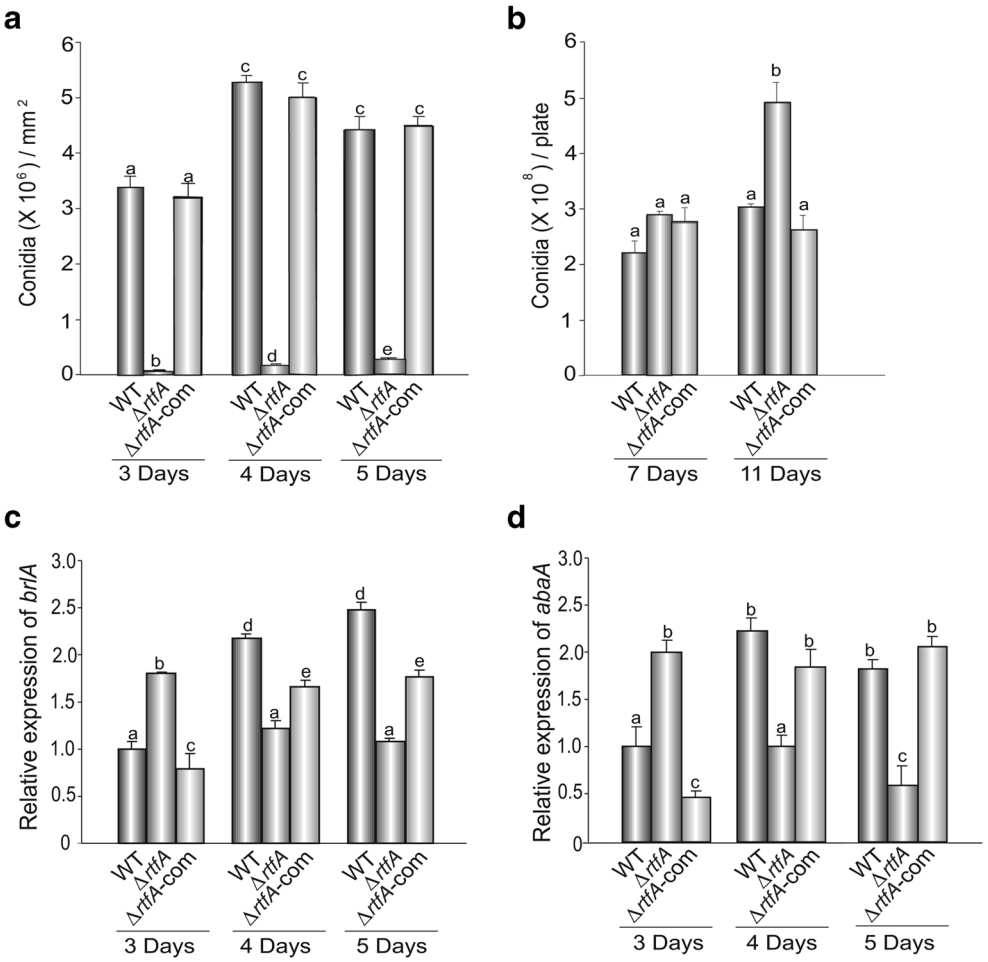

Fig. 3 Role of *rtfA* on *A. flavus* conidial production. The wild-type (WT), Δ*rtfA*, and complementation (Δ*rtfA*-com) strains were point inoculated on YGT medium and incubated at 30 °C in the dark in triplicate. Quantification of conidia after 3, 4, and 5 days **a** and 7 and 11 days **b** of incubation. Relative expression of *brlA* **c** and *abaA* **d**. *Error bars* represent standard error. *Different letters on the columns* indicate values that are statistically different ($p < 0.05$)

levels of *nsdC*, known to be involved in sclerotial formation (Cary et al. 2012) in YGT cultures. After an abnormal increase in the expression of this gene in the Δ*rtfA* strain at 3-days incubation, a significant decrease in its expression was observed when compared to the wild-type and Δ*rtfA*-com controls after 4 and 5 days of incubation (Fig. 5b).

rtfA is necessary for normal aflatoxin B_1 biosynthesis

In *A. nidulans*, *rtfA* influenced the production of the AF-related mycotoxin ST (Ramamoorthy et al. 2012). In the current study, the effect of the *rtfA* homolog on AFB_1 production in *A. flavus* was examined. AFB_1 biosynthesis was greatly reduced or completely abolished in the Δ*rtfA* strain when compared to the wild-type and Δ*rtfA*-com controls (Fig. 6a and Fig. S2a in the ESM). Interestingly, it was also noted in the TLC analyses that the synthesis of several unknown metabolites was also *rtfA* dependent (Fig. S2 in the ESM). Due to the effects observed on AF biosynthesis, an expression analysis of the ST-AF pathway-specific transcription factor gene, *aflR*, was carried out. We also examined the expression of the aflatoxin pathway biosynthetic genes *ver1* and *omtA* as indicators of AF cluster activation. Expression of *aflR*, *ver1*, and *omtA* were up regulated in the *A. flavus* Δ*rtfA* strain at the 3-day time point and down regulated at the 4- and 5-day time points (Fig. 6b–d).

rtfA regulates the expression of the global regulatory genes *veA* and *laeA*

Ramamoorthy et al. (2012) showed that in *A. nidulans rtfA* is functionally dependent on *veA*. In our current study, we examine whether *rtfA* regulated *veA* expression as well as the expression of *laeA*, whose gene product is a VeA-interacting protein (Bayram et al. 2008). The gene expression analysis revealed increases of *veA* and *laeA* expression in the *A. flavus* Δ*rtfA* strain after 3 days of incubation, while *veA* and *laeA* expression levels decreased levels of expression at after 4 and 5 days compared to the isogenic control strains (Fig. 7a–b).

rtfA is necessary for normal growth under light and temperature stresses

Since absence of *rtfA* results in a slight reduction of fungal growth, we investigated whether environmental stresses such

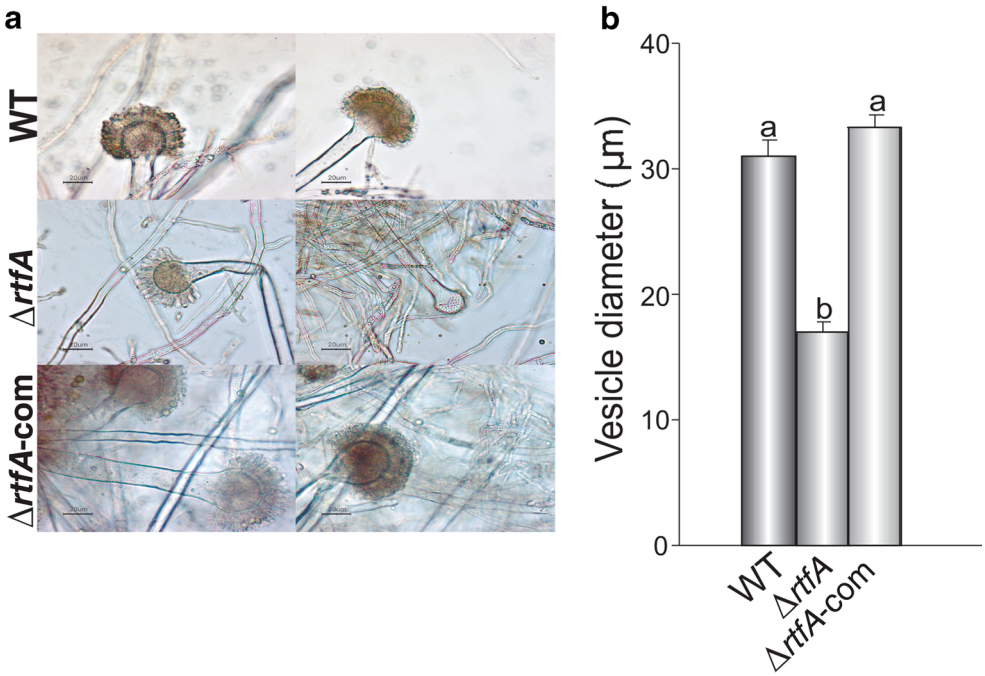

Fig. 4 Effects of *rtfA* on conidiophore structure. **a** The wild-type (WT), Δ*rtfA*, and complementation (Δ*rtfA*-com) strains were point inoculated on YGT medium and incubated at 30 °C in the dark for 3 days. Sample was taken 1 cm from the center of the colony and mounted on a slide before being photographed at ×400 magnification using a Nikon E-600 microscope. **b** From the samples in panel **a**, the diameter of 30 conidiophore vesicles was measured. *Error bars* represent standard error. *Different letters on the columns* indicate values that are statistically different ($p < 0.05$)

as light or temperature might further impact this phenotype. Our results indicated that light and higher temperatures had only a small impact on the radial colony growth of the Δ*rtfA A. flavus* strain (Fig. 8a); however, combination of light and temperatures of 42 and 44 °C further accentuated the reduction of growth in the *rtfA* mutant more notably than in the control strains. Conidiation was also reduced in the *rtfA* mutant with respect to the controls at 42 °C. A temperature of 44 °C suppressed conidiation in all strains.

Discussion

Consumption of AF-contaminated food has been associated with hepatotoxicity, liver cancer, teratogenicity, immunotoxicity, and death (Dvorackova and Kusak 1990; Payne and Brown 1998; Probust et al. 2010; Sweeny and Dobson 1999; Trail et al. 1995). In developed countries, AF contamination can lead to significant adverse economic impacts. In the U.S.A. alone, AF contamination of crops can result in losses of hundreds of millions USD annually, especially during years where there has been significant drought in the midwest corn belt (Wu et al. 2014). Searching for novel genetic targets could lead to new strategies to reduce the detrimental effects of AF contamination as well as decreasing dissemination and survival of *A. flavus*. In this study, we characterized the role of the *rtfA* gene in development and secondary metabolism in this understudied and agricultural and medically relevant AF-producer fungus. *rtfA* was first studied in the model fungus *A. nidulans* where it was shown to positively influence morphogenesis and the biosynthesis of ST and other metabolites, including penicillin (Ramamoorthy et al. 2012). Our results revealed partial functional conservation

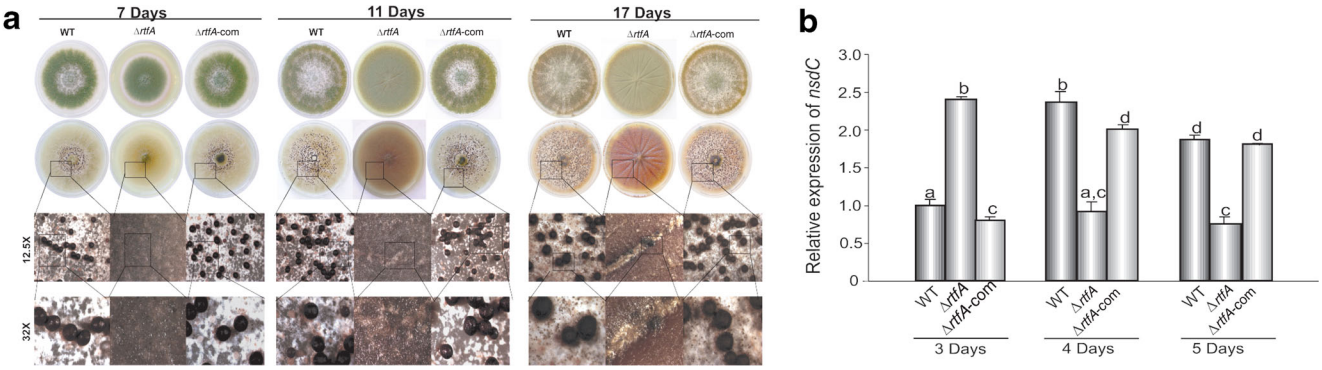

Fig. 5 *Aspergillus flavus rtfA* is necessary for normal sclerotial production. **a** The wild-type (WT), Δ*rtfA*, and complementation (Δ*rtfA*-com) strains were point inoculated on YGT medium and incubated for 7, 11, and 17 days at 30 °C in the dark. Photographs of the colony before and after ethanol wash to visualize sclerotia production are shown. Micrographs of cultures taken after the ethanol wash. **b** Relative expression of *nsdC*. Experiments were carried out in triplicate. *Different letters on the columns* indicate values that are statistically different ($p < 0.05$)

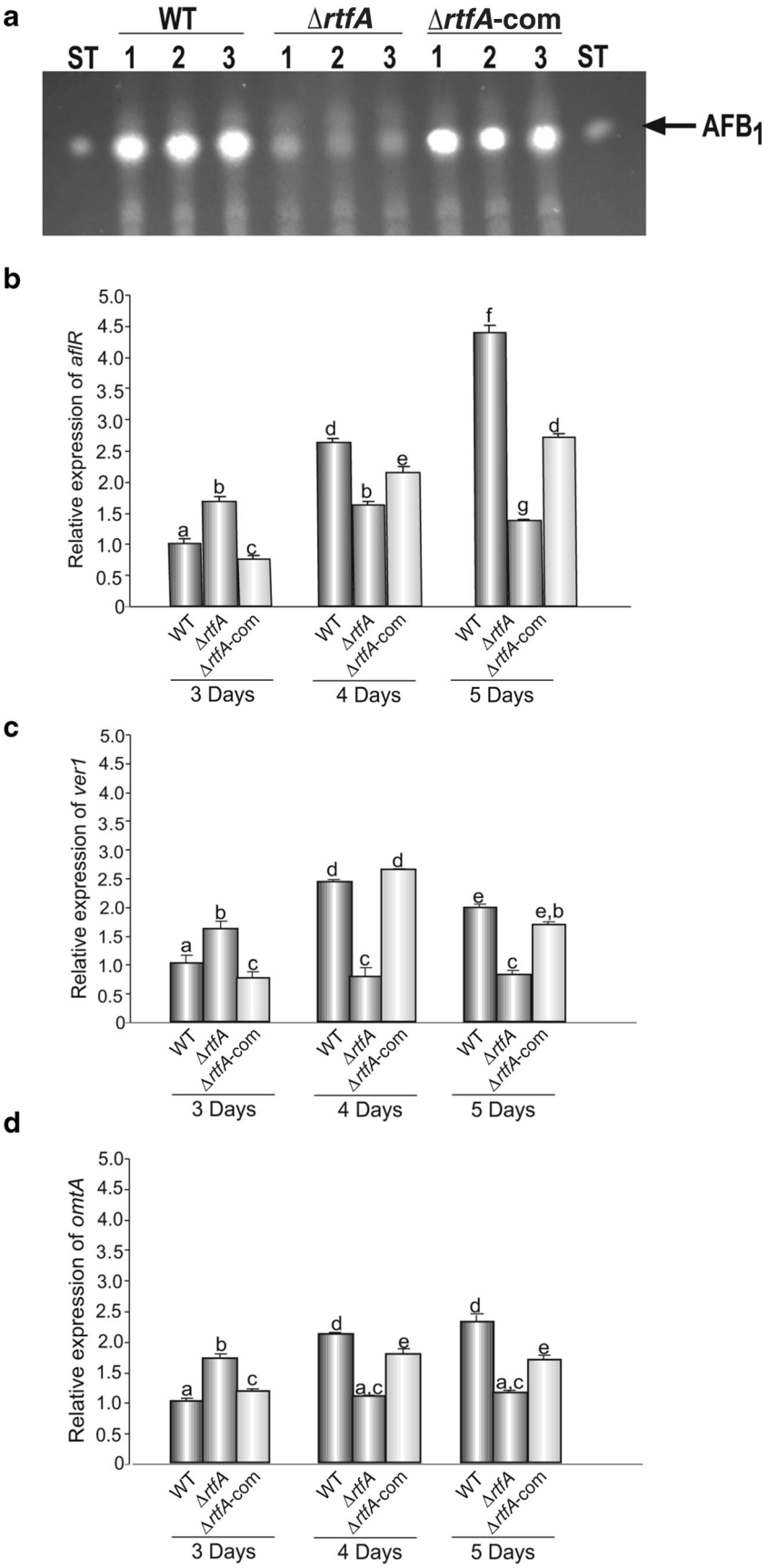

Fig. 6 *rtfA* is necessary for normal aflatoxin B_1 biosynthesis **a** TLC analysis of AFB_1 production in YGT top-agar inoculated cultures incubated for 7 days at 30 °C in the dark. Experiments were carried out in triplicate. Relative expression of *aflR* **(b)** *ver1* (**c**) and *omtA* (**d**) from top-agar inoculated cultures. Experiments were carried out in triplicate. *Different letters on the columns* indicate values that are statistically different ($p < 0.05$)

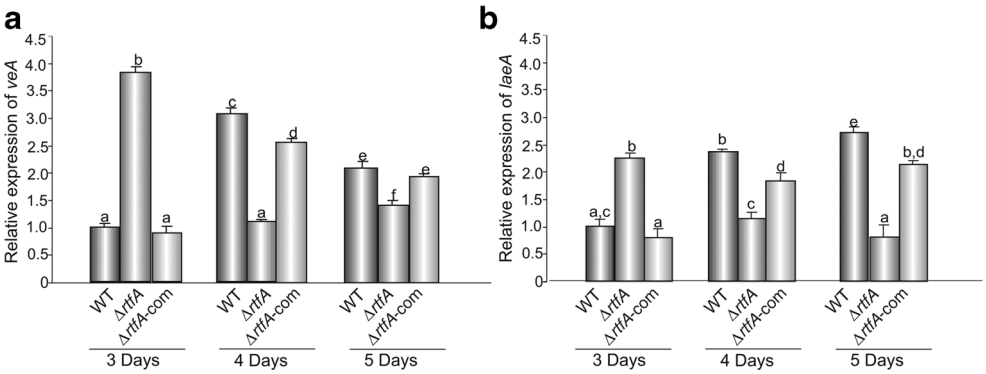

Fig. 7 *rtfA* regulates *veA* and *laeA* expression. **a** Relative expression of *veA*. **b** Relative expression of *laeA*. Experiments were carried out in triplicate. *Different letters on the columns* indicate values that are statistically different ($p < 0.05$)

between *A. flavus* and *A. nidulans rtfA*. As in *A. nidulans*, *rtfA* positively affects vegetative growth. The reduction in colony growth was not as notable in the *A. flavus* mutant as was described for the Δ*rtfA* strain of the model fungus. However, the reduction in growth observed in the *A. flavus* mutant was intensified by environmental stresses, such as light and high temperatures, particularly when the mutant was exposed simultaneously to these stress factors.

In addition, *A. flavus rtfA* was shown to influence conidiation as in *A. nidulans* (Ramamoorthy et al. 2012). In both cases, a decrease in conidial production in the absence of *rtfA* was observed, suggesting that *rtfA* is necessary for normal asexual spore production. The decrease in conidiation was particularly exacerbated when the cultures were exposed to high temperature, rendering aconidial colonies. Interestingly, the capacity to produce conidia was regained and even surpassed the wild-type level in considerably older cultures. It is possible that in older cultures, environmental factors, such as water availability or nutrient starvation, may override reduced conidiation due to the absence of *rtfA* by triggering asexual development through an *rtfA*-independent mechanism in an attempt to escape an environment no longer conducive to growth. It is also possible that *rtfA* is necessary for normal activation of asexual development (Adams et al. 1998), which could be delayed in the mutant strain as suggested by reduced levels of *brlA* and *abaA* at the 4- and 5-day time points compared to the control. In addition, our results revealed that *rtfA* is also important for proper condiophore formation in *A.*

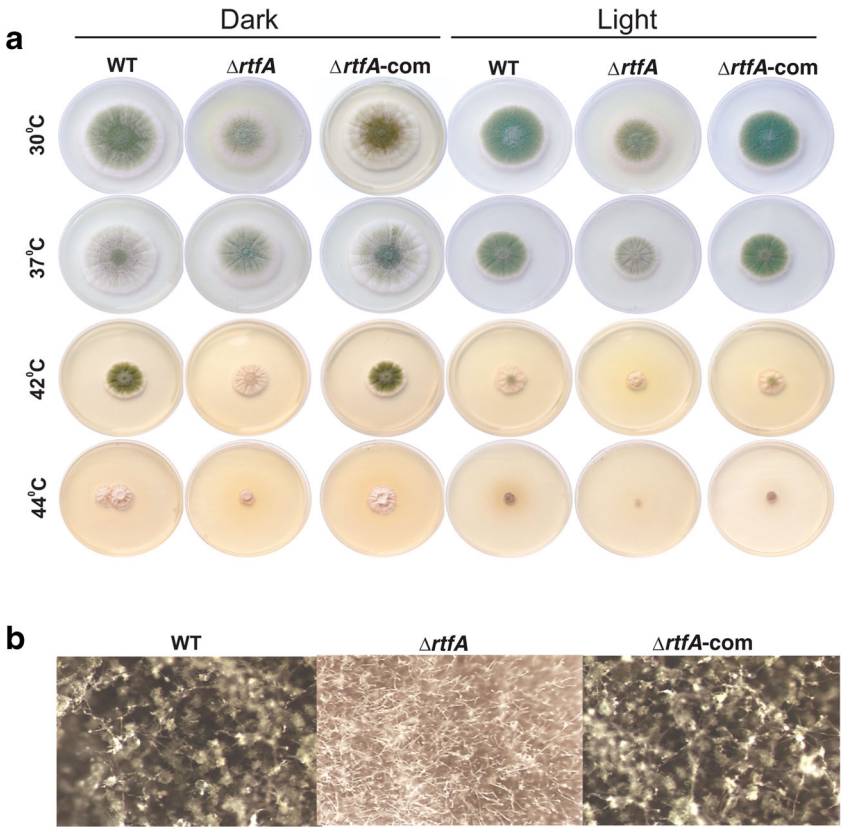

Fig. 8 *rtfA* influences the resistance of *A. flavus* to light and temperature stresses. **a** *A. flavus* wild-type (WT), Δ*rtfA* and complementation (Δ*rtfA*-com) strains were point inoculated on YGT medium and incubated at 30, 37, 42, and 44 °C under light or dark conditions. Cultures were incubated for 5 days. **b** Micrographs of cultures grown at 42 °C in the dark (×50 magnification). All experiments were carried out in triplicate

flavus. The *rtfA* deletion mutant presents a statistically significant reduction of the conidiophore vesicle size when compared to those in the isogenic control strains. It is a fact that *rtfA* is necessary for normal conidiophore formation and conidial production is relevant, since conidia are the most efficient form of dissemination for this fungus.

A. flavus rtfA is also necessary for the formation of sclerotia. Similarly, the *rtfA* homolog in *A. nidulans* in necessary for cleisthothecial production (Ramamoorthy et al. 2012). Previous reports support that sclerotia in *A. flavus* might be vestigial cleistothecia that lost the capacity to produce ascospores (Geiser et al. 1996). Furthermore, the complementary alpha- and HMG-domain *MAT* genes have been investigated in *A. flavus* and *A. parasiticus* (Ramirez-Prado et al. 2008). Also, the presence and function of mating type genes in *Aspergillus oryzae* was also described, indicating a possible heterothallic breeding system (Wada et al. 2012). Additionally, in some isolates ascospore-bearing ascocarps embedded within sclerotia of *A. flavus* and *A. parasiticus* have been found (Horn et al. 2009, 2014). The fact that *rtfA* governs the production of cleistothecia and sclerotia in *A. nidulans* and *A. flavus*, respectively, provides additional evidence that conserved genetic regulatory pathways, without significant evolutionary modification of its output, govern the formation of both structures. *rtfA*-dependent regulation of sclerotial morphogenesis in *A. flavus* is relevant due to the fact that these structures are critical for fungal survival under harsh environmental conditions, providing a source of inoculum for crop infections during consecutive seasons.

Morphological development is genetically linked to secondary metabolism (reviewed by Bayram and Braus 2012; Calvo et al. 2002; Calvo 2008). Fungal secondary metabolites play a diversity of roles, for example, as signaling molecules (Yim et al. 2007), virulence factors (Myung et al. 2012; Watanabe et al. 2003; Stanzani et al. 2005; Comera et al. 2007) or as part of a defense chemical arsenal against insect predators or other soil microbes competing for the same resources (Losada et al. 2009; König et al. 2013). Previously, *A. nidulans rtfA* was shown to be necessary for normal production of the mycotoxin ST, as well as the β-lactam antibiotic PN (Ramamoorthy et al. 2012). Similar to the *A. nidulans* study, our current work indicates that the *A. flavus rtfA* homolog also affects the biosynthesis of the secondary metabolite, AF. Production of AFB_1 was dramatically decreased in the *rtfA* mutant compared to the wild-type. Along with the effect of *rtfA* on AFB_1, our chemical analyses indicated that several unknown metabolites detected in wild-type cultures were not synthesized, or synthesized at different levels in the Δ*rtfA* strain with respect to the controls. This indicates that *rtfA* is not only a regulator of AFB_1 biosynthesis, but also governs the production of other secondary metabolites in *A. flavus*, possibly by an epigenetic control mechanism similar to that previously described in *S. cerevisiae* (Ng et al. 2003).

Our results revealed the *rtfA* affects the transcription of developmental regulators, such as *brlA*, *abaA*, and *nsdC* (Adams et al. 1998; Cary et al. 2012) as well as AF genes *aflR*, *ver1*, and *omtA*. Interestingly, the expression pattern of these genes was altered in the same manner in the absence of *rtfA*, showing an initial abnormal higher expression followed by a notable decrease with respect to the wild type over time. Biological processes are temporally and spatially regulated. This has been demonstrated in great detail in *A. nidulans* morphogenesis studies (Adams et al. 1998). It is likely that *rtfA* epigenetic regulation of morphogenesis and secondary metabolism contributes in setting these temporal and spatial parameters, and that abrogation of *rtfA* function could lead to abnormal expression patterns that delay/suppress the process they control. In yeast, Rtf1 has been described to be involved in silencing of some genome regions (Ng et al. 2003). Absence of *rtfA* in *A. flavus* could potentially cause this initial abnormal genetic derepression, followed by a rapid repression/blockage by an unknown epigenetic control system.

Furthermore, our study indicates that *rtfA* influences the expression of the global regulators *veA*, known to be functionally connected to *rtfA* in *A. nidulans* (Ramamoorthy et al. 2012), as well as *laeA*, encoding a well characterized VeA-interacting protein (Bayram et al. 2008; Chang et al. 2013). Like VeA, LaeA is involved in epigenetic regulation of secondary metabolism and development (Calvo et al. 2016; Amare and Keller 2014). The expression of *veA* and *laeA* also followed the same expression pattern as the genes described above. It is likely that the effect of *rtfA* on developmental and secondary metabolic genes is, at least in part, *veA*- and *laeA*-dependent, and that alterations in *veA*, *laeA*, and possibly *nsdC* expression caused by modifications of the *rtfA* locus could result in pleiotropic effects affecting many cellular processes, including development and secondary metabolism in *A. flavus*.

In conclusion, this study contributes to the characterization of the *rtfA* gene in the opportunistic plant pathogen *A. flavus*. This work revealed that *rtfA* is necessary for multiple functions, including normal vegetative growth, conidiophore structure, conidial production, formation of sclerotia, and resistance to light and temperatures stresses. Importantly, our study also indicated that *rtfA* regulates chemical development, such as the biosynthesis of the highly toxic and carcinogenic compound AFB_1, as well as the synthesis of other secondary metabolites. Furthermore, *rtfA* appears to have a broad regulatory scope in *A. flavus*, capable of modulating the expression of the global regulators such as *veA*, *laeA* and *nsdC* possibly through an as of yet uncharacterized role in epigenetic modification of chromatin in this agriculturally important fungus. For these reasons, *rtfA* shows potential as a genetic target for future strategies designed to reduce the incidence of mycotoxin contamination of food and feed crops destined for consumption by humans and animals.

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Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors."

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Conflict of interest All authors declare that they have no conflict of interest.

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