Ann Microbiol (2013) 63:763–769
DOI 10.1007/s13213-012-0531-7

ORIGINAL ARTICLE

5-Azacytidine inhibits aflatoxin biosynthesis in *Aspergillus flavus*

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Received: 14 February 2012 / Accepted: 9 August 2012 / Published online: 16 September 2012

Abstract Aflatoxins, mainly produced by *Aspergillus flavus* and *A. parasiticus*, are a group of potent mycotoxins with carcinogenic, hepatotoxic, and immunosuppressive properties. Many studies have been devoted to investigating their biosynthesis mechanism since they were discovered half a century ago. 5-Azacytidine (5-AC), a derivative of the nucleoside cytidine and an inactivator of DNA methyltransferase, is widely used for studies in epigenetics and cancer biology, and has also been used for studying secondary metabolism in fungi. In this study, 5-AC was applied to investigate its effect on the development and aflatoxin biosynthesis of *A. flavus*. The results indicate that 5-AC inhibits the ability to produce aflatoxin and also causes a fluffy aconidial phenotype. Further studies revealed that 5-AC affects gene expression of *A. flavus* to a limited degree, and the unique homolog of DNA methyltransferase gene (*DmtA*) expressed constitutively during different developmental stages of *A. flavus* irrespective of 5-AC. This work may provide some basic data to elucidate the role of 5-AC in aflatoxin biosynthesis and the development of *A. flavus*.

Keywords 5-azacytidine · Aflatoxin · *Aspergillus flavus* · Gene expression

Introduction

Aflatoxins (AF), a group of polyketide-derived furanocoumarins mainly produced by *Aspergillus flavus* and *A. parasiticus*, contaminate various food commodities and pose a serious health risk due to their hepatotoxic and immunosuppressive properties. Since the discovery of their detrimental effects on human health half a century ago, many studies have been devoted to investigating the mechanism of AF biosynthesis (Kensler et al. 2011).

With the rapid development of *Aspergillus* molecular genetics, many studies have reported that more than 25 AF biosynthesis pathway genes are clustered within a 70-kb DNA region in the *A. flavus* genome (Yu et al. 2004). Some genes located outside the pathway gene cluster have been found to play a part in AF biosynthesis, and numerous environmental factors are also closely related to AF biosynthesis (He et al. 2007).

Inhibitory compounds such as alkaloids, antibiotics, bioflavonoids, Ca^{2+} channel blockers, coumarins, flavonoids, and hydroxamic acids have been employed to decipher the mechanism of AF biosynthesis and can thus be used to identify regulatory networks that control the process (Holmes et al. 2008). Piperlongumine, isolated from *Piper longum* (Lee et al. 2002) and glyceollin (Song and Karr 1993) inhibits aflatoxin B_1 (AFB_1) production in *A. flavus*. β-Carotene inhibits AFB_1 biosynthesis of strain *A. flavus* KS7F 12/11 but not of other *A. flavus* strains, suggesting that the inhibition of AF biosynthesis differs within strains of the same species (Norton 1997).

5-Azacytidine (5-AC) is a derivative of the nucleoside cytidine that can be incorporated into DNA, resulting in the trapping and inactivation of DNA methyltransferase. It rapidly depletes cellular DNA methyltransferase and reduces the methylation level of the genomic DNA (Lyko and Brown 2005). Therefore, 5-AC has been widely used as a potent DNA methyltransferase inhibitor to study epigenetics, cancer biology (Lyko and Brown 2005), and secondary metabolism in fungi (Williams et al. 2008). Recent research reported that 5-AC inhibited production of AF in *A. parasiticus* (Wilkinson et al. 2011). However, previous research in our laboratory using bisulfite sequencing has

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reported that *A. flavus* is a species with no methylation (Liu et al. 2012), thus making it unclear whether the DNA demethylating drug 5-AC could play a role in AF biosynthesis of *A. flavus*.

To investigate the role of 5-AC in the development and AF biosynthesis in *A. flavus*, changes in phenotype, AF production, and gene expression of *A. flavus* treated by 5-AC were investigated. This work may pave a way for further understanding on AF biosynthesis of *A. flavus*.

Materials and methods

Fungal strain and culture conditions

Conidia of *A. flavus* NRRL 3357, a producer of aflatoxins B_1, B_2, G_1, and G_2, were collected from plates using 0.05 % Triton X-100. Then, 10^5 CFU conidia were inoculated on 3.9 % (w/v) potato dextrose agar medium (PDA; Difco) or Czapek Dox agar medium (CA, 30 g/L sucrose, 3 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4$ $7H_2O$, 0.5 g/L KCl, 0.01 g/L $FeSO_4$ $7H_2O$, 15 g/L agar) and cultured as a stationary culture. In the liquid medium culture, conidia (10^6CFU/mL medium) were inoculated in 2.4 % (w/v) potato dextrose broth (PDB; Difco) and cultured in a shaker at 200 rpm. All cultures were kept at 30 °C and kept away from light to avoid its effect on AF biosynthesis or 5-AC stability.

Detection of AFB_1

AFB_1 was detected using modified thin layer chromatography (TLC) as described by Hicks et al. (1997). Forty μL of chloroform was added into a 1.5-mL centrifuge tube containing 100 μL of *A. flavus* mycelium-free culture, shaken at 200 rpm for 20 min, and centrifuged at 2,000 rpm for 3 min. Twenty μL of the organic phase was transferred into a new tube, dried in a 70 °C water bath, and resuspended in 20 μL of acetone. Each extract was separated on a toluene:ethyl acetate:acetic acid (8:1:1, v/v/v) solvent system and was observed under long wave (365 nm) UV light.

Quantification of AFB_1

One hundred μL of chloroform was added into a 1.5-mL centrifuge tube containing 100 μL of *A. flavus* mycelium-free culture, and thoroughly vortexed. The organic phase was transferred into a new tube. The extracting process was repeated and the organic phase was collected and dried in a 70 °C water bath, and resuspended in 400 μL methanol-PBS (2:8, v/v). The ELISA assay of AFB_1 quantification was performed according to the manufacturer's instruction (BrinsBio, China).

Measure of the dry weight of fungal mycelia

Spores (10^7) were cultured at 200 rpm, at 30 °C for 3 days, then the fungal mycelia were obtained through filtered the culture adequately using vacuum filtration. The mycelia were roasted in an 80 °C oven for more than 6 h to wipe the water off completely, and the dry mycelia were weighed to evaluate the growth of the fungus.

Preparation of fungal total RNA

Total RNA was extracted from the fungal mycelia using a modified hot acid phenol method (Salter and Conlon 2007). A total of 2 g of the fine powder was promptly scraped into a 50-mL pre-cooled tube containing 15 mL extraction buffer (10 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 0.5 % (w/v) SDS) and 15 mL acid phenol. After incubation in the water bath at 65 °C for 15 min, the tube was thoroughly vortexed and centrifuged at 11,000 rpm at 4 °C for 10 min. The aqueous phase was then transferred into a pre-cooled tube with 15 mL acid phenol:chloroform:isoamylol (25:24:1, v/v/v), vortexed, and centrifuged at 10,000 rpm at 4 °C for 10 min. This step was repeated several times to completely remove the impurities. Finally, only the aqueous phase was transferred to a new pre-cooled tube, and isopropanol (100 %) was added to the tube at 1:1 (v/v) to precipitate RNA at −20 °C overnight. The mixture was centrifuged at 10,000 rpm at 4 °C for 15 min to collect the RNA. After washing with 70 % ethanol twice, the RNA was resuspended in DEPC-treated water. Three biological replicates were prepared for RNA preparation and RT-PCR.

RT-PCR

Reverse transcription was carried out with total RNA (treated with DNase) according to the M-MLV first-strand cDNA synthesis protocol (Invitrogen) for RT-PCR. Genes and corresponding primers used for the expression analysis are listed in Table 1. The PCR protocol used is as follows: an initial denaturing step of 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C (*aflE*, *aflR, aflW, laeA*) or 55 °C (other genes), and 1.5 min at 72 °C, and a final extension step at 72 °C for 10 min. Each PCR contained 10 μL of 2×HS PCR Reaction Mix (Dongsheng Biotech, China), 0.2 μL of Taq DNA polymerase, 0.8 μL of each primer (10 μM), and 1 μg of cDNA in a total volume of 20 μL. The housekeeping gene *tub-1* coding β-tubulin was chosen as the internal control. PCR products were visualized on a 1.0 % agarose gel.

Table 1 Gene-specific primers used for RT-PCR

Gene[a]	Accession no.	Primer sequence (5′-3′)		PCR product
aflC (pksA)	XM_002379910	FP	TTTCTTGACCAGTCCCACTATGT	1,176 bp
		RP	CCCTTTGTTTCGTGCTTTCC	
aflD (nor-1)	XM_002379908	FP	ACGGATCACTTAGCCAGCAC	812 bp
		RP	CTACCAGGGGAGTTGAGATCC	
aflE (norA)	XM_002379901	FP	GATGGCACAACGGCAGAAT	868 bp
		RP	TTCGATCGGCTGTTGCTTG	
aflG (avnA)	XM_002379896	FP	TCTACACGAGCAATCCGGAG	803 bp
		RP	TACCGGTGGATACAGACGCA	
aflI (avfA)	XM_002379893	FP	TATTCACTGGGGCCACTGGCT	885 bp
		RP	TAATGGCAAGCTCTCGGCG	
aflO (omtB)	XM_002379892	FP	GCCTTGACATGGAAACCATC	1,331 bp
		RP	CCAAGATGGCCTGCTCTTTA	
aflP (omtA)	XM_002379891	FP	GCCTTGCAAACACACTTTCA	1,241 bp
		RP	AGTTGTTGAACGCCCCAGT	
aflR (apa-2)	XM_002379905	FP	ATTCAACTCGGCGACCATCA	678 bp
		RP	TGCTCAGCAAGTAGCCATCC	
aflV (cypX)	XM_002379888	FP	CGCAAGATTCCTGGTCCC	812 bp
		RP	CCAGCTAGGAGCAACGC	
aflW (moxY)	XM_002379887	FP	GAAGACCGCGGAGAATGG	911 bp
		RP	GGCCCAATGACACTGCC	
aflY (hypA)	XM_002379885	FP	TGCCCTAGAGTTTCAACAGC	504 bp
		RP	ATCAGGTCAGAGCGTCCTTT	
aflYa (nadA)	XM_002379884	FP	CCAGCACTGTCAACCGAAAC	706 bp
		RP	GCCAGGTATCTTGCCACTCA	
laeA	XM_002374798	FP	TATCATGCGTACCGCAAAGG	759 bp
		RP	CGAAATGCGCCTGATTCTGT	
DmtA	XM_002378337	FP	ATGTCCTCAATCCAGGCTC	1,404 bp
		RP	TTACCCAGAAGCAATGACG	
Tub-1	M38265	FP	GCTTTCTGGCAAACCATCTC	1,198 bp
		RP	GGTCGTTCATGTTGCTCTCA	

[a]Alternative gene names, if any, are given in parentheses

Results

5-AC induces a “fluffy” phenotype of *A. flavus*

After conidia of *A. flavus* were inoculated on PDA plates with or without 1 mM 5-AC at 30 °C for 120 h, nearly all the 5-AC treated mycelia converted to a “fluffy” phenotype lacking green conidia (Fig. 1b). This “fluffy” phenotype remained even under extended incubation. However, the phenotype could not be propagated when the fluffy mycelia were transferred onto a fresh PDA medium without 5-AC, and some green conidia reappeared (Fig. 1d). A similar phenomenon was shown on Czapek-Dox agar (CA) medium (Fig. 1e~h).

5-AC prevents aflatoxin production of *A. flavus*

When a total number of 10^7 conidia were incubated in a 150-mL conical flask containing 30 mL of PDB with or without 1 mM 5-AC at 30 °C, 200 rpm, we found that 5-AC remarkably reduced AF production compared to the control through detection by TLC. Similar results were obtained in each of four separate experiments, each with three replicates cultured from 1 through 10 days. The levels of accumulated AF remained low through the 10th day of culture in the presence of 5-AC (Fig. 2a). However, the biomass showed that the growth of *A. flavus* was almost unaffected by 5-AC. The dry weight of fungal mycelia of 3-day culture without 5-AC was 81.4±6.8 mg, compared to 77.3±5.9 mg when 5-AC was present.

In order to quantify the inhibitory effect of AFB_1 by 5-AC, an ELISA assay was carried out. On the whole, AFB_1 accumulated steadily in the culture without 5-AC, and the concentration of AFB_1 reached 957 ng/mL after culture for 120 h (Fig. 2b). In contrast, the AFB_1 level accumulated in the culture with 5-AC treatment was almost invariably lower than 5 ng/mL from 0 through 120 h (Fig. 2b). A small amount of AFB_1 was brought in when spores were inoculated since there was an initial level of AFB_1 in the culture at time 0 h. This result is consistent with the TLC analysis shown in Fig. 2a.

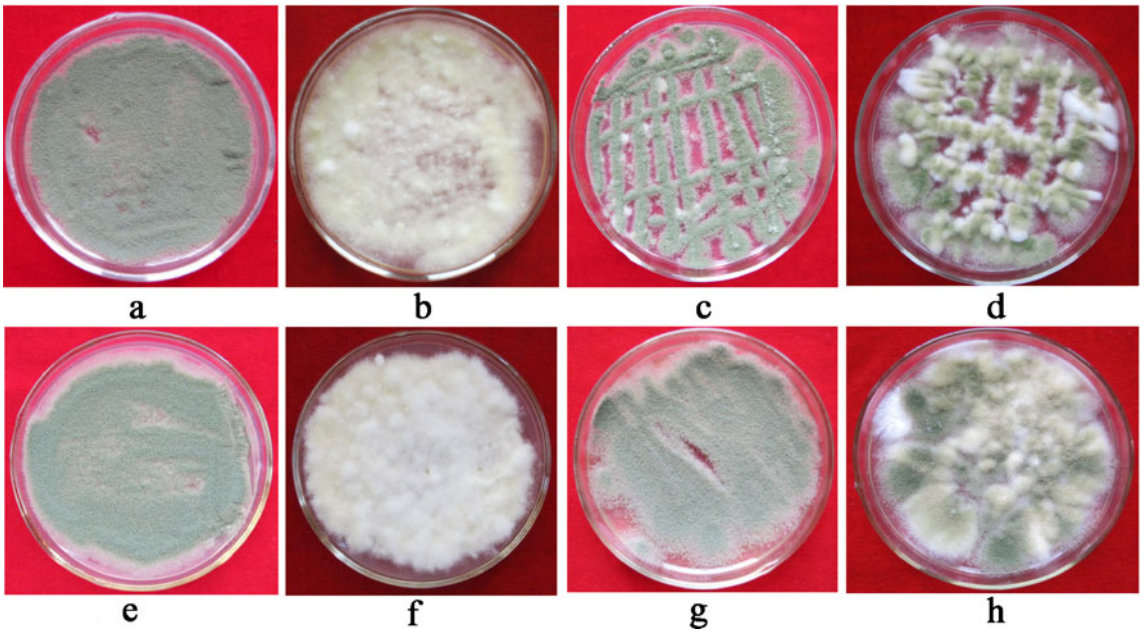

Fig. 1 "Fluffy" phenotype of *A. flavus* induced by 5-AC**. a**–**d** PDA medium; **e**–**h** CA medium. **a**, **e** Without 5-AC; **b**, **f** with 1 mM 5-AC; **c**, **d** propagation cultures of (**a**) and (**b**) on basic PDA medium, respectively; **g**, **h** propagation cultures of (**e**) and (**f**) on basic CA medium, respectively

5-AC has limited effects on gene expression of *A. flavus*

To investigate whether the inhibitary effect of 5-AC on the AF production is due to its disturbance to the expression of genes related with AF biosynthesis, the expression pattern of 72-h-old mycelia was investigated by RT-PCR. A total of three separate RT-PCR measurements were carried out with RNA from independent

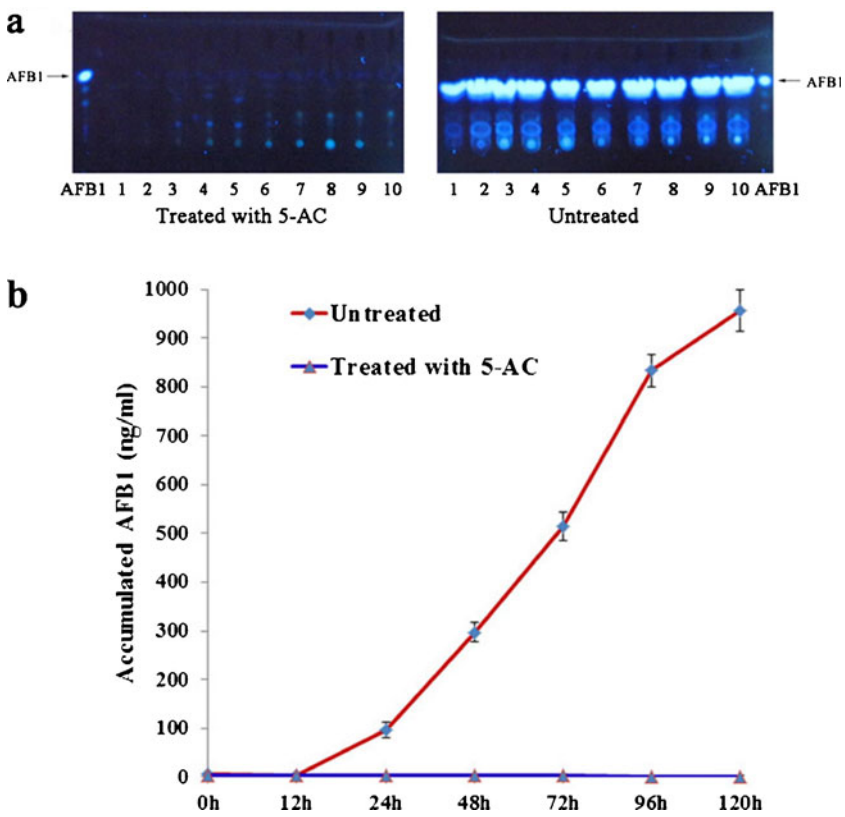

Fig. 2 The inhibitory effect of AF biosynthsis in *A. flavus* by 5-AC. **a** *Left* PDB medium plus 1 mM 5-AC; *right* PDB medium without 5-AC. *Lanes 1–10* represent culture time (days). *AFB1* aflatoxin B_1 (Alexis). **b** Quantification of aflatoxin in *A. flavus* culture by ELISA. The data are the mean ± SE from *n*= 2 samples for each time point from three separate experiments

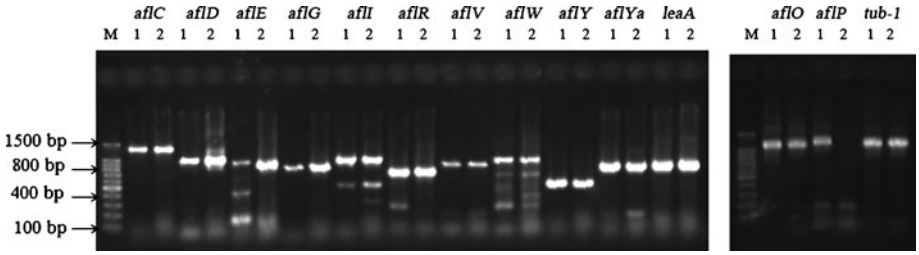

Fig. 3 Effect of 5-AC on gene expression of *A. flavus*. *M* DNA maker; *1* and *2* represent untreated or treated with 5-AC, respectively

mycelia with and without 5-AC treatment, to ensure the reproducibility of results. Amongst 13 genes tested, 12 are involved in the AF biosynthetic pathway and 1 is the secondary metabolism pathway regulator gene *laeA* (Table. 1). The results showed that only the expression of *aflP*, a gene that encodes the O-methyltransferase A, was suppressed by 5-AC. The other genes detected in this experiment, including the AF pathway regulatory gene *aflR*, the global secondary metabolism pathway regulator gene *laeA,* and the 10 AF biosynthesis pathway genes (Table 1), did not show significant change in expression in response to 5-AC (Fig. 3).

O-methyltransferase, one of the 25 genes located in the AF biosynthetic pathway gene cluster and encoded by *aflP* gene, is responsible for the conversion of sterigmatocystin (ST) to methylsterigmatocystin and dihydrosterigmatocystin to dihydro-O-methylsterigmatocystin (Yu et al. 2004). However, further experiments found that the precursor ST does not accumulate when *A. flavus* is treated with 5-AC (data not shown).

Expression of the *DmtA* homolog is constant irrespective of 5-AC

Extensive literature mining and investigation into the genome of *A. flavus* indicated that only one DNA methyltransferase homolog exists in the genome of this fungus (Liu et al. 2012). In order to investigate if the unique homolog of *DmtA* in *A. flavus* plays a role in AF biosynthesis, RT-PCR was performed to study its expression pattern during different developmental stages, with or without 5-AC treatment. We found that the expression level of this homolog showed no difference among conidia, 12-h-, or 72-h-old mycelia without 5-AC, or 72-h-old mycelia treated with 5-AC (Fig. 4). This constitutive expression throughout all developmental stages suggests that the homolog of *DmtA* in *A. flavus* is not affected by the presence of 5-AC.

Discussion

A number of experiments applying 5-AC have indicated that this well-known DNA methylation inhibitor may be a regulator in fungal secondary metabolism. Ten out of 12 fungi studied by Williams et al. (2008), including *A. flavus* and *A. westerdijkiae,* are responsive to 5-AC and produce new or enhanced levels of secondary metabolites. *N. crassa* treated with 5-AC at concentrations lower than 30 μM overproduces carotenoids and suppresses its carotenoid levels at higher concentrations (100 and 300 μM) (Kritsky et al. 2002). Our findings in this paper show that 5-AC has the ability to reduce AF production in *A. flavus*, which is similar to what Wilkinson et al. (2011) found in *A. parasiticus*.

Relationships exist between a "fluffy" colony phenotype and second metabolism in the *Aspergillus* family. *A. niger*, *A. nidulans*, and *A. fumigatus*, when treated with 5-AC, developed an inheritable "fluffy" phenotype and the overproduction of various enzymes (Tamame et al. 1983a; Ben-Ami et al. 2010). Tamame et al. considered that 5-AC induced the *A. nidulans* "fluffy" mycelia phenotype through the mutagenic effect on the 5-AC-sensitive gene *fluF*, which is located on the right arm of chromosome VIII (Tamame et al. 1983b, 1988). Ben-Ami et al. found that the transcriptional profiling of the 5-AC-induced *A. fumigatus* "fluffy" variant showed differential expression of multiple genes involved in G-protein signaling (Ben-Ami et al. 2010). Our research showed that 5-AC converted the mycelia of

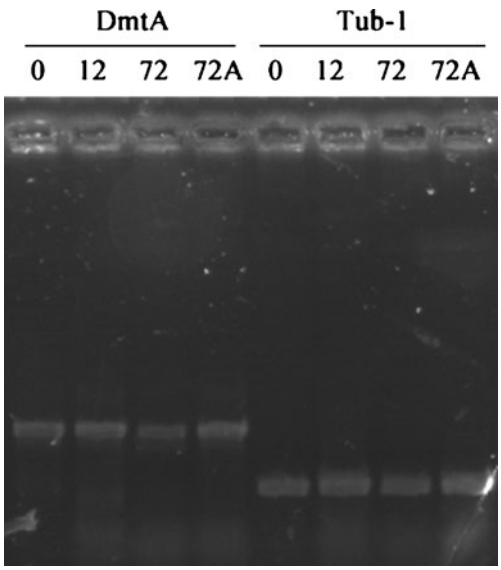

Fig. 4 The expression of homolog of *DmtA* in *A. flavus*. *0* Conidia; *12* 12-h-old mycelia; *72* 72-h-old mycelia; *72A* 72-h-old mycelia cultured with 5-AC

A. flavus to a "fluffy" phenotype. However, our work does not provide information on the relationship between the fluffy phenotype and AF production.

Wilkinson et al. (2011) considered that loss of the ability to produce AF in *A. parasiticus* treated by 5-AC is not a result of transcription inhibition of AF biosynthesis genes or translation of the mRNA, and that the most likely explanation for the non-aflatoxigenicity of the treated cultures is their inability to form the proper vesicle structure for coordinated enzymatic conversion of AF precursors to stable metabolites. Our results in *A. flavus* showed that, within 13 genes tested, only the expression of *aflP* gene, which encodes the O-methyltransferase A protein, was suppressed to a significant degree by 5-AC. However, the precursor ST was not found to accumulate in our experiment. These observations lead us to propose that the incapacity of AF production in *A. flavus* treated by 5-AC is not a result of blocking-up of transcription in the AF biosynthesis pathway genes or the global secondary metabolism pathway regulator genes. The role of 5-AC in the inhibition of AF production in *A. flavus* needs further studies.

Our previous work using bisulfite sequencing indicates an absence of DNA methylation in *A. flavus* (Liu et al. 2012), so there is no possibility that 5-AC suppresses AF biosynthesis as a demethylating drug. How does 5-AC affect AF production of *A. flavus*? In addition to its role as a DNA methyltransferase inhibitor, 5-AC has also been proposed to be a histone methylation regulator (Wada et al. 2005; Komashko and Farnham 2010). When an *Inf1* gene-silenced strain of *Phytophthora infestans* is treated with 5-AC, the *inf1* gene is reactivated and its chromatin is remodeled to a less condensed state (van West et al. 2008). Since no DNA methylation in this gene was detected by restriction analysis or BS-PCR-Seq, the authors concluded that 5-AC is a histone methylation inhibitor instead of a DNA methylation inhibitor. Similarly, we suggest that alteration of the phenotype and inhibition of aflatoxin production by 5-AC in *A. flavus* is likely achieved through reducing histone methylase activity or altering chromatin structure.

We have found that the DNA methyltransferases possessed by the *Aspergillus* members are closely related to the repeat-induced point-mutation defective (RID) of *Neurospora* and the Masc1 of *Ascobolus immersus*. The DmtA of the *Aspergillus* members might not be a true DNA methyltransferase, but may possibly be an enzyme responsible for repeat-induced point-mutation (RIP) (Liu et al. 2012). Further research in this study confirmed that the unique homolog of the DNA methyltransferase gene *DmtA* in the genome of *A. flavus* is actively expressed throughout the developmental stages of *A. flauvs,* even in the presence of 5-AC. The constitutively expressed *A. flavus DmtA* homolog may be correlated with RIP mutation and is not responsible for the methylation of genomic DNA in *A. flavus*.

Acknowledgements This work was supported by National Natural Science Foundation of China (Grant no. 31170044 and 30870024) and Science and Technology Planning Project of Guangdong Province, China (grant no. 2011B020305005). We thank Prof. T.W. Hill (Rhodes College, Memphis, TN, USA) for suggestions and amendments to the paper.

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