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Fusion expression and anti-*Aspergillus flavus* activity of a novel inhibitory protein DN-AflR

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ABSTRACT

The regulatory gene (*aflR*) encodes AflR, a positive regulator of transcriptional pathway that activates aflatoxin biosynthesis. It has been demonstrated in our laboratory that L-Asp-L-Asn (DN) extracted from *Bacillus megaterium* inhibited the growth of *Aspergillus flavus*. We fused gene encoding DN with the gene encoding specific dinuclear zinc finger cluster protein of AflR, then fusion protein competed with the AflS-AflR complex for the AflR binding site and significantly improved anti-*A. flavus* activity (growth of *A. flavus* and biosynthesis of aflatoxin B_1) of DN. The fusion gene *dn-aflR* was cloned into pET32a and recombinant plasmid was introduced into *Escherichia coli* BL21. The highest expression was observed after 10 h induction and fusion protein was purified by affinity chromatography column. Compared with DN, the novel fusion protein DN-AflR significantly inhibited the growth of *A. flavus* and biosynthesis of aflatoxin B_1 ($P < 0.05$). This study promoted the use of competitive inhibition of fusion proteins to reduce the expression of regulatory genes in the biosynthetic pathway of aflatoxin. Moreover, it provided more supports for deep research and industrialization of such novel anti-*A. flavus* bio-inhibitors and biological control of microbial contamination.

1. Introduction

Aflatoxin is one of the most potent naturally occurring toxic and carcinogenic compounds, which is a mycotoxin that poses serious threats to human health (Cleveland et al., 2008). Aflatoxin contamination has serious influence on export of agricultural products, income of farmers and economic development (Atehnkeng et al., 2015; Wu and Hasan, 2012). Biological methods, especially use of antagonistic microorganisms to inhibit aflatoxin biosynthesis gradually became hot spot in recent years (Mishra and Das, 2003). Palumbo et al. isolated one strain of *Bacillus* from almonds, which inhibited the growth of *Aspergillus flavus* (Palumbo et al., 2006). Early studies found that *Mycobacterium smegmatis* and *Rhodococcus erythropolis* produced F420 H2-dependent reductases to degrade aflatoxin (Lapalikar et al., 2012; Taylor et al., 2010;). Some studies isolated antifungal compounds from *Bacillus* and verified their inhibitory effects on growth of *A. flavus* (Bottone and Peluso, 2003; Zhang et al., 2008). Above all, antagonistic microorganisms produce metabolites or enzymes to inhibit expression of regulatory genes, or degrade aflatoxin.

Aflatoxin biosynthetic pathway has been studied for years and is one of the best understood fungal secondary metabolic pathways. The whole-genome sequencing of *A. flavus* has been accomplished, and we could better control aflatoxin contamination through deep research of the regulatory genes and mechanisms. For example, a strain of *Bacillus megaterium* reduces the amount of aflatoxin synthesis by inhibiting expression of some aflatoxin biosynthesis-regulated genes (Kong et al., 2010). Up to now, at least 34 genes have been identified as members of the aflatoxin pathway gene cluster (Cleveland et al., 2009). On the 70-kb biosynthetic gene cluster of aflatoxin, *aflR* and *aflS* (formerly known as *aflJ*) are genes involved in pathway regulation (Yu et al., 2004). *aflR* is necessary for transcription of some genes in *Aspergillus* gene cluster (Chang et al., 1995; Matsushima et al., 2001; Woloshuk et al., 1994). This gene encodes a specific DNA binding protein (AflR) containing 444 amino acids, and the 29th to 56th amino acids constitute a binuclear zinc finger cluster protein with sequence Cys-Xaa2-Cys-Xaa6-Cys-Xaa6-Cys-Xaa2-Cys-Xaa6-Cys, which is the key region of AflR to activate gene transcription and determines AflR-binding specificity (Burger et al., 1991). AflR possesses typical DNA-binding domain of the GAL4-type positive regulatory protein family in fungi (Ehrlich et al., 1999). A common feature of fungal gene clusters, including those for secondary metabolites, is the presence of specific regulatory genes, which have been found to encode members of the zinc binuclear cluster protein family typified by GAL4 (Giniger et al., 1985; Marmorstein et al., 1992; Todd and Andrianopoulos, 1997). AflS is found to be involved in the regulation of transcription (Chang, 2003). Between *aflR* and *aflS* is intergenic region, where promoter region is located. AflS-AflR complex

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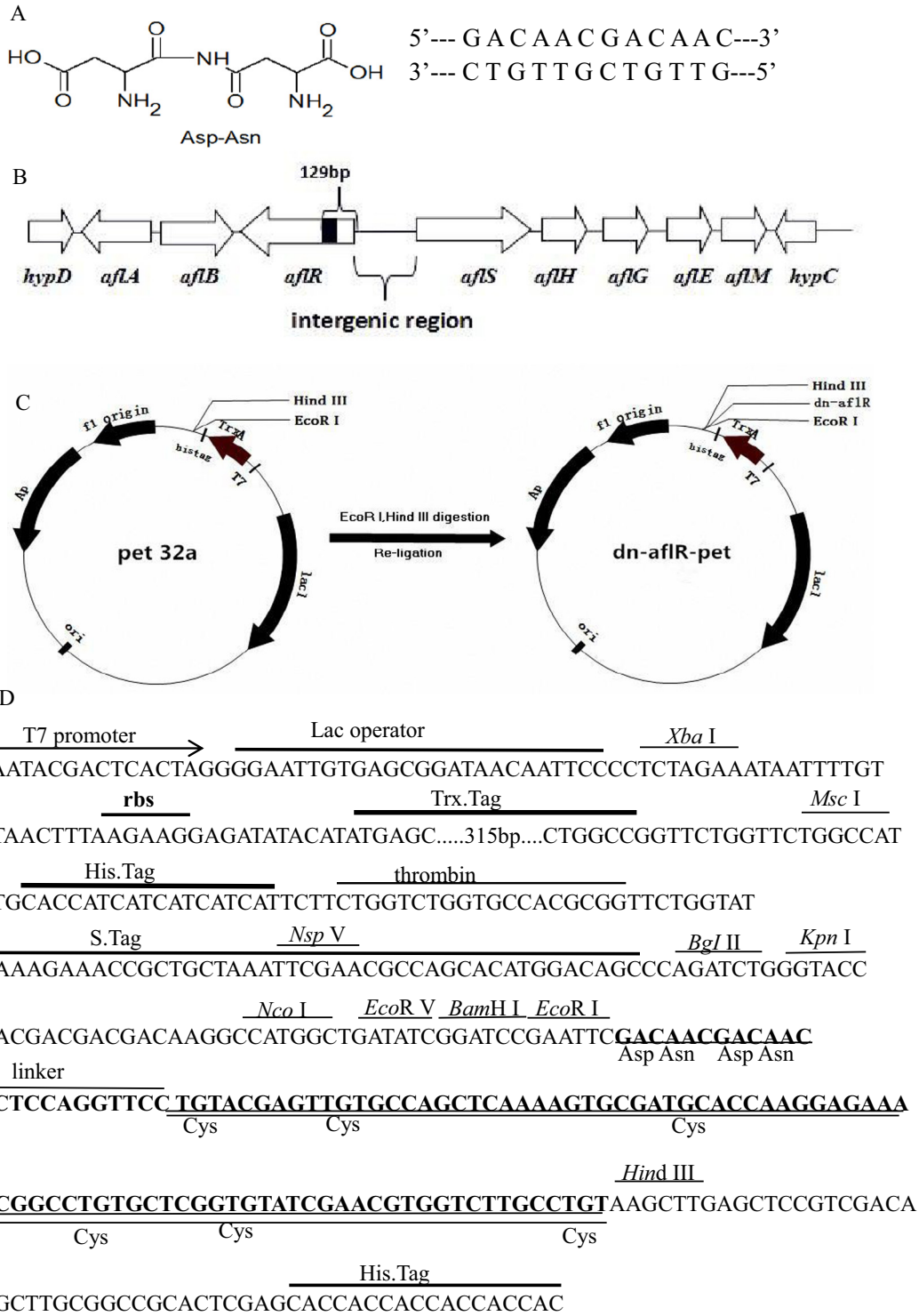

Fig. 1. Molecular modified fragments and characteristics of *E. coli* expression plasmid dn-aflR-pET32a. (A) The chemical formula and base sequences of DN. (B) The aflatoxin biosynthetic pathway and clustered genes, AflR binding site (black area) and intergenic region. (C) Construction of expression plasmid. DNA fragment was digested with Hind III and EcoR I, and inserted into downstream of the thioredoxin (Trx) and then ligated with T4 DNA ligase. DN-AflR was expressed as a fusion protein with Trx and 6His. Ar, an ampicillin resistance gene. (D) Base composition of the inserted fusion gene (thick base) containing genes encoding DN (under line) and zinc finger cluster protein (double line). 6His tag, Trx tag and unique restriction sites (over line) were also indicated.

binds to AflR binding site and activates aflatoxin biosynthesis. The decrease in the amount of aflatoxin biosynthesis is mainly through inhibiting the expression of *aflR* or *aflS* (acting on the intergenic region), preventing the formation of a potent AflS-AflR complex and inhibiting activation of aflatoxin biosynthetic pathway (Cleveland et al., 2009).

Recently, L-Asp-L-Asn (DN) which was identified from *Bacillus megaterium* has robust inhibitory effect on *A. flavus* growth and aflatoxin B_1 production (Chen et al., 2019). To improve the inhibitory effect of DN, we transformed the genes encoding DN and GAL4-type zinc finger cluster protein (specifically binds to the AflR binding site) by gene fusion. We hypothesized the fusion protein could compete with AflS-AflR complex by acting on AflR binding sites to inhibit the

activation of AflS-AflR complex and improve anti- *A. flavus* activity of DN.

2. Materials and methods

2.1. Materials, strains, and culture conditions

A. flavus NRRL 3357, preserved in School of Food Science and Engineering, Ocean University of China, was maintained at 4 °C on potato dextrose agar (PDA; Bio-way technology, Shanghai, China). For liquid culture, *A. flavus* was transferred into 250 ml Erlenmeyer flask containing 100 ml of minimal glucose medium (MM) at 28 °C. *Escherichia coli* DH5α and *E. coli* BL21 (Ruibio Biotech, Beijing, China) were used as hosts for plasmid amplification and gene expression, respectively. *E. coli* was grown in LB medium (10 g/l Tryptone, 5 g/l Yeast Extract, 10 g/l NaCl) containing 50 μl/ml ampicillin (Solarbio, Beijing, China) at 37 °C with shaking at 180 rpm.

2.2. Effects of DN on mycelial morphology of A. flavus

2.2.1. Preparation of spore suspension

Spores of *A. flavus* were washed off with sterile distilled water containing 0.1% Tween-80 from PDA medium and filtered with a cotton slag to prepare spore suspension. The number of spores was counted by hemocytometer and diluted to the desired concentration.

2.2.2. TEM and SEM

0.8 mg/ml DN (synthesized in GL Biochem, Shanghai, China) and 10^5 spores/ml were added in 50 ml MM medium and cultivated at 28 °C with 180 rpm for 48 h. Control group didn't contain DN. Then the mycelia were fixed with 2.5% glutaraldehyde, dehydrated in ethanol and embedded in an epoxy resin to be observed by Transmission Electron Microscope (TEM; JEOL, Tokyo, Japan) (Vigneshwaran et al., 2007), and the mycelia were also observed by Scanning Electron Microscope (SEM; JEOL, Tokyo, Japan) after dispersion by ultrasonic wave (Theis et al., 2005).

2.3. Expression and identification of fusion protein

2.3.1. Expression vector and its construction and transformation into E. coli BL21

The fusion gene *dn-aflR* containing genes encoding DN and zinc binuclear cluster protein (Cys-Xaa2-Cys-Xaa6-Cys-Xaa6-Cys-Xaa2-Cys-Xaa6-Cys, based on AAM03003.1, NCBI) was synthesized by Hongxun Biotech (Suzhou, China), and amplified with PCR (forward primer 5'-GAATTCGACAACGACAACT-3′, reverse primer 5′- AAGCTTACAGGCA AGACCA-3′). The GAATTC of restriction site EcoR I was sequentially inserted into the 5′-end of the fusion gene, and AAGCTT of the restriction site Hind III was sequentially added to the 3′-end. Modified gene was obtained from the Plasmid Mini Kit (OMEGA, Omega bio-tek, Shanghai, China). Restriction enzymes Hind III and EcoR I were used to digest the *dn-aflR* and pET32a (+), then the digested fragments were separated and identified on 1% agarose gel and recovered from gel by using Agarose DNA Extraction Kit (OMEGA, Omega bio-tek, Shanghai, China). The digested fragments were ligated at 4 °C with T4 DNA ligase (Thermo Fisher Scientific, New York, USA) (Fig. 1). Then recombinant plasmid dn-aflR-pET32a was transformed into *E. coli* DH5α and single colony was picked for colony PCR. The recombinant plasmid extracted from suspension and empty plasmid were both transformed into *E. coli* BL21 by heat shock, and cultivated on solid LB medium containing 50 μg/ml ampicillin (Sambrook et al., 2001). After 16 h, single colony was inoculated into LB liquid medium and sent to Ruibio Biotech (Beijing, China) for DNA sequencing.

2.3.2. Protein expression and analysis

A single colony (containing dn-aflR-pET32a) was inoculated into 10 ml LB medium and cultured at 37 °C. Then overnight culture was inoculated into fresh 300 ml LB media in 1:5 ratio. When optical density (OD600) reached 0.6, the final concentration of 0.8 mM IPTG was added to induce the expression. After 10 h induction at 16 °C under 160 rpm, the cells were washed with buffer (20 mM Tris, 30 mM NaCl, 10 mM imidazole, pH = 7.5) and then lysed by sonication (JY92-IIN, Ningbo Scientz Biotech, Ningbo, China; ultrasonic power 300 w, ultrasound work 4 s, stop 6 s, 60 times). Supernatant was collected and identified by SDS-PAGE.

2.3.3. Protein purification

The presence of His tag in the recombinant protein meant that the purification was performed with Ni^{2+}-NTA affinity chromatography. Briefly, samples were passed through a column and extensively washed with the elution buffer (100 mM Tris base, 500 mM NaCl, and 200 mM Imidazole, pH = 8). Purified fusion protein was demonstrated by SDS-PAGE and relative quantity was determined by Braford protein assay at a wavelength of 595 nm by standard protein BSA (bovine serum albumin) (Solarbio, Beijing, China).

2.4. Effects of DN and DN-AflR on growth of A. flavus

2.4.1. Anti-A. flavus activity

Fusion protein was evaluated by agar disk diffusion experiment. The diameter of inhibition zone indicated the anti-*A. flavus* activity. 0.1 ml spore suspension (10^5 spores/ml) was coated on PDA plate and Oxford Cups filled with 100 μl DN (600 μg/ml) and DN-AflR (600 μg/ml) were put on it, respectively. The Oxford Cup on control plate was filled with 100 μl sterile water.

2.4.2. MIC and MFC

A series of solutions containing DN-AflR or DN at the concentrations of 30, 50, 100, 150, 400, 600 and 900 μg/ml were prepared by serial dilution. 200 μl of above solutions was added into each well of 96-well plates containing PDA medium, respectively. Pure PDA without DN-AflR and DN was the control. Then 5 μl suspension (10^5 spores/ml) was added to each well and placed at 28 °C for 48 h (Wiegand et al., 2008). By definition, the minimum concentration of compound that completely inhibited growth of *A. flavus* was the minimum inhibitory concentration (MIC) (non-visible *Aspergillus* hyphae). The culture was further incubated for 8 days to get the MFC, while the lowest fungicidal concentration (MFC) was the lowest concentration which no spore could germinate at.

2.5. Effects of DN and DN-AflR on AFB_1 biosynthesis

DN or DN-AflR (final concentrations of 30, 60 and 90 μg/ml) were added into 30 ml MM medium, respectively, and 100 μl of suspension (10^5 spores/ml) was inoculated to each group and incubated at 28 °C with 200 rpm. Each experiment was repeated 3 times. After culturing for 48 h, samples were centrifuged at 8000 *g* for 20 min at room temperature. Five milliliters of supernatant were diluted with 5 ml ultrapure water. Next, 5 ml of the diluted sample was extracted in immune affinity columns (Huaan Magnech BioTech Co., Ltd., Beijing, China) and then eluted with 1 ml of methanol at a flow rate of 1 drop per second. The eluent was evaporated under a gentle stream of nitrogen at 45 °C up to dryness condition, and then derivatized with 200 μl hexane and 100 μl trifluoroacetic acid (TFA) for 15 min. After being evaporated to dryness again, the eluent was redissolved in 200 L water–acetonitrile (85:15, v/v).

AFB_1 was analyzed according to retention time in HPLC system equipped with a ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5um, Agilent, Palo Alto, CA, USA) and a 470 fluorescent detector (G1321A, Agilent, USA) (λexc 360 nm; λem 440 nm) using a mobile phase solvent of 10% acetonitrile, 40% methanol, and 50% water. The flow rate was 0.8 ml min^{-1} and injection volume was 20 μl (Zhou et al.,

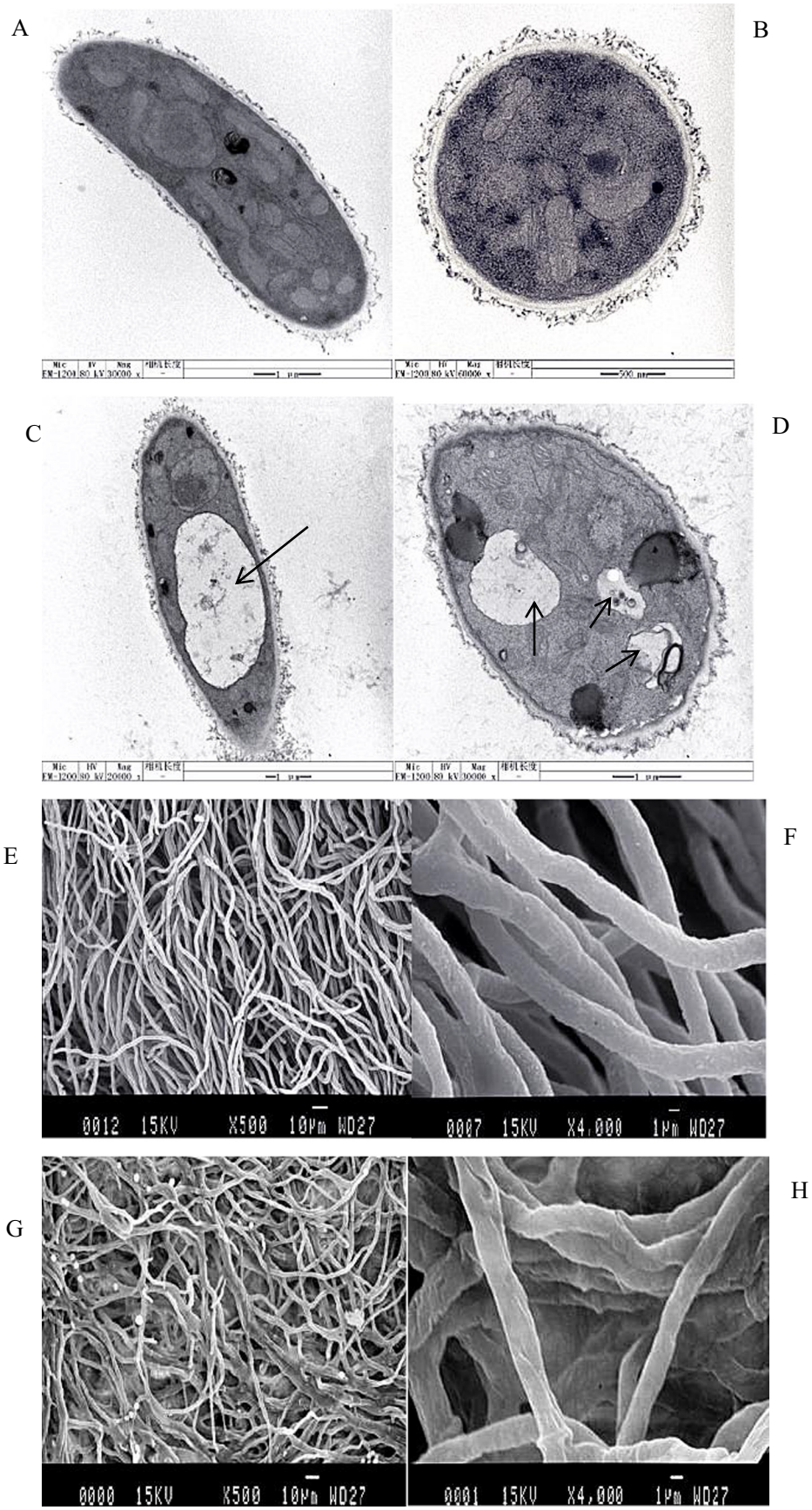

Fig. 2. Effects of DN on mycelia morphology of *A. flavus*. (A), (B), (C), (D): TEM; (E), (F), (G), (H): SEM; (A), (B), (E), (F): Control groups; (C), (D), (G), (H): DN treatment groups.

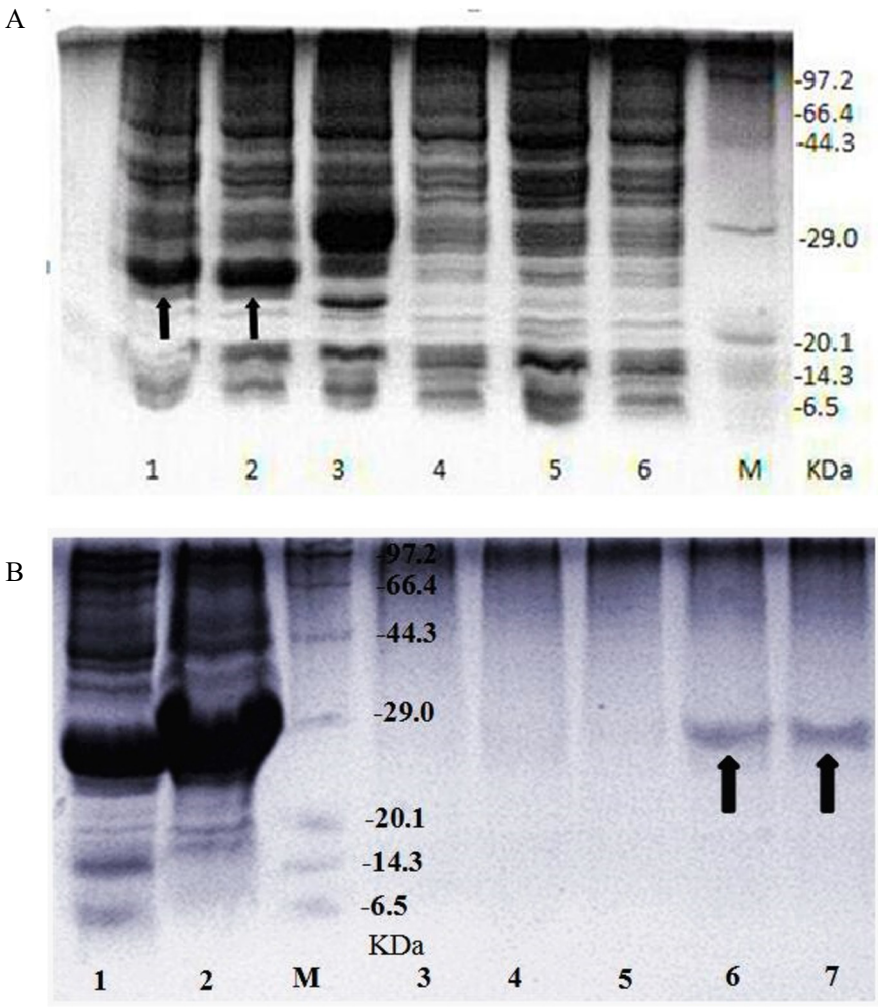

Fig. 3. SDS-PAGE analyzed the expression of DN-AflR. (A) M: molecular weight marker (Takara). Lanes 1 and 2: protein bands of *dn-aflR*-pET32a expression. Lane 3: protein band of empty pET32a expression. Lanes 4, 5 and 6: negative control (before inducted). The position of the fusion protein was indicated with an arrowhead. (B) Analysis of purified fusion protein DN-AflR. M: marker. Lane 1: expressed supernatant after ultrasonic breaking. Lane 2: precipitate after 8 mM urea dissolved. Lanes 3, 4 and 5: 50 mM imidazole buffer band, 100 mM imidazole buffer band and 300 mM imidazole buffer band, respectively. Lanes 6 and 7: 200 mM imidazole elution buffer band. The position of the purified protein was indicated with an arrowhead.

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2.6. Statistical analysis

All data were presented as mean ± S.D. One-way analysis of variance (ANOVA) and Duncan's multiple comparison test were used and carried out with SPSS software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of DN on mycelia morphology of A. flavus

3.1.1. TEM

In control group, the internal structure of cells grew normally and various organelles were clearly visible (Fig. 2A and B). However, cell structures of mycelia treated by DN were obviously abnormal: organelles were degenerated. Vacuole showed by black arrow in Fig. 2C and D became significantly larger, expanded and fractured.

3.1.2. SEM

The mycelia of control groups were straight, neatly arranged, and intact and smooth (Fig. 2E and F). While the mycelia treated with DN were abnormal, and the morphology of mycelia was deformed and became uneven thickness and partially broken (Fig. 2G and H).

3.2. Expression and identification of DN-AflR

The expected DNA and vector fragments were observed by 1% agarose electrophoresis after digesting with EcoR I and Hind III. Colony PCR and DNA sequencing showed that target fragment (*dn-aflR*) was successfully inserted into the vector. The molecular weight of empty plasmid (Trx-His-pET32a) and fusion protein (Trx-His-DN-AflR) were predicted to be 19 kDa and 22 kDa, respectively. Expression vector with fusion gene had a distinct protein band (24 kDa, Fig. 3A, lanes 1 and 2). The empty plasmid did not express the band at the corresponding position and had its own specific band (Fig. 3A, lane 3). Results illustrated successful expression of the fusion protein DN-AFLR. The supernatant after sonication (Fig. 3B, lane 1) and the precipitate after 8 mM urea dissolution (Fig. 3B, lane 2) showed distinct protein bands, which were consistent with the predicted molecular weight of the expected protein (Fig. 3A). There were no obvious protein bands in wash buffer (Fig. 3B, lanes 3–5), and there was only one obvious protein band in elution buffer (Fig. 3B, lanes 6 and 7), which corresponded to the target protein. ImageJ software (https://imagej.nih.gov/ij/) was used to compare the density of the bands on the gel, the fusion protein accounts for approximately 57.4% of the total cellular protein; the relative content of fusion protein was about 600 μg/ml.

3.3. Inhibitory effect of DN-AflR on growth of A. flavus

Plate treated by DN had only a small inhibition zone of 5.0 ± 0.2 mm (Fig. 4A, plate a). The control group (Fig. 4A, plate c) and 200 mM imidazole treated group (Fig. 4A, plate b) showed no

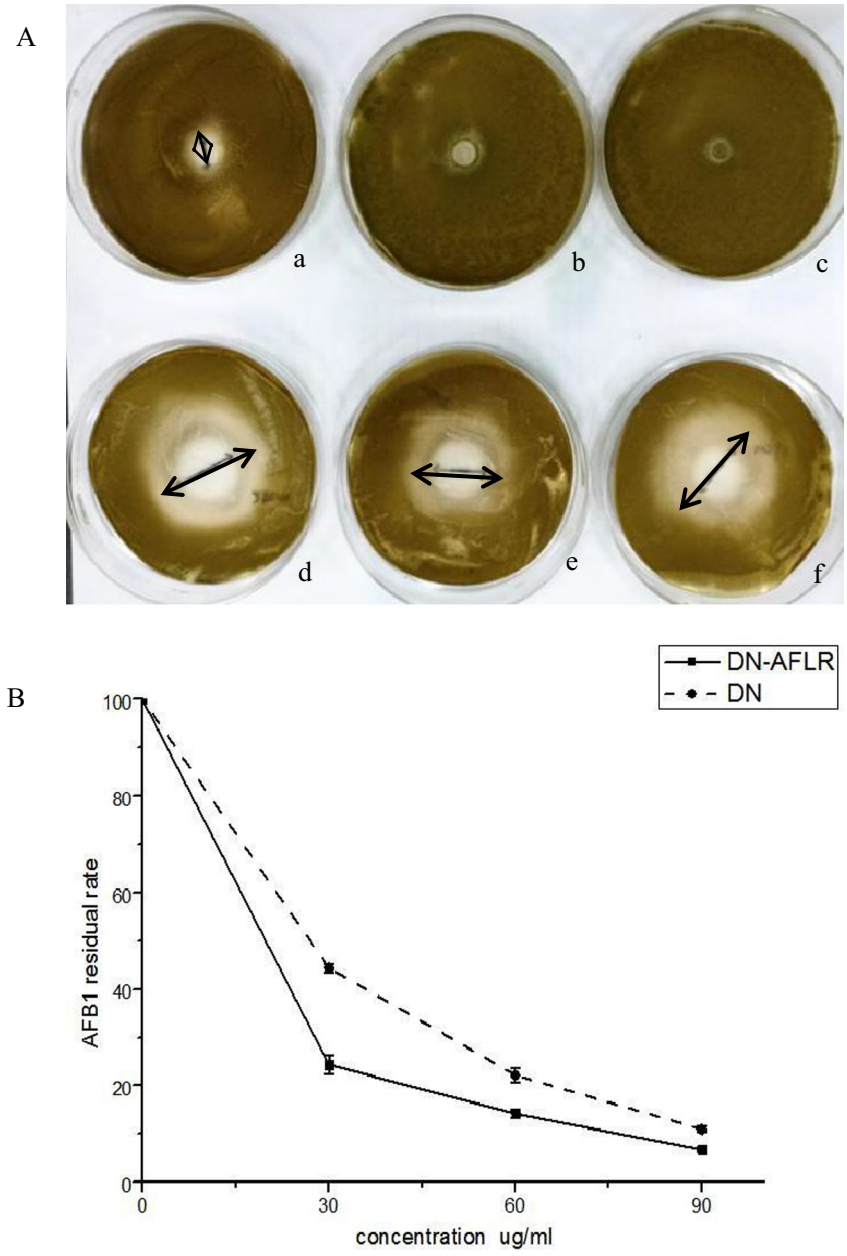

Fig. 4. Anti- *A. flavus* activity and degradation of AFB_1. (A) Plate a: treated with DN. Plate b: treated with 200 mM imidazole. Plate c: control group with no treatment. Plates d, e and f: treated with DN-AflR, the circle diameters of them were around 24, 19 and 21 mm, respectively. (B) The abscissa represented the final concentration of DN (dotted line) and DN-AflR (solid line), the ordinate represented the percentage residual AFB_1 (without any treatment as 100%). The final concentrations of inhibitory proteins in the three experimental groups were 30, 60 and 90 μg/ml, respectively.

Table 1
The MICs and MFCs of fusion protein DN-AflR and DN.

	Concentrations (μg/ml)							MIC (μg/ml)	MFC (μg/ml)
	30	50	100	150	400	600	900		
DN-AflR	+	+	+	−	−	−	−	150	400
DN	+	+	+	+	+	−	−	600	> 900
Negative control	+	+	+	+	+	+	+	/	/

Negative control: no inhibitory protein.
+: observed fungal growth.
−: no fungal growth observed.
/: no inhibition.

Table 2
The basic properties prediction of DN-AflR.

Number of amino acids	36
Theoretical pI	8.33
Formula	$C_{146}H_{250}N_{50}O_{54}S_6$
Total number of atoms:	506
Aliphatic index	40.83
Total number of negatively charged residues (Asp + Glu)	4
Total number of positively charged residues (Arg + Lys)	6
Grand average of hydropathicity (GRAVY)	−0.581

Basic informations were analyzed by ExPASy (http://web.expasy.org/protparam/).

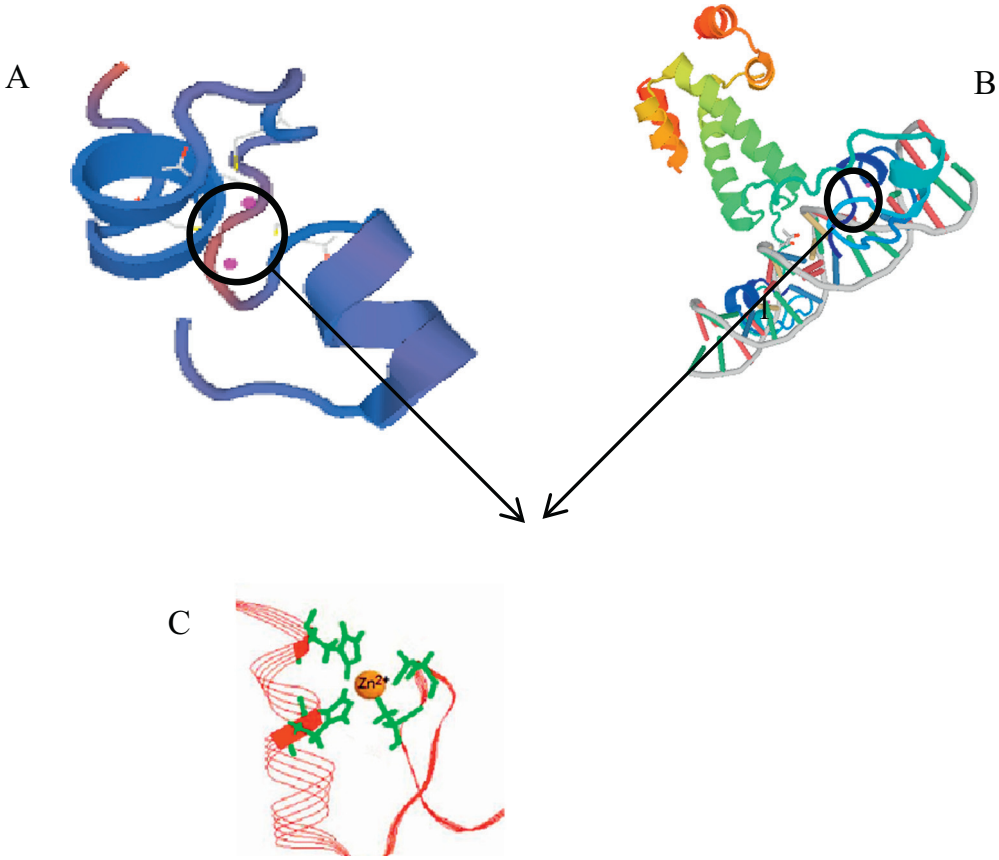

Fig. 5. (A) Spatial structure prediction of DN-AflR. (B) Structure of GAL4. Marked position in circle: zinc finger structure. (C) Zinc finger structure.

significant zone of inhibition. Diameter of inhibition zone treated with fusion protein was as high as 21.3 ± 0.2 mm (Fig. 4A, plates d-f). Inhibitory ability of DN-AflR was much stronger than that of DN. The MICs of DN-AflR and DN were 150 μg/ml and 400 μg/ml, respectively. MFC of DN-AflR was 400 μg/ml. Results of two repeated experiments were consistent (Table 1). The MIC and MFC of fusion protein were lower than that of DN, indicating that the fusion protein had stronger inhibitory effect on growth of *A. flavus* at a lower concentration.

3.4. Inhibitory effect of DN-AflR on aflatoxin B_1 biosynthesis

Two groups of experiments were performed using DN-AflR and DN with final concentrations of 30, 60, and 90 μg/ml. The percentage of residual AFB_1 after fusion protein treatment was 24.37%, 14.21% and 6.74%, respectively, and the percentage of residual AFB_1 after DN treatment was 44.39%, 22.19% and 10.99%, respectively (Fig. 4B). Compared with DN, fusion protein had stronger inhibitory effect on biosynthesis of aflatoxin B_1 ($P < 0.05$). At 30 μg/ml of DN-AflR, > 75% aflatoxin B_1 was inhibited.

3.5. Physical properties and molecular structure of DN-AflR

Amino acid sequence, isoelectric point and other basic informations were analyzed by ExPASy (http://web.expasy.org/protparam/; Table 2). Spatial structure was predicted by SWISS-MODEL. The fusion protein DN-AflR carries a positive charge, and has a relatively strong hydrophobic effect. By analyzing the predicted structures of DN-AflR (Fig. 5A) and GAL4 (Fig. 5B), DN-AflR also has the zinc finger DNA-binding functional structure (Fig. 5C).

4. Discussion

At present, more and more microorganisms and their metabolites were reported to inhibit the growth of *A. flavus* and degrade aflatoxins (Kong et al., 2014; Palumbo et al., 2006). Albert et al. transformed the laccase gene of *T. versicolor* into recombinant *A. niger* by gene cloning, and the inhibition rate on aflatoxin of laccase (118 U/l) after recombinant expression as high as 55% (Alberts et al., 2009). Study showed that Fh8 tag fusion expression could significantly improve the ability of *E. coli* to express soluble exogenous proteins (Costa et al., 2013). Douillard et al. demonstrate that novel L. *lactis* fusion partner expression vectors allow high-level expression of soluble heterologous proteins (Douillard et al., 2011). Genetic engineering could highly express the active proteins and other metabolites in prokaryotic or eukaryotic hosts, which is the great way to cut down costs.

However, there was no report on the inhibition of growth of *A. flavus* by molecular modification of positive regulatory genes in aflatoxin biosynthesis. Fusion expression has been continuously applied to the process of expressing recombinant protein in order to improve functions of the active protein (Schückel et al., 2012; Terpe, 2003). TEM and SEM are effective applications to analyze the characteristics and morphology of samples. SEM showed that DN caused changes in mycelial morphology of *A. flavus*. TEM showed that ruptured vacuoles accounted for most of cell space and destroyed the function and balance of other organelles, which affected normal growth of the whole cell (Fig. 2). So that DN had the ability of inhibiting the growth of *A. flavus*.

In order to improve the anti-*A. flavus* effect of DN, we fused the genes encoding DN and specific sequences of zinc finger cluster protein (specifically binds to the AflR binding site), then successfully constructed a recombinant plasmid *dn-aflR*-pET32a (Fig. 1). In the process of *E. coli* expression, when expressed in the host system at a high level, the recombinant protein was easy to form inclusion bodies (Lilie et al.,

1998). So in order to avoid the above situation as much as possible, according to the reports (Pal and Srivastava, 2013), we used 16 °C, 160 rpm as the conditions for inducing expression. To get a deeper comprehension of DN-AflR, its physical properties and spatial structure were predicted. Studies showed that the outer membrane of most cells is negatively charged, while most antifungal proteins are positively charged and they bind to the cell surface through electrostatic attraction (Carvalho and Gomes, 2009), especially, DN-AflR contains the zinc finger cluster protein structure (compare with GAL4). These properties make DN-AflR more convincing for the inhibitory ability on growth of *A. flavus* and biosynthesis of aflatoxin. Compared with DN, the fusion protein DN-AflR had stronger inhibitory effect on growth of *A. flavus* (Fig. 4A). Simultaneously, it also had significant advantages in inhibiting aflatoxin B_1 biosynthesis (Fig. 4B), especially, under 30 μg/ml concentration of DN-AflR, > 75% aflatoxin B_1 was inhibited. Fusion protein is mainly compete with AflS-AflR complex by acting on AflR binding sites to inhibit the activation of aflatoxin, the off-target effect did not occur in the current experiment. If the off-target effect occurs, we need find whether the incorrect of gene sequence causes fusion protein to lose its inhibitory effect. Then the wrong designed DNA sequence should be amended by gene editing technology. Gene editing technology is a worthwhile solution, which is essential for the better control of infection, virulence, and drug resistance and plays a vital role in creating gene knock-outs, knock-ins, and replacements of sequences (Singh et al., 2017). In addition to accuracy of the gene, for the expression product, mass spectrometry can be used to more accurately locate whether the off-target effect caused by the incorrect expression of the fusion protein.

The available data indicate that aflatoxin contamination in cereals including corn and peanut is still a public health problem, and methods for aflatoxin decontamination involving microorganisms or related products such as enzymes offer an attractive alternative for food industry, considering their “natural” appeal and the increasing rejection of the consumer to chemical treatments of foods (Alberts et al., 2009). The application of biocontrol agents to corn by incorporation of seed coating is explored and results suggest that seed coating may be a useful approach to deliver biocontrol agents for reducing aflatoxin contamination in corn (Accinelli et al., 2018; Wu and Guclu, 2013). Next step, fusion protein (DN-AflR) is scheduled to be kept adherent to the seed surface by a starch-based bioplastic or contact with the seed surface directly to assess the extent to aflatoxin contamination of crops. Furthermore, in order to lower the cost, this fusion protein need to be highly expressed by the aid of gene engineering.

To the best of our knowledge, this is the first report about fusing the genes encoding inhibitory peptides and specific zinc finger cluster protein, and fusion expressed the novel anti-*A. flavus* protein. Results showed the modified novel protein reduced aflatoxin biosynthesis and growth of *A. flavus*, which could control aflatoxin contamination. Compared with DN, the inhibitory ability of novel protein has been improved significantly.

This work promoted deep researches for fusion expression of antifungal proteins it also showed fusion expression of anti-fungal proteins, such as DN-AflR, is a promising method to economically improve the inhibitory activity of bio-inhibitors for *A. flavus*. Furthermore, this study also provided a new idea about enhancing the anti-*A. flavus* ability of inhibitory substances and more theoretical basis and technical support for the further bio-control of *A. flavus* and other microbial contamination.

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